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Comparison of nested polymerase chain reaction (PCR), real-time PCR and viral culture for the detection of cytomegalovirus in subgingival samples

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Introduction: The purpose of this study was to compare nested polymerase chain reaction (PCR), real-time PCR, and shell vial for the detection of human cytomegalovirus (HCMV) in subgingival samples in periodontitis patients.
Methods: A group of 44 patients and 24 individuals without periodontitis were included in the study. A full periodontal examination was conducted in each subject. Gingival crevicular fluid (GCF) was collected by pocket lavage and used for viral culture (shell vial). Additional subgingival samples were obtained with paper points and used for molecular analysis. Nested PCR and real-time PCR were used to detect and quantify HCMV. Student's *t*-test and chi-squared test were used to compare groups. The sensitivity and specificity for the tests were calculated on 2 × 2 tables considering the nested PCR as the gold standard.
Results: The detection of HCMV was greater using nested PCR than with either real-time PCR or shell vial ($P < 0.0001$). However, the frequency detection of both molecular techniques was higher than in viral culture ($P < 0.0001$). Only one case of chronic periodontitis was positive by viral culture. Agreement between nested PCR and real-time PCR was observed 47.7% and 4.1% of the time in the periodontitis and control groups, respectively. The sensitivity of real-time PCR was 60%, compared with 2.8% for the shell vial technique.
Conclusions: In conclusion, this study confirmed that active HCMV infection occurs in human periodontitis; however, its frequency seems to be low. In contrast, latent periodontal HCMV infection seems to be a more frequent event.

Key words: human cytomegalovirus; nested polymerase chain reaction; periodontitis; real-time polymerase chain reaction; shell vial

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In recent years, attention has been drawn to the detection of human cytomegalovirus (HCMV) in subgingival samples and gingival tissue in patients with periodontitis. HCMV has been reported as the most frequently found virus of the Herpesviridae family (15) in periodontal pockets. However, there have been conflicting results for virus detection. The number of viral copies and the frequency of detection vary greatly between subjects and between clinical

conditions (9, 13, 14). No consensus for the role of HCMV in periodontitis has been achieved to date.

The first technique used to detect HCMV in periodontal pockets was the nested polymerase chain reaction (nested PCR) used by Parra and Slots (12) and Contreras and Slots (4). The test is relatively easy to perform and provides information on the presence or the absence of a specific sequence of the genomic DNA from the virus. The method uses two amplification rounds so the sensitivity of detection and specificity of this nested PCR are high. Later, real-time PCR was employed to quantify the viral load in the periodontal pocket and tissue (8, 9, 13). The results can be interpreted as the presence or absence of virus and as the number of viral copies. However, no information on the replicative state of the virus is obtained from the results of a PCR. One rapid way to establish the viability and infectious state of the virus is by viral culture using the Shell Vial system (5); this detects the expression of viral immediate-early antigens, in a short-term *in vitro* cell culture, by immunofluorescence. The shell vial approach has been used for years for the detection of HCMV in different types of fluids and tissues (5, 17). However, it has not been used for the detection of HCMV in gingival crevicular fluid (GCF).

The purpose of this study was to compare the nested PCR, real-time PCR, and shell vials for the detection of HCMV in subgingival samples in periodontitis patients.

Materials and methods

The study protocol was revised and approved by the Institutional Review Committee for Human Research of the Universidad del Valle according to the Helsinki Declaration of 1975, as revised in 2000. Patients attending the dental clinics at the School of Dentistry between January 2005 and April 2007 were invited to participate in the study. Each patient signed a written informed consent before inclusion in the study. Participants had good general health and had at least 20 teeth present (excluding third molars). Diabetes, heart disease, human immunodeficiency virus infection, pregnancy, heavy cigarette smoking (>15 cigarettes/day), previous periodontal treatment (6 months), and previous antibiotic intake (4 months) served as exclusion criteria.

