*Oral Microbiology Immunology 2006: 21: 73–78*
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ORAL MICROBIOLOGY
AND IMMUNOLOGY

# Detection and quantification of herpesviruses in Kostmann syndrome periodontitis using real-time polymerase chain reaction: a case report

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*Yildirim S, Yapar M, Kubar A. Detection and quantification of herpesviruses in Kostmann syndrome periodontitis using real-time polymerase chain reaction: a case report. Oral Microbiol Immunol 2006: 21: 73–78.* © Blackwell Munksgaard, 2006.

**Background/aims:** Kostmann syndrome, or severe congenital neutropenia, is an autosomal recessive disease of neutrophil production and is associated with severe periodontal pathology. The aim of this study was to determine whether human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) contribute to the pathogenesis of Kostmann syndrome periodontitis.
**Methods:** Supragingival plaque and saliva samples were taken from a 6-year-old boy and his 3-year-old sister suffering from Kostmann syndrome, and from two age- and gender-matched healthy children serving as controls. The samples were taken before and 24 months after periodontal treatment. Real-time polymerase chain reaction (TaqMan Real-Time PCR) assay was used to quantify HCMV and EBV DNA.
**Results:** EBV was detected in baseline samples from the Kostmann syndrome patients but not in samples from the healthy control subjects. HCMV was only detected in the saliva of the boy with Kostman syndrome at baseline. Herpesviruses numbers decreased dramatically in the post-treatment samples.
**Conclusion:** EBV and HCMV were detected in the two subjects with Kostmann syndrome periodontitis. The results of the study indicate that nonsurgical treatment of Kostmann syndrome periodontitis can reduce supragingival and salivary herpes viral loads.

Key words: cytomegalovirus; Epstein-Barr virus; herpesvirus; Kostmann syndrome; severe congenital neutropenia; periodontal disease

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Accepted for publication July 17, 2005

Severe congenital neutropenia, or Kostmann syndrome, is a rare hereditary kind of severe neutropenia and was originally reported as an autosomal recessive disease of neutrophil production. Affected individuals are usually children with severe neutropenia with an absolute blood neutrophil count of less than $0.2 \times 10^9$/l (1, 4). Infections in Kostmann syndrome patients include cellulitis, perirectal abscess, stomatitis, meningitis, pneumonia, and sepsis. Although they suffer from severe bacterial infections since the first years of life, unfortunately the patients can never be diagnosed as having an important systemic disease (26). Granulocyte-colony stimulating factor therapy is generally used to reduce the frequency of life-threatening infections and to increase survival (16). The underlying genetic defects in Kostmann syndrome and the exact pathogenic mechanisms increasing susceptibility to infections remain unclear. Although neutrophil function is not completely normal in patients with severe congenital neutropenia, there may be enough redundant neutrophil bactericidal capacity to promote normal host response to inflammation (40).

Periodontitis can be a manifestation of systemic diseases including certain hematologic disorders such as acquired neutropenia and leukemia, and various genetic disorders (6). As neutrophils have been

suggested to provide the first line of host defense against bacterial invasion, chronic neutropenia is generally associated with increased prevalence and severity of infections, including oral infections and periodontal diseases (3, 13, 31, 39). Defraia & Marinelli (10) have stated that severe periodontal pathology, which is similar to aggressive periodontitis in the primary dentition (formerly called prepubertal periodontitis), can be considered a characteristic finding in Kostmann syndrome. Some studies have reported that nonsurgical treatment of periodontal disease in severe congenital neutropenia will improve the systemic immune system (15, 32); others, however, have found that patients successfully treated for agranulocytosis continue to suffer from oral infections such as chronic periodontitis (4, 30, 46).

Recent studies have demonstrated that herpesviruses appear as putative pathogens in various types of periodontal diseases (35). Slots (35) stated in his excellent review that herpesviruses and periodontopathic bacteria may cause periodontal pathosis in defective neutrophil functions associated with aggressive disease by infecting and perturbing neutrophils. It has been shown that at least two members of herpesviruses, human cytomegalovirus (HCMV) and Epstein-Barr Virus (EBV), occur with a high frequency in actively progressing periodontitis lesions (22, 33, 45).

