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TT Virus Infection of Periodontal Tissues: A Controlled Clinical and Laboratory Pilot Study

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Background: A novel single-strand, circular DNA virus has been recently isolated and named TT virus (TTV). It has been demonstrated that peripheral blood cells harbor TTV DNA, suggesting that the virus might replicate in lymphoid cells and contribute to lymphocyte imbalances with consequent immunosuppressive effects. The purpose of this study was to investigate the prevalence of TTV DNA in healthy and periodontally compromised subjects, evaluating the presence of the virus in the gingiva and saliva, and comparing virological results with clinical data.

Methods: Twenty-one patients (seven males and 14 females, aged 25 to 76 years) were enrolled in the study. Eleven subjects were diagnosed with moderate periodontitis, while 10 were periodontally healthy. A sample of saliva was taken from each patient before recording the periodontal data; subsequently, a gingival biopsy was performed. A real-time polymerase chain reaction was used to quantify the presence of TTV DNA in saliva and gingival specimens.

Results: A statistically significant association was found between TTV in gingival tissue and the presence of periodontitis ($P = 0.0351$), while no association was observed between TTV in saliva and the presence of periodontitis ($P = 0.4762$).

Conclusions: A new DNA virus (TTV) was first identified in the gingival tissue and was found to be significantly associated with the presence of periodontitis. These findings need to be investigated in further studies. *J Periodontol* 2004;75:1216-1220.

KEY WORDS

Clinical trials; DNA, circular; gingiva/virology; periodontitis/etiology; saliva/virology; transfusion-transmitted virus.

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A novel DNA virus has been recently isolated from the serum of Japanese patients with post-transfusion non-A-E hepatitis and named TT virus (TTV) from the initials of one of the patients.¹ Its molecular features have suggested that TTV might belong to the family of *Circoviridae*, characterized by a circular molecule of negative, single-stranded DNA of about 3,800 nucleotides. The natural history of TTV infection is still poorly understood. Circulating viral DNA has been detected in the plasma of healthy individuals at rates ranging from 1% to 92%, as a possible result of uneven geographic distribution and/or, more probably, of differences in the sensitivity of the polymerase chain reaction (PCR) methods used for detection.² In fact, whenever wide-range PCR sensitive assays have been used, two-thirds of the global population carries TTV in plasma regardless of disease status and geographical location. TTV infection is already prevalent in the early months of life, although in some surveys the viremia rates tended to increase with age.^{3,4} However, the level of virus replication may vary among different individuals and this may represent an important marker of a possible pathogenetic role for TTV.

How TTV spreads is still unclear. That the virus can be transmitted by blood and blood products has been documented.⁴ However, the existence of other more effective modes of diffusion, such as oral-fecal, transplacental, and sexual routes of transmission,⁵⁻⁷ has been suggested. In a recent report,⁸ the authors found high TTV loads in the nasal secretions of infants suggesting that the virus may be airborne.

The biological cycle and the replicative mechanisms of TTV are still unknown. Also

the sites and cell types, as well as the definitive tissues and organs, where TTV undergoes primary amplification and/or persists and from which it sheds into the circulation are unknown. Nevertheless, the frequent identification of TTV in peripheral mononuclear blood cells (PMBC) may suggest that the virus replicates in lymphoid cells.⁹ If this observation is confirmed, TTV could represent an ideal candidate agent for unexplained immunosuppressive syndrome.

Based on these recent findings, the aim of this study was to investigate the prevalence of TTV in healthy and periodontally compromised subjects by evaluating the presence of the virus in the gingiva and saliva and comparing the laboratory results with the actual periodontal conditions.

MATERIALS AND METHODS

Study Population

The study population consisted of 21 subjects consecutively selected by a single clinician in a private practice. Subjects included seven males and 14 females, of Caucasian heritage, from middle economic levels and a mean age of 43.76 ± 12.36 years (range 25 to 76). Ten subjects were periodontally healthy while 11 subjects were diagnosed with periodontitis. Patients defined as affected by periodontitis (test group) were those who showed clinical attachment level loss ≥ 6 mm in two or more teeth and one or more sites with periodontal probing depth ≥ 5 mm, according to the definition of established periodontitis by Machtei and coworkers.¹⁰ Subjects had to satisfy the following criteria: 1) no previous active periodontal treatment in the last 5 years (only maintenance or preventive care); 2) non-smokers; and 3) no systemic antibiotic therapy in the last 6 months. The protocol of the study was approved by a human experimentation committee and the patients agreed to participate in the study and signed the appropriate consent forms.