Periodontitis group

A group of 44 patients (37 with chronic periodontitis, seven with aggressive periodontitis), with mean age 40.3 years, were included in the study. As part of the study design, patients with chronic and aggressive periodontitis were considered in the same group for analysis. Periodontal diagnosis was conducted according to the consensus report of the American Academy of Periodontology (1, 6, 16). Subjects with chronic periodontitis presented with pocketing (≥4 mm), clinical attachment loss (≥4 mm), bleeding on probing, and bone loss as evidenced by radiographic analysis. Subjects with aggressive periodontitis had severe attachment loss, pocketing, bone loss, and plaque and calculus deposits that were inconsistent with the severity of the periodontal destruction. They were systemically healthy people under 30 years of age. A full-mouth clinical examination was conducted in each patient. Probing depth (PD in mm), clinical attachment level (CAL in mm) at sampled sites and subject level were recorded using a computerized periodontal probe (Florida Probe; Florida Probe Corporation, Gainesville, FL). The percentage of bleeding on probing (BOP) sites and the plaque index (PI) (11) were also recorded.

Control group

A group of 24 individuals without periodontitis (mean age 30.8 years) were included as controls. In general, subjects showed no evident clinical signs of gingival inflammation, no evidence of attachment loss of >3 mm at more than one site, or PD >3 mm, no detectable periodontal pockets (PD ≥ 4 mm) and bone loss evidenced by radiographic examination. Individuals in this group exhibited isolated sites with bleeding during probing but for study purposes, only sites with no bleeding on probing were sampled.

The same clinical examination that was conducted in patients with periodontitis was carried out in control subjects.

Clinical sampling

Plaque samples were obtained from the six deepest sites in periodontitis patients and from six healthy sites in the healthy controls by inserting a paper point at the sample site and keeping it in place for 20 s. Previous supragingival plaque was eliminated using sterile gauze. The paper points were pooled in microcentrifuge vials and stored at −70°C until DNA extraction.

The GCF was collected on a different day from the plaque sample. After isolation of the site using sterile gauze, the tip (0.5 mm diameter) of a micropipette (0.1–10 μl) was inserted 1–2 mm into the periodontal pocket and 10 μl α-minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA) were instilled inside the pocket and then collected. The procedure was repeated 10 times in each of the six sites sampled. After pooling the diluted GCF, approximately 250 μl was collected. Caution was taken to not induce gingival bleeding and GCF samples that were contaminated with blood were discarded. Immediately after sampling, GCF was resuspended in 500 μl α-MEM supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) and centrifuged at 1811 *g*. for 15 min at 8°C. The supernatant was carefully transferred to a new centrifuge vial and used immediately for viral culture.

DNA extraction

DNA extraction from the clinical samples was carried out according to Parra and Slots (12) and Contreras and Slots (4). Briefly, 500 μl TE buffer (10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid, pH 7.5) was added to the microcentrifuge vial containing the paper points and vortexed for 10 min. Nucleic acid was bound to silica particles in the presence of guanidinium thiocyanate (GuSCN) lysis buffer. Nucleic acid/silica was recovered by centrifugation (12,000 ***g*** for 10 min); it washed first in buffer (GuSCN–Tris–HCl), then twice in 70% ethanol and once in acetone and then dried in a heating block at 56°C for 10 min. The nucleic acid/silica pellet was resuspended in 100 μl TE buffer, incubated at 56°C for 10 min and centrifuged (12,000 ***g*** for 2 min) and the supernatant was stored at −70°C.

Nested PCR

Nested PCR was conducted to detect HCMV in clinical samples according to Parra and Slots (12) and Contreras and Slots (4). Primer sequences and PCR conditions (4, 12) are described elsewhere. Appropriate negative and positive controls were used in each amplification round. The detection limit of the nested PCR was established by the amplification of HCMV DNA isolated from 10-fold serial dilutions of a viral stock solution. Amplification products (136 bp) were resolved by

electrophoresis on 2% agarose gels, stained with ethidium bromide (0.5 μg/ml) and observed under ultraviolet light (300 nm) transilluminator. Data are presented as the frequency detection (%) of HCMV-positive subjects.