Epstein-Barr virus is a member of the *Herpesviridae* family, subfamily gamma *Herpesvirinae*, genus *Lymphocryptovirus*. Primary infection of EBV, which is primarily transmitted by saliva, actively replicates in the epithelial cells of the oropharynx and can subsequently infect recirculating B lymphocytes, which may lead to acute infectious mononucleosis (5, 9, 12). EBV has a narrow tissue tropism limited to B lymphocytes, T lymphocytes and epithelial cells of primate origin. By the time they reach their 20s, more than 90% of humans have become seropositive for EBV. In normal individuals, latent EBV infection is controlled by humoral immunity, cytotoxic T cells, and the interferon system (12, 21).

Cytomegalovirus has the largest genome of all herpes viruses and appears to replicate only in human cells. HCMV infection is found in a significant proportion of the population. As with EBV, seropositivity increases with age. The virus is spread through most bodily secretions, particularly saliva, urine, vaginal secretion, and semen. HCMV causes no symptoms in children and generally only mild disease in adults. It elicits both humoral antibodies and cell-mediated immunity; but the infection cannot be cleared. Although suppressed, the virus may later reactivate. Particularly in cases of immunosuppression, infection by the virus can be immunosuppressive in itself (12).

To the best of our knowledge, no studies have investigated the occurrence of human viruses in periodontitis lesions of Kostmann syndrome. The purpose of this pathophysiological study was to investigate the presence of HCMV and EBV in supragingival plaque and saliva samples in two siblings with Kostmann syndrome.

## Material and methods

Baseline clinical features, bone marrow, cytogenetic examinations and mutational analysis, and the treatment of the 6-year-old boy (Case 1) and his 3-year-old sister (Case 2) with Kostmann syndrome have been described previously (17). In the Case 1, generalized periodontal inflammation characterized by severe gingival inflammation, edema and attachment loss was observed in the primary dentition. After eruption of his permanent incisors and first molars, inflammation was observed to be slight but the attachment loss remained the same. Case 2 also presented generalized periodontal inflammation. Scaling and professional toothcleaning was performed monthly in conjunction with 0.2% chlorhexidine rinses. Restorative treatments were also done where necessary. After 24 months, the patients' oral hygiene was found to be satisfactory. As a part of their treatment, long-term antibiotics (5 mg trimethoprim, 25 mg sulfamethoxazole/kg/24 h orally, for 3 consecutive days each week) was prescribed. Granulocyte-colony stimulating factor therapy was not considered because systemic condition of the patients was stable (17).

### Sample collection

All procedures were approved by the local ethics committee and written informed consent was obtained from the parents after full explanation of the procedures.

Supragingival plaque and saliva samples were collected from both patients and from two other age- and gender-matched healthy children (control subjects). Samples were obtained from the patients at the beginning (baseline samples) and 24 months after the initial periodontal treatment. Periodontal treatment included mechanical debridement and antimicrobial therapy.

A sterile toothbrush was used to collect dental plaque from teeth as previously described by Okada et al. (27). In brief, the children brushed their teeth for 1 min under the supervision of a dental professional. The toothbrush was washed vigorously in a plastic vial containing 1 ml sterile deionized water. Saliva from the subjects was collected by having the children spit into an empty and sterile plastic vial.

### Sample preparation for real-time polymerase chain reaction (PCR) assay

The sample preparation and TaqMan Real-Time PCR assay conditions were described previously (24). In brief, samples were suspended in 50 µl of TE buffer (10 mM Tris-hydrochloride, 1 mM EDTA, pH 8). After vigorous vortex mixing, 100-µl aliquots of the specimens were exposed to 200 µl K buffer and 10 µl protease (68 mg/ml) (Sigma-Aldrich Corp., St. Louis, MO) for 60 min at 42°C for lysis of the specimens. DNA was extracted from specimens using alkali phenol-chloroform-isoamyl alcohol (25 : 24 : 1). Extracted DNA was resuspended in 100 µl of distilled water, and 5 µl of the DNA solution was used in the PCR analysis.

### New oligo designs for TaqMan Real-Time PCR assay

The primers and probes (oligos) used in this study were designed by OLIGOWARE 2.0 software, the new version of OLIGOWARE 1.0 (25). OLIGOWARE 2.0 is able to detect one of the highly conserved regions of EBV and HCMV, using its own database of the sequences downloaded from GenBank databases.

### Quantification of viral DNA

Quantification of viral load was previously explained in detail (23–25). In brief, the EBV and HCMV genomes were amplified and the amplicons cloned with TOPO-TA Cloning Kit (Invitrogen, Eugene, OR). These plasmids were used for EBV and HCMV quantification. Serial dilutions of plasmid DNA ($10^1$–$10^8$ plasmids/ml) were used to determine the dynamic range of quantification. Plasmid standards was calculated using OLIGOWARE 2.0 (25).