Data Collection

Missing teeth, plaque index (PI), bleeding on probing (BOP), periodontal probing depth (PD), gingival recession (Rec), clinical attachment level (CAL), mobility, lesion of root furcation, full-mouth plaque score (FMPS), and full-mouth bleeding score (FMBS) were recorded.

Salivary Withdrawal

Saliva was withdrawn and placed into a sterile pipette (about 200 μ l) and kept in a freezer at -10° – -15° C.

Gingival Biopsy

A gingival biopsy about $4 \times 2 \times 2$ mm was taken from the area between the upper left second premolar and the first left molar of all of the participants. This area was chosen for esthetic reasons. The gingival tissue was then washed in a sterile solution, put into a sterile pipette, and kept in a freezer at -10° – -15° C.

Laboratory Procedures

Real-time PCR assay. TTV DNA extraction was carried out on 200 μ l of saliva or weighed tissue samples using a commercially available kit[†] according to the manufacturer's protocol. Extracted viral DNA was eluted in 50 μ l TE buffer and stored at -80° C until PCR amplification. Primers and probe were designed from a portion of the untranslated region (UTR), which was found to be highly conserved among all TTV sequences available in the National Institutes of Health, GenBank at the time of writing. The oligonucleotide sequences are as follows: AMTS (forward primer 5'-GTGCCGAGGTGAGTTTA-3', position 177-194), AMTAS (reverse primer 5'-AGCCCGGCCA GTCC-3', position 226-239), and AMTPTU (TaqMan probe 5'-TCAAGGGGCAATTCGGGCT-3', position 205-223). The probe was labeled by 6-carboxy-fluorescein (FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA) at its 5' and 3' ends, respectively. Reactions were carried out in a 25 μ l format as previously described.⁸ Real-time PCR was monitored by a sequence detection system[§] and proprietary sequence detector software.^{||} Each run contained several negative controls (no template) as well as the reference template (positive control) at 10^1 to 10^6 copies/10 μ l. Both controls and samples were assayed in triplicate and at least two independent DNA extractions for each sample were tested. Samples were considered positive whenever TTV DNA was detected in at least two of three replicates. All samples positive in only one replicate and/or with a coefficient of variation of 50% or greater were re-extracted and tested again in triplicate. Procedures for quantification of copy number and evaluation of intra- and inter-assay precision and reproducibility of the assay have been previously reported.^{11,12} The lower limit of sensitivity of the assay was 1.0×10^3 copies per ml of saliva or per gram of tissue.

Statistical Analysis

The descriptive statistic analysis was expressed as a mean \pm standard deviation. Two Fisher exact tests were performed to evaluate the association between the presence of periodontitis and the presence of TTV in gingiva and saliva. TTV DNA titre was considered quantitatively negative (absent) if $<1.00 \times 10^3$ copies/g/ml. In addition, two *t* test analyses were done to verify a possible relationship between the presence of periodontitis and the natural logarithm of TTV titre (TTV titre/1,000) in gingiva and saliva. The TTV titers were changed into natural logarithms (TTV titre/1,000) to satisfy the assumption of normality and homoscedasticity.

A simple linear regression was built up between natural log TTV titre/1,000 and age. Another simple linear regression analysis was also performed to investigate

[†] QIAgen, Chatsworth, CA.

[§] ABI Prism 7700, Applied Biosystems, Foster City, CA.

^{||} Version 1.6.3, Applied Biosystems.

Table 1.