Real-time PCR

A *Taq*Man assay was conducted to quantify HCMV in clinical samples (13). Primer sequences were: left 5′-GGACGCTGTTTCCGAATA-3′ and right 5′-GGACGCTACTTTCCGATCCT-3′ (Invitrogen). The probe was a Universal probe that uses locked nucleic acid nucleotide (LNA) chemistry (Universal Probe Library, probe # 52 catalog # 4688490; Roche Applied Science, Indianapolis, IN). The probe sequence was (FAM) 5′-GGGAGGAG–(TAMRA). PCR reaction mixture was adjusted to 25 μl: 12.5 μl *Taq*Man Universal Master Mix (Applied Biosystems, Foster City, CA), 5 pmol primers, and 4 pmol probe. The amplification program was set to 2 min at 50°C, 10 min at 95°C and then 45 cycles each consisting of a step at 95°C for 15 s followed by 60°C for 1 min. Amplification efficiency was calculated from a standard curve generated by the amplification of 10-fold serial dilutions (10^{-1} to 10^{-6}) of a concentrated HCMV DNA (710,000 copies/μl). Data were reported as copies/μl. The procedure was standardized with an amplification efficiency of 100% (slope −3.01) and detected 0.71 copies/μl HCMV using a known quantity of DNA (Fig. 1). Results were presented as positive or negative to indicate the presence of the virus. Detection was carried out in an ABI Prism 7500 Sequence detection system (Applied Biosystems, Foster City, CA).

Viral culture (shell vial)

Infectious HCMV in GCF was detected by the expression of the viral immediate-early protein pp72 (IEpp72) in a cell line of gingival fibroblasts using the shell vial technique described previously (17).

Gingival fibroblasts were obtained from healthy gingival tissue during a crown-lengthening procedure from a 25-year-old female patient according to Hakki et al. (7) and a cell line of gingival fibroblasts was generated after 10 continuous cell passages in culture. For isolation of HCMV from GCF, 100,000 gingival fibroblasts were seeded on 2-ml glass vials containing a round glass slide in the bottom and were grown to a 90% confluence cell monolayer at 37°C in 5% CO_2. Before GCF sample inoculation, medium (a-MEM supplemented with 10% fetal bovine serum) was removed and 200 μl GCF was added directly on to the cells and incubated at 37°C in 5% CO_2 for viral adsorption for 30 min. After adsorption, 800 μl α-MEM supplemented with 10% fetal bovine serum was added and centrifuged at 805 *g* for 45 min at 25°C. Inoculated vials were incubated for 48 h at 37°C, fixed with cold acetone for 10 min and processed for immunofluorescence to detect viral antigens.

Fixed glass slides were removed from the vials and a primary antibody directed to the IEpp72 (Virogen Corp., Watertown, MA) was added and incubated at 37°C. Slides were washed three times with phosphate-buffered saline and a fluorescein isothiocyanate-conjugated mouse anti-human immunoglobulin G (Sigma, St Louis, MO) was added as secondary antibody and incubated for 1 h at 37°C. Slides were washed three times with phosphate-buffered saline and mounted on a microscope glass slide and observed under a fluorescence microscope. Positive cells exhibiting green fluorescence staining inside the nucleus were identified and counted per field (Fig. 2). Positive and