### TaqMan Real-Time PCR Assay

This assay was performed in a Perkin-Elmer 7700 Sequence Detection System (Applied Biosystems, Foster City, CA).

*Table 1.* The novel oligos designed for this study

| Infectious agents | Primers | |
|---|---|---|
| HCMV | Forward primer | 5′ TTG AGC CCG GCG GTG GT 3′ |
| | Reverse primer | 5′ AGC TCA CCG ATC ACA GAC ACC 3′ |
| | TaqMan probe | 5′ FAM AGA GAA GCG CCA CAT ACA GCG CAA AC TAMRA 3′ |
| EBV | Forward primer | 5′ ACC TGG TCA TCC TTT GCC A 3′ |
| | Reverse primer | 5′ GTG CTT CGT TAT AGC CGT AGT 3′ |
| | TaqMan probe | 5′ FAM CAG TAC GAG TGC CTG CGA CCA G TAMRA 3′ |

The amplification mix contained 1X TaqMan Buffer A (500 mM KCl, 100 mM Tris-HCl, 100 mM EDTA, 600 nM passive reference A, pH 8.3 at room temperature), 4 pmol of probe, 5 pmol of each primer, 0.2 mM of each dNTP, 6 mM $MgCl_2$, 1.0 U hot start *Taq* polymerase (AmpliTaq Gold® DNA Polymerase, Applied Biosystems) and 5 µl template in total volume of 25 µl. The reaction conditions for all templates were 2 min at 50°C, 10 min at 95°C, 40 cycles with 15 s at 95°C and 1 min at 60°C.

The first row of five wells contained serial dilutions of plasmid DNA ($10^2$, $4 \times 10^3$, $3 \times 10^5$, $10^7$, $10^8$; A1–5) and second row contained four wells with four controls without templates. The third row contained the samples C5–12 and D5–12. All PCR assays were performed in triplicate.

### Classical PCR assay for periodontopathic bacteria

PCR primers for *Porphyromonas gingivalis* were 5′ AGG CAG CTT GCC ATA CTG CG 3′ and 5′ ACT GTT AGC AAC TAC CGA TGT 3′; for *Prevotella intermedia* were 5′ TTT GTT GGG GAG TAA AGC GGG 3′ and 5′ TCA ACA TCT CTG TAT CCT GCG T 3′; for *Treponema forsythia* 5′ GCG TAT GTA ACC TGC CCG CA 3′ and 5′ TGC TTC AGT GTC AGT TAT ACC T 3′; for *Campylobacter rectus* 5′ TTT CGG AGC GTA AAC TCC TTT TC 3′ and 5′ TTT CTG CAA GCA GAC ACT CTT 3′; and for *Actinobacillus actinomycetemcomitans* 5′ AAA CCC ATC TCT GAG TTC TTC TTC 3′ and 5′ ATG CCA ACT TGA CGT TAA AT 3′ (2).

PCR was performed with a final volume of a 50-µl mixture containing 30 pmol of each primer (MWG-Biotech, Ebersberg bei München, Germany), 2 U *Taq* DNA polymerase (Bioron, Ludwigshafen, Germany), 1.5 mM $MgCl_2$, 0.1 mM dNTP mix, 5 µl of 10x Reaction Buffer (Bioron, Ludwigshafen, Germany), and 5 µl of extracted DNA sample. PCR procedures included a 40-round amplification process and were performed in three steps covering denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and an extension at 72°C for 40 s. Detection of PCR products was performed by electrophoresis in a 2% agarose gel containing 0.5 µg/ml ethidium bromide. Gels were analyzed using QUANTITY ONE software (Bio-Rad Laboratories, Hercules, CA).

## Results

In this study novel primers and probes were designed for EBV and HCMV (Table 1). Pre- and post-treatment saliva and supragingival plaque samples were positive ($10^6$ and $10^3$, respectively) in the two patients (Table 2). HCMV was only detected in the saliva sample of the Case 2 at baseline Fig. 1 shows amplification plots of EBV DNA with the standard dilutions (A1–5) and the samples (C5–12). Only one positive sample (D5) was found in the amplification plots of HCMV DNA (Fig. 2). The calibration curves from the TaqMan Real-Time PCR assay indicate a linear detection range of $10^8$ and $10^2$ plasmids and a correlation coefficient of $r = 0.99$ (Figs 3 and 4). These curves also confirm the assay sensitivity and the range of detection of the assay. As the real-time PCR measures logarithmically, and the differences between the plaque samples cannot be 10-fold, the weight difference of the plaque samples should have no effect on the quantitative test result.