Individual Patient Data

Patients	Periodontitis	TTV in Gingiva	TTV in Saliva	Gender	Age	FMPS	FMBS	Missing Teeth (N)	PD (mean)	CAL (mean)	CAL Interproximal†
1	No	2.1 × 10 ⁸	1.9 × 10 ⁷	M	43	15	14	5	1.97	1.97	2.0
2	No	<1.0 × 10 ³ *	2.2 × 10 ⁷	F	34	27	34	2	1.72	1.72	2.0
3	No	<1.0 × 10 ³ *	3.0 × 10 ⁷	M	41	21	29	2	1.91	1.91	2.5
4	No	3.9 × 10 ⁷	5.5 × 10 ⁶	F	31	9	22	4	1.80	1.80	2.0
5	Yes	1.3 × 10 ⁹	2.1 × 10 ⁷	F	56	62	49	15	2.30	3.09	4.5
6	No	<1.0 × 10 ³ *	<1.0 × 10 ³ *	M	46	36	41	4	2.07	2.17	2.5
7	Yes	1.9 × 10 ⁸	5.9 × 10 ⁷	F	54	61	41	7	2.66	3.19	6.0
8	No	<1.0 × 10 ³ *	8.0 × 10 ⁶	F	25	8	31	4	1.82	1.83	2.0
9	No	8.3 × 10 ⁷	1.6 × 10 ⁷	F	52	35	4	6	2.22	3.03	3.0
10	Yes	2.3 × 10 ⁹	1.3 × 10 ⁹	M	49	86	91	3	4.64	7.14	14.0
11	Yes	5.6 × 10 ⁶	5.4 × 10 ⁶	F	29	58	57	1	2.19	2.19	3.5
12	Yes	3.0 × 10 ⁹	1.8 × 10 ⁹	F	42	67	85	1	3.28	3.44	5.0
13	Yes	2.0 × 10 ⁷	7.8 × 10 ⁷	M	46	21	21	1	2.92	3.82	5.5
14	Yes	9.3 × 10 ⁷	7.1 × 10 ⁵	F	47	12	14	4	3.54	4.02	4.5
15	Yes	5.0 × 10 ⁷	1.4 × 10 ⁶	F	47	38	93	10	2.42	2.75	4.0
16	No	9.7 × 10 ⁷	1.4 × 10 ⁸	M	37	10	11	7	2.51	3.39	4.0
17	Yes	3.5 × 10 ⁷	3.3 × 10 ⁸	F	60	14	10	12	3.21	3.34	5.5
18	No	4.2 × 10 ⁷	9.6 × 10 ⁷	F	26	33	58	6	1.93	2.06	2.0
19	Yes	5.8 × 10 ⁷	5.5 × 10 ⁶	F	76	50	11	3	4.10	4.39	8.5
20	No	2.2 × 10 ⁸	1.9 × 10 ⁸	F	31	65	52	1	2.34	2.36	2.0
21	Yes	4.4 × 10 ⁸	3.5 × 10 ⁸	M	47	18	20	3	3.49	4.05	4.5

* TTV DNA titer considered quantitatively negative (<1.00 × 10³ copies/g/ml).

† Mean value of CAL calculated in correspondence to the distal site of the upper left second premolar and the mesial site of the first left molar.

the association between the mean value of clinical attachment level at the sites where a gingival biopsy for TTV analysis was performed (distal point of the upper left second premolar/mesial point first molar) and natural log TTV titre in the gingiva.

RESULTS

Of the 21 patients, 17 had positive results from the TTV DNA analysis performed on gingival tissue (>1.00 × 10³ copies/g), while 20 patients were positive in the TTV DNA analysis of the saliva samples (>1.00 × 10³ copies/ml). Only one subject (patient 6 in Table 1) was completely negative at the gingiva and saliva TTV DNA analysis (Table 1). Individual patient data are shown in

Table 2.

Descriptive Statistical Analysis

Patient	N	Gender (female)	Age	PD (mean ± SD in mm)
Healthy	10	6	36.60 ± 8.83	2.03 ± 0.26
Periodontitis	11	8	50.27 ± 11.73	3.16 ± 0.77
P value (t test)*		0.6594	0.0075	0.0003

* Fisher exact test.

Table 1. Descriptive statistical analyses and *t*-tests are reported in Table 2.

The Fisher exact test resulted in a statistically significant association between the presence of TTV in gingiva and periodontitis ($P = 0.0351$). However, a statistically significant association was not demonstrated between the presence of TTV in saliva and periodontitis ($P = 0.4762$). In addition, a statistically significant association was found between the natural logarithm of TTV titre (TTV titre/1,000) in gingiva and the presence of periodontitis ($P = 0.0153$), although no association was observed between the natural logarithm of TTV titre (TTV titre/1,000) in saliva and the presence of periodontitis ($P = 0.3252$).

Age was not correlated to the natural logarithm of TTV DNA in gingiva ($P = 0.1437$), nor was gender found to be significantly associated with the natural logarithm of TTV DNA in gingiva (*t* test; $P = 0.6364$). Moreover, clinical attachment levels at the sites where gingival biopsies for TTV analysis were performed were associated to the natural log TTV titre in gingival tissue ($P = 0.0413$).