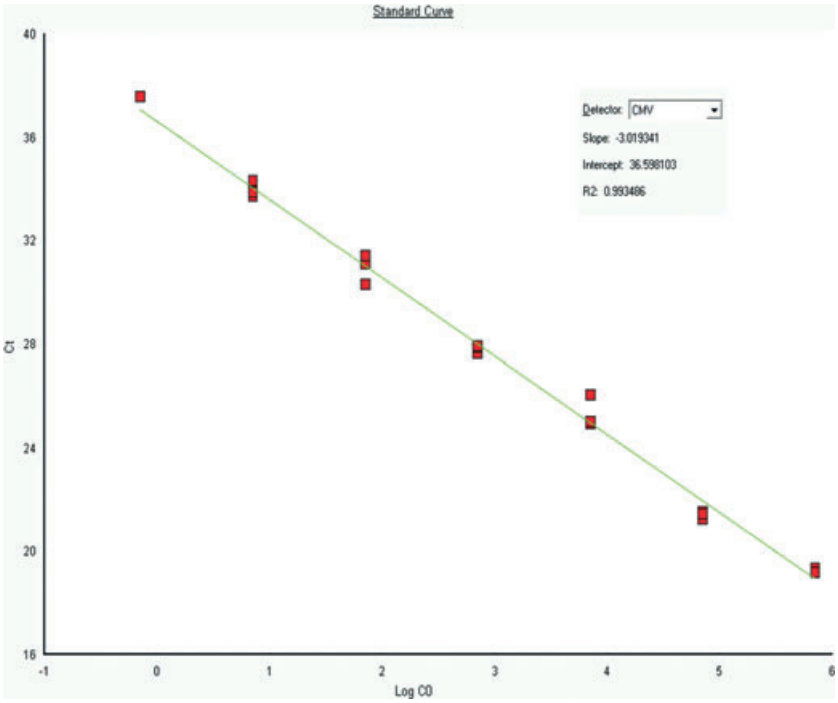

Fig. 1. Standard curve generated for Real time PCR for the detection and quantification of HCMV in subgingival plaque samples. The standard curve shows the input level of HCMV DNA on the x-axis and the cycle threshold (Ct) on the y-axis. The assay is sensitive as to few 0.71 copies/μl of template DNA and is linear across 6 orders of magnitude from 0.71 to 710.000 copies/μl per PCR.

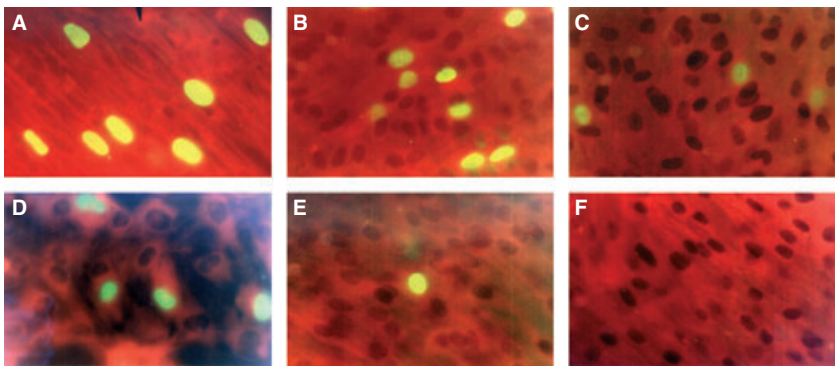

Fig. 2. Shell vial detection of HCMV. The IE pp72 antigen was detected by means of immunofluorescence and it can be observed as the green labeling inside the nucleus of gingival fibroblasts. Serial dilutions of a viral stock solution were used: (A) 71,000 c/μl, (B) 7,100 c/μl, (C) 710 c/μl, (D) 71 c/μl, (E) 7.1 c/μl. A negative control was used (F).

negative control slides were used for comparison.

To establish the minimum number of viral copies detected by the shell vial (detection limit), a controlled infection test was carried out using different amounts of HCMV. First, a viral stock solution was produced by infecting gingival fibroblasts (100% confluency) in 25-cm^2 culture flasks at a 10^{-6} dilution from the concentrated viral stock (HCMV Towne strain). The HCMV Towne strain was kindly provided by Dr Mark Stinski and Phil Lashmit from the University of Iowa. Cells were maintained with α-MEM supplemented with 10% fetal bovine serum until a cytopathic effect of >90% was observed (approximately 2 weeks). Supernatant was collected and stored at −70°C for further quantification by real-time PCR. Aliquots of the supernatant were quantified in duplicates for HCMV using a commercially available kit (LC CMV kit; Roche Diagnostics, Indianapolis, IN) in a Roche LightCycler version 2.0 (Roche Diagnostics). After establishing the quantity of HCMV in the stock solution, gingival fibroblast shell vials (as described above) were inoculated in duplicates with serial dilutions of the viral stock (in copies/μl): 710,000, 71,000, 7100, 710, 71, 7.1, and 0.71. Infected cells were maintained for 48 h and stained for IEpp72 as described previously. The shell vial technique was consistently able to detect viral inocula as low as the equivalent to 7.1 coplies/μl HCMV in three different assays (Fig. 2).