*Table 2.* The mean EBV and HCMV counts in positive samples

| | | Mean EBV counts/ml samples | |
|---|---|---|---|
| | | Pretreatment | Post-treatment |
| Case 1 | Saliva | $1.7 \times 10^6$ | $3.4 \times 10^3$ |
| | Supragingival plaque | $1.5 \times 10^6$ | $3.1 \times 10^4$ |
| Case2 | Saliva | $3.2 \times 10^6$ | $3.6 \times 10^4$ |
| | Supragingival plaque | $2.0 \times 10^6$ | $2.4 \times 10^5$ |
| | | Mean HCMV counts/ml samples | |
| | | Pretreatment | Post-treatment |
| Case 1 | Saliva | Negative | Negative |
| | Supragingival plaque | Negative | Negative |
| Case2 | Saliva | $2.7 \times 10^4$ | Negative |
| | Supragingival plaque | Negative | Negative |

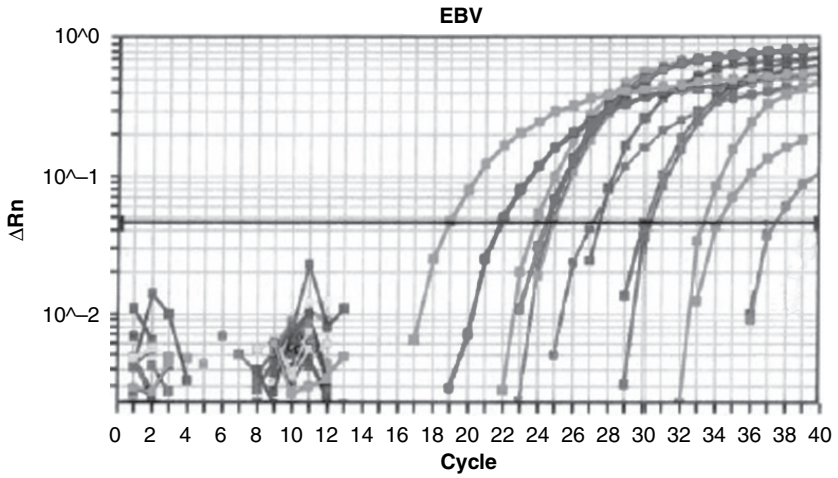


*Fig. 1.* Amplification plots of EBV DNA. A1(108), A2(107), A3(105), A4(102), A5(103): standard dilutions. C5, C6, C7, C8, C9, C10, C11, C12: samples.

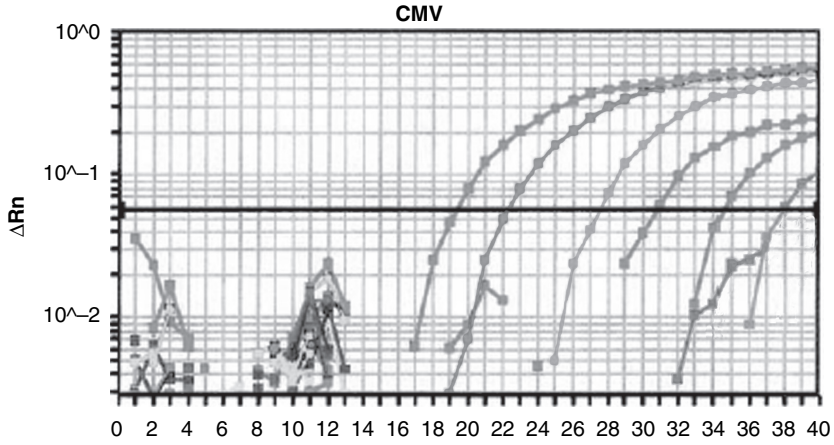


*Fig. 2.* Amplification plots of HCMV DNA. A1(108), A2(107), A3(105), A4(102), A5(103): standard dilutions. D5: only one positive sample.