DISCUSSION

Periodontitis is an infectious disease that involves specific microbiota and characteristic host responses determining periodontal tissue destruction. Severe conditions of periodontitis afflict about 5% to 20% of the population, even though moderate or mild forms of periodontitis are more widespread.¹³

Recently, Contreras et al.¹⁴⁻¹⁸ proposed a new etio-pathogenetic hypothesis of periodontal disease based on a strong association observed between periodontal disease and viral infection of periodontal tissue. The viral species identified by the PCR method are herpes simplex virus 6, 7, and 8 (HSV 6-7-8); cytomegalovirus (HCMV); and Epstein-Barr virus type 1 (EBV-1) belonging to the *Herpetoviridae* family. The authors described a cytopathic effect of HCMV and HSV infection, where the main target cells are monocytes and macrophages. This process leads to an upregulation of tumor necrosis factor- α and interleukin-1 gene expression. How-

ever, its pathogenic role still remains unclear. Other studies identified specific HSV-1 antigens in the sulcular epithelial cells of patients who were undergoing periodontal treatment.^{19,20}

The first aim of this study was to investigate the presence of TTV, a recently identified widespread DNA virus in humans, in the gingiva and in the saliva of 21 subjects, 10 periodontally healthy and 11 with periodontitis. Overall, the results have shown that most of the subjects examined (test or control group) harbored TTV in their mouth and that viral titres were often very high, thus suggesting that this area of the body may represent an important site of viral persistence and continual excretion. In addition, the recent finding of higher levels of TTV in the saliva suggests that this biological fluid might represent an efficient vehicle for virus transmission.⁷ Although a few studies²⁻⁴ have reported that TTV viremia tends to increase with age, the results of the present investigation showed a non-statistically significant association ($P = 0.1437$) between age and TTV titre in gingiva; nor was gender found to be associated with TTV titre ($P = 0.6364$). Similar results have been obtained in a study by Masia et al.,²¹ in which the prevalence of TTV DNA was not significantly different in males and females or in different age groups.

A second aim of this study was to test a possible association between the TTV titre and the presence of periodontitis. In fact, TTV levels in the gingival tissue were significantly higher in subjects with periodontitis than in those without disease ($P = 0.0153$), while no association was observed between the TTV titre in saliva and the presence of periodontitis ($P = 0.3252$). The significance of such a finding is still unclear, but it could suggest a possible role for TTV in the pathogenesis of periodontal disease.

Moreover, it was interesting to note that high TTV levels were statistically associated with high values of clinical attachment loss ($P = 0.0413$). A possible explanation for this can be ascribed to the ability of TTV to replicate in locally stimulated resident lymphoid cells.

Table 2. (continued)

Descriptive Statistical Analysis

CAL (mean \pm SD in mm)	CAL 2nd Premolar/ 1st Molar (mean \pm SD in mm)	FMPS (% \pm SD)	FMBS (% \pm SD)	Missing Teeth (SD)	Natural Log TTV in Gingiva (copies TTV DNA/g)	Natural Log TTV in Saliva (copies TTV DNA/ml)
2.22 \pm 0.56	2.40 \pm 0.66	25.90 (17.53)	29.60 (17.52)	4.10 (1.97)	6.86 \pm 5.93	9.29 \pm 3.47
3.77 \pm 1.29	5.95 \pm 2.98	44.27 (25.10)	44.73 (32.77)	5.45 (4.87)	11.91 \pm 2.04	10.65 \pm 2.69
0.0024	0.0016	0.0694	0.2095	0.4224	0.0153	0.3252

However, it is equally possible that increased replication of the virus may enhance gingival inflammation while not causing periodontitis. It is also plausible that the periodontal inflammatory process attracts *in situ* peripheral lymphocytes that contain TTV. Thus, the increased TTV titre in subjects with periodontitis could be due to the influx of circulating lymphocytes, rather than to active replication of the virus in locally present cells. On the other hand, saliva samples from subjects with periodontitis contained TTV levels similar to those from healthy individuals and, importantly, from patients harboring TTV in the saliva but not in the gingiva. These data suggest that at least part of the virus present in the mouth was produced locally. It is clear that these findings do not define an etiologic role for TTV in periodontitis, but they certainly pique interest in clarifying the significance of TTV in disease pathogenesis and whether this is mediated via immune system function.

In conclusion, a new DNA virus (TTV) was identified in the gingival tissue and in the saliva of 10 periodontally healthy and 11 periodontally affected subjects, and it was found to be significantly associated with the presence of periodontitis.

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