In addition, we established that the nested PCR used here reliably detected as few as 0.71 copies/μl HCMV by serially diluting (10-fold) aliquots of the HCMV stock solution and then proceeding to extract the DNA and PCR (explained above) in three independent experiments. Both nested PCR and real-time PCR were able to detect as few as 0.71 copies/μl HCMV; however, nested PCR continue to detect below 0.71 copies/μl HCMV (10^{-7}, 10^{-8}, and 10^{-9} dilutions, data not shown) although results were less consistent than 0.71 copies/μl. In contrast, real-time PCR only detected to 0.71 copies/μl HCMV. The nested PCR was considered as the reference in this study because it was more sensitive than real-time PCR for the detection of HCMV.

Statistical analysis

Demographic and clinical variables are presented as mean and standard deviation (SD). To test for differences for clinical variables, the *S*tudent's *t*-test and the chi-squared test were used to compare groups. The frequency detection of HCMV between diagnostic tests and subject groups was analyzed using the chi-squared test where indicated. The sensitivity, specificity, and predictive values were calculated on 2 × 2 tables considering the nested PCR as the gold standard. Statistical significance was assumed when $P \leq 0.05$. Data were analyzed using statistical software (STATA, statistical software for Windows StataCorp LP, College station, TX).

Results

A real-time quantitative PCR assay targeting the HCMV polymerase gene assay was adapted for use on an ABI 7000 instrument. Assay amplification efficiency was evaluated on serial dilutions of purified HCMV DNA as shown in Fig. 1. The assay was sensitive to as few as 0.71 copies/μl HCMV.

Forty-four patients with periodontitis (mean age 40.3 years old) and 24 subjects without periodontitis (mean age 30.8 years old) were included in the study. Overall, subjects with periodontitis presented increased PD and CAL compared to control subjects ($P < 0.05$). In addition, plaque accumulation and BOP were more frequent in patients with periodontitis ($P < 0.05$).

No consistent correlation between higher copies of HCMV and the severity of periodontal destruction in periodontitis patients was observed. For example, one subject presenting with PD 9.2 mm and CAL 9.3 mm had 3099.2 copies/μl, whereas a subject with PD 8.3 mm and CAL 8.1 mm had 2.94×10^1 copies/μl. In contrast, only one subject from the control group was positive for HCMV by real-time PCR in which viral copies were very low (6.45×10^4 copies/μl, PD sampled site = 3 mm, CAL sampled site = 2.5 mm).

The frequency detection of HCMV according to test and clinical group is presented in Table 1. Patients suffering from periodontitis had a higher frequency of HCMV ($P < 0.0001$) as detected by nested PCR (79.5%) and real-time PCR (47.7%) in comparison to healthy subjects (25% nested PCR, 4.1% real-time PCR). It was observed that the detection of HCMV was lower with real-time PCR than with nested PCR in both groups of patients. Only one patient (2.3%) in the periodontitis group was identified as positive for HCMV using the shell vial technique (Fig. 3). In general, the detection of HCMV was greater using nested PCR than with real-time PCR and shell vial

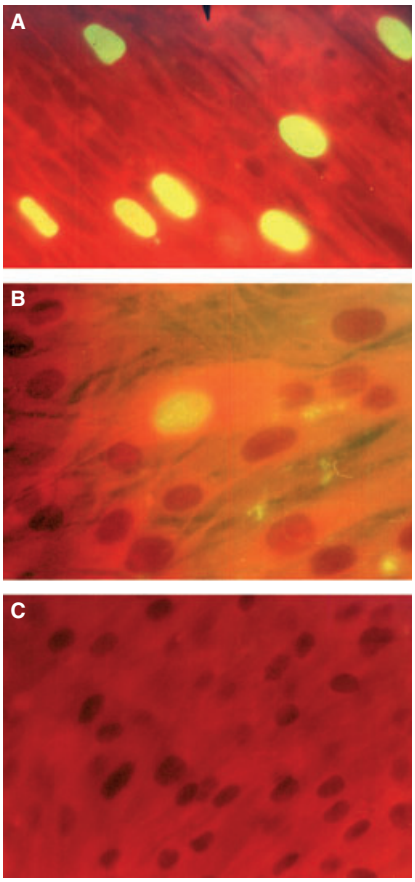

Fig. 3. HCMV detection by shell vial in one subject with chronic periodontitis. (A) positive control, (B) GCF form ChP patient and (C) negative control. Infected cells are identified by the green fluorescence inside the nucleus.