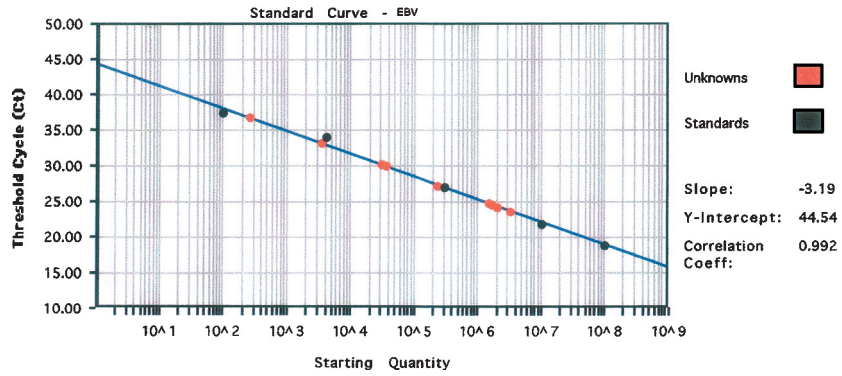


*Fig. 3.* Linear relationship between starting quantity of EBV DNA and threshold value ($C_t$).

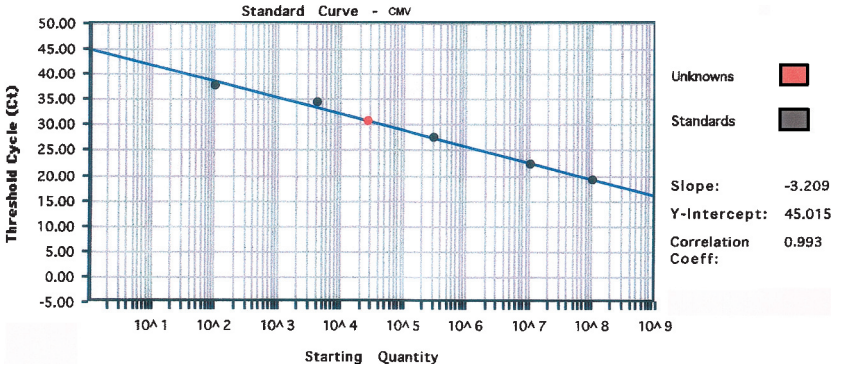


*Fig. 4.* Linear relationship between starting quantity of HCMV DNA and threshold value ($C_t$).

The specificity of the assay was confirmed by several herpesvirus strains and no cross-reactivity was observed (23, 25). The specificity of the oligos, on the other hand, was confirmed by controlling the DNA sequence of the HCMV and EBV; no cross-reactivity with other viruses or cells was detected with OLIGOWARE 2.0.

Of the five periodontopathogens studied, only *C. rectus* was detected in the baseline supragingival plaque sample of Case 1.

## Discussion

Periodontal destruction in the primary dentition may be associated with hematologic and immunologic disorders that may have genetic background. Review of the literature for aggressive periodontitis (formerly called prepubertal periodontitis) indicates that several systemic diseases, including those involving leukocytes (e.g. neutropenia, leukemia, and leukocyte adhesion defects), may exist in the background of periodontal disease (41). Understanding the independent roles of herpesviruses, bacteria and the host defense system may help to clarify periodontal pathogenesis. As periodontal diseases are a manifestation of hematologic and/or immunologic disorders, the study of periodontal pathologies is of value. In this respect, the cases of periodontal pathophysiology in Kostmann syndrome presented an opportunity to study such a relationship.

This is the first known report of a study, using a Real-Time PCR-based method, aimed at identifying and quantifying the oral herpesviruses in Kostmann syndrome periodontitis. The periodontal disease status of the patients showed a good match with the supragingival and salivary HCMV and EBV loads, suggesting that EBV and, possibly, HCMV may participate in the pathophysiology of Kostmann syndrome periodontitis.

Active herpesvirus infections may contribute to the pathogenesis of Kostmann syndrome periodontitis via several mechanisms. Latent herpesvirus may become active following the suppression of the host immune system. Incidents have been reported of HCMV reactivation leading to a severe infection, which presents acute periodontal inflammation and tissue loss (11). Studies investigating the presence of human HCMV, EBV, HSV, and human papillomavirus show that there is a higher prevalence of one or more of these viruses in periodontitis lesions than in gingivitis (29). On the other hand, Slots (35) reported that most clinical manifestations in immunocompetent individuals are secondary to cellular or humoral immune responses.