Table 1. Frequency detection (%) of human cytomegalovirus as detected by polymerase chain reaction (PCR), real-time PCR and shell vial in subjects with and without periodontitis

	PCR *n* (%)	Real-time PCR *n* (%)	Shell vial *n* (%)
Periodontitis group (*n* = 44)	35 (79.5%)[1,4]	21 (47.7%)[2,5]	1 (2.3%)
Control group (*n* = 24)	6 (25%)[3]	1 (4.1%)	0

[1]Significantly different compared to real-time PCR and shell vial, $P < 0.0001$, chi-squared test.
[2]Significantly different compared to shell vial, $P < 0.0001$, chi-squared test.
[3]Significantly different compared to real-time PCR, $P < 0.0001$, chi-squared test.
[4]Significantly different compared to control group, $P < 0.0001$, chi-squared test.
[5]Significantly different compared to control group, $P < 0.0001$, chi-squared test.

Table 2. Agreement between diagnostic techniques for the detection of human cytomegalovirus in subjects with and without periodontitis

	PCR/real-time PCR	PCR/shell vial	PCR/real-time PCR/shell vial
Periodontitis group (n = 44)	21 (47.7%)	1 (2.27%)	1 (2.27%)
Control group (n = 24)	1 (4.16%)	0	0

Data are presented as the number of samples (%) that were positive for the respective test.
PCR, polymerase chain reaction.

Table 3. Sensitivity, specificity, and predictive values of real-time polymerase chain reaction (PCR) and shell vial for the detection of human cytomegalovirus in subgingival samples

Test	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Real-time PCR	60	100	100	39
Shell vial	2.8	100	100	20

PCR was considered as the reference for the detection human cytomegalovirus.

($P < 0.0001$). However, the frequency detection of both molecular techniques was higher in contrast to viral culture ($P < 0.0001$).

Agreement between the three techniques was observed in only one case (2.27%) in the periodontitis group (Table 2). Agreement between nested PCR and real-time PCR was observed 47.7% and 4.1% of the time in the periodontitis and control groups, respectively. In addition, if we considered the nested PCR as the gold standard for the detection of HCMV in subgingival samples, the sensitivity of real-time PCR was 60% while the specificity was 100% (Table 3). In contrast, the shell vial technique had lower sensitivity (2.8%) but high specificity (100%). The probability of having the virus when the test was positive (positive predictive value) was 100% for real-time PCR and shell vial (Table 3). In contrast, the probability of not having the virus when the test was negative (negative predictive value) was <40% for both techniques (Table 3).

Discussion

To further delineate the nature of periodontal HCMV infection, the present study determined, by means of viral culture, that active HCMV infection occurred in at least one of 44 (2.27%) patients with chronic or aggressive periodontitis. No viral activation was detected in the control group. In the same group of patients, this study also determined the presence of genomic DNA from HCMV by nested PCR in 79.5% and by real-time PCR in 47.7% of periodontitis patients; detection frequencies were lower in individuals without periodontitis (Table 1). These results reveal that active HCMV infection appears to be an infrequent event in periodontal pockets. The molecular analysis used in this study did not make it possible to determine if there was active replication because HCMV quantification by real-time PCR demands at least two separate samples taken over a period for comparison. However, because samples were analyzed in parallel using molecular and culture detection, low agreement between techniques suggests that HCMV was probably in a latent state in most cases. It is likely that HCMV reactivation is limited to a few periodontal sites in periodontitis patients.

Another explanation for these differences is the manipulation and specific characteristics of GCF. Dilution of GCF during sampling, presence of substances that inhibit viral infection, sample quality, and low viral copies could be taken into consideration to explain the lack of viral activation in most periodontitis patients. Contrasting with this result, a previous study from Contreras and Slots (3) demonstrated active HCMV infection in four of nine subjects studied by messenger RNA analysis to detect the late major capsid protein. Nowzari et al. (10) also tested for active replication of HCMV using real time reverse transcription PCR (HCMV pp67 messenger RNA) and found few transcripts (<20%) in patients with periodontitis who were undergoing renal transplants. To explain the contrast, paper-point samples (subgingival biofilm samples) were used in the previous studies while in this study viral activation was determined in a GCF wash.

While HCMV is well known as a cause of serious illness in immunocompromised patients, it is now being discussed as a pathogen of emerging importance for non-systemically compromised patients. Generally, active HCMV infection is not recognized in such patients because they are not routinely monitored for HCMV infection. Because of low viral loads, the incidence of active HCMV infection could be easily underestimated by a less sensitive assay, such as the shell vial. Quality and sample type seem to be important to HCMV detection. Shell vial was rarely positive for HCMV in patients with septic shock, a finding that differed for patients who had received a kidney transplant (2).

Whether active HCMV infection is related to increased periodontal destruction is still to be determined. We found no correlation between the number of copies of HCMV and PD and CAL at sampled sites in periodontitis patients (data not shown). In fact, the results varied greatly between subjects and support the aforementioned statements. Real-time PCR quantified the number of viral copies of HCMV but the question as to whether that quantity of virus is actually infectious remains to be answered. Our results showed that only one patient with 113,039 copies/μl in the periodontitis group showed active viral replication in cultured gingival fibroblasts (Fig. 3). HCMV is therefore capable of infecting gingival fibroblasts and the number of viral copies may be of importance but needs to be studied further. This is interesting because it did not correlate with the highest number of HCMV copies. Our shell vial technique detected 7.1 copies/μl viable virus but the same assumption could not be made for periodontal pockets. This was the case for samples in which the number of HCMV copies was >1000/μl but that still yielded no positive result in culture. Possible explanations are that the virus is not in an active state of replication and/or infected cells are just carrying latent viral DNA particles. Moreover, the results from the controlled infection during shell vial standardization presented here showed that infection occurred more frequently with elevated copies of the virus (71,000 copies/μl vs. 0.71 copies/μl). This is relevant if we want to consider the impact of periodontal therapy on HCMV counts.

The study design was cross-sectional so investigators could not rule out that viral activation may be occurring more frequently than was observed. Shell vial sensitivity was low in this study, meaning that false-negative cases could be undetected. Another possible explanation is that HCMV activation might occur in a few periodontal sites and at different times during the cyclic nature of periodontal disease and so even the actual negative sites could become positive later. It could be interesting to follow a group of patients

with periodontitis before and after treatment in the long term to determine if this hypothesis is true and to validate the other proposal that HCMV reactivation may be linked to periodontal disease exacerbation and pathogenesis.

Nested PCR was a more sensitive detecting subgingival HCMV than real-time PCR. In samples where nested PCR was positive but real-time PCR was negative, very low quantities of HCMV, or double amplification rounds with nested PCR may have accounted for the differences. For example, some clinical samples showed very low copies of HCMV (6.45×10^4 copies/μl) and samples that were negative could have been below this value but were detected by nested PCR. Under the laboratory conditions used in this study, both nested PCR and real-time PCR showed good detection limits of HCMV, but some cases may remain underestimated by the latter technique. This may be because 70 cycles of amplification were used in nested PCR (two amplification rounds) compared with 45 cycles in real-time PCR. Discrepancies in this study could also be attributed to the different detection of amplification products between nested and real-time PCR. Since the limit of detection was calculated by using DNA from a reference strain of HCMV, it may be possible that, although molecular sensitivity is reported to be similar for nested PCR and real-time PCR, some differences may occur for biological specimens. The simultaneous use of both techniques may be necessary in some instances. The use of nested PCR to screen for the presence of HCMV does not underscore the enormous usefulness of real-time PCR to quantify HCMV in subgingival samples.

In conclusion, within the limitations of this study, it was confirmed that active HCMV infection occurs in human periodontitis; however, its frequency seem to be low. In contrast, latent periodontal HCMV infection seems to be a more likely and frequent event. Longitudinal clinical and virological studies are warranted to clarify the relationship between HCMV and the pathogenesis of periodontitis.

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