In this study, HCMV was detected only in the saliva of Case 2. However, EBV was detected in all samples from the two study patients, decreasing significantly after the periodontal treatment. Recent studies have shown an association between oral EBV and periodontal diseases such as chronic periodontitis, juvenile periodontitis (aggressive periodontitis), and Down's syndrome periodontitis (8, 20). It has been reported that HCMV reactivation leads to a severe infection and that HCMV was detected more frequently in deep periodontal pockets than in shallow ones (7, 11, 34, 38). The chronic state of the periodontal disease and the absence of deep

periodontal pockets in the present subjects may explain why HCMV was not detected in supragingival samples. On the other hand, the levels of EBV in Case 2 were 10 times higher than in Case 1 post-treatment samples, perhaps indicating that the periodontal status of Case 2 might have been unstable at the time of preoperative sampling.

The importance of the immune system in periodontal pathogenesis is well known and in this context severe congenital neutropenia is clearly a risk factor for periodontal disease. Even though immunodeficiency is of key importance in the development of Kostmann syndrome periodontitis, it is likely that the dual occurrence of HCMV and EBV is a contributory factor of this rare and aggressive form of periodontitis. Herpesviruses may have participated in the periodontal pathogenesis of the severe congenital neutropenia as a consequence of immunosuppression via reactivation.

As patients with severe congenital neutropenia have a significant risk of developing acute myelogenous leukemic transformations, we collected supragingival plaque from the patients using a toothbrush as the least inconvenient, fastest and most reliable technique. This method was developed by Okada et al. (27). They reported that the method is easy to use and does not cause any anxiety in children, and that the average amount of genomic DNA collected is sufficient for carrying out PCR analysis. This technique is also considered suitable for detection of periodontal pathogens as, in the case of subgingival plaque in children, there are limited gingival sites and hence limited numbers of pathogens present (19). Periodontopathogens are more readily detectable in subgingival areas affected by the aggressive periodontal disease and supragingival plaque sampling may therefore have failed to detect all the targeted periodontopathogens in the present sampling. Nonetheless, it has been shown that periodontal pathogens can be detected in supragingival plaque, and in healthy persons as well (14, 42). The supragingival plaque in children can harbor periodontopathic bacteria and the increase in probing depth between the ages of 6 and 69 is apparently not affected by the occurrence of these bacteria (36, 37). Although the difference between health and disease has been shown to be more pronounced in the subgingival plaque sampling, suspected periodontal pathogens do occur at supragingival sites, albeit sometimes in low amounts or proportions (43).

It has been shown that careful supragingival plaque control leads to a decrease in the amount of subgingival plaque and/or the levels of specific supragingival species or morphotypes. In addition, the microbial changes were accompanied by improvements in clinical parameters and in some cases long-term stability of the disease (44). In the present study, nonsurgical periodontal therapy decreased gingival inflammation and, after 2 years, the periodontal statuses were healthy and stable in both cases. Baseline EBV genomic counts were as high as $10^6$ per ml in both saliva and supragingival plaque samples, and as the 24-month post-treatment samples show, the virus could not be totally eliminated. However, the viral levels appear to have been well tolerated by the host. No information is available on the relationship between herpesvirus load and the risk of periodontal disease. Kubar et al. (24) stated that quantifying viral loads is important to differentiate the clinically significant from the latent herpesvirus loadings. Quantitative PCR assay may be beneficial in this context as a means to monitor the disease prognosis. Although our cases were systemically stable throughout the follow-up period, it remains to be seen whether any changes in viral and/or bacterial load in the episodic attacks of the Kostmann syndrome occur.

As with many other neutrophil defects, antibiotic therapy was the main option for these patients. After long-term application of antibiotic prophylaxis, only one of the five periodontopathogens studied, *C. rectus*, was found, and in only one sample; this can be attributed to the antibiotic therapy. and although trimethoprim and sulfamethoxazole therapies do not have direct antiviral effects, it is reasonable to suggest that antibacterial, nonsurgical periodontal therapies and oral hygiene motivation of the patients may lead to a reduction in the level of periodontitis. Thus, the decrease of virus counts may be due to removal of periodontal pockets, which function as reservoirs for viruses (45).

A reduction of gingivitis and a decrease in the levels of herpesviruses have also been reported for periodontitis lesions of Down's syndrome, Papillon-Lefèvre syndrome and Trisomy 21 (18, 28). Based on the data from our study it seems reasonable to suggest that in patients with compromised immunity, herpesvirus(es) might be involved in periodontal pathologies in the absence of periodontopathic bacteria.

The results of the present study might help to explain why the clinical characteristics of periodontitis as a manifestation of Kostmann syndrome are so aggressive. Because the clinical parameters and the dramatically decreased viral load parameters were well adjusted, the present study might provide quantitative proof that EBV and possibly HCMV may play a role in Kostmann syndrome periodontitis.

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