Hepatitis C virus (HCV) infection represents a major public health problem in the world today. The infection primarily causes liver disease; however, HCV infection has also been associated with extrahepatic abnormalities, including mixed cryoglobulinemia, malignant lymphoma, Sjögren’s syndrome, and oral lichen planus (2, 12, 18, 19, 34, 39). Lymphotropism of HCV has been observed, and several laboratories have detected the virus in blood mononuclear cells (BMC) (16, 22, 26, 28, 35, 38). Common risk factors for HCV infection include blood transfusion from unscreened donors as well as injection drug use. Although sexual and vertical transmissions have also been reported, there remain a large number of HCV carriers in whom no route of infection has been identified.

Epidemiological surveys demonstrate that body fluids other than blood, including saliva, might be potential sources of HCV infection. Experimental inoculation of saliva obtained from chronic HCV carrier chimpanzees has been reported to transmit hepatitis to recipient animals (1). Several studies have demonstrated HCV RNA in the saliva of hepatitis C patients by reverse transcription (RT)-nested PCR. However, the detection rates of viral RNA within saliva have varied widely, and some groups have failed to demonstrate HCV RNA in their GCF. Most patients (20 of 26; 77%) had higher HCV RNA levels in their GCF than in their saliva. Although there was not a statistically significant correlation between the serum viral load and HCV level in saliva or GCF, patients with low serum HCV loads were less likely to have detectable HCV in their saliva. These findings have important implications for medical personnel and suggest that epidemiological studies designed to understand the significance of the oral route of transmission of HCV are warranted.

The search for hepatitis C virus (HCV) in body fluids other than blood is important when assessing possible nonparenteral routes of viral transmission. However, the role of oral fluids in HCV transmission remains controversial. Here we quantitatively determined HCV RNA in saliva and gingival crevicular fluid (GCF) of anti-HCV-positive patients. Most patients (14 of 18; 78%) whose saliva specimens were negative had HCV RNA in their GCF. Most patients (20 of 26; 77%) had higher HCV RNA levels in their GCF than in their saliva. Thus, we examined the presence of HCV RNA in the saliva and GCF of anti-HCV antibody-positive patients using realtime quantitative RT-PCR.

**MATERIALS AND METHODS**

**Sample collection.** Twenty-six dental patients attending the hospital of Nippon Dental University at Tokyo were studied. All of the patients were anti-HCV antibody seropositive on the basis of screening using a second-generation enzyme immunoassay (Abbott HCV PHA, Abbott Diagnostics, Abbott Park, IL). This study protocol was approved by the Ethics Committee of the hospital and was conducted according to *Ethic Guideline for the Studies on Human Genome and Gene Analysis*. Written informed consent was obtained from each patient participating in the study.

Blood samples were collected and centrifuged for 20 min at 5,000 rpm to separate the serum. Patients spit into a cup to obtain saliva samples. Whole saliva samples (approximately 2 ml) were then transferred into sterile containers. None of the samples were macroscopically observed to contain blood. GCF specimens were collected by first drying the gingival surface with sterile cotton, after which the area was isolated in order to prevent contamination with saliva. A paper strip (2 by 5 mm) was then subgingivally inserted for 30 s to collect specimens (approximately 50 µl). If there was visible contamination of the sample with blood, another sample without macroscopic blood contamination was taken from another site. The depth at gingival crevices was then measured by a periodontal probe, and the presence of bleeding on probing was examined. Serum, saliva, and GCF samples were collected simultaneously and were stored at −80°C before use.

**RNA extraction.** Total RNA was extracted from 100 µl of serum or saliva specimens and from paper strips with collected GCF using a QiAamp viral RNA kit (QIAGEN, Valencia, CA). In preliminary experiments using various amounts of serum, saliva, and GCF samples in the presence or absence of paper strips, we confirmed that (i) sample volumes of >40 µl yielded the same efficiencies of RNA extraction from each specimen and (ii) inclusion of a paper strip described above in the lysis buffer did not influence the efficiency of RNA extraction.

**Quantitation of HCV RNA.** To determine the quantity of HCV RNA, real-time RT-PCR involving single-tube reactions was performed using TaqMan EZ RT-PCR Core reagents (PE Applied Biosystems, Foster City, CA), as previously described (3). Briefly, the reaction mixture contained 1× TaqMan EZ buffer, 500 nM concentrations of each primer from the HCV 5’ noncoding region (5’-GAG TGT CGT GCA GCC TTC A-3’ and 5’-CAC TCG CAA GCA CCC TAT CA-3’), a 200 nM concentration of fluorogenic probe (5’-(6-carboxyfluorescein)
TABLE 1. Clinical and virological characteristics of 26 patients examined in this studya

<table>
<thead>
<tr>
<th>Age</th>
<th>Gender</th>
<th>ALT level (IU/liter)</th>
<th>AST level (IU/liter)</th>
<th>HCV antibody titer (2°)</th>
<th>Genotype</th>
<th>Oral disease(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>68</td>
<td>F</td>
<td>30</td>
<td>48</td>
<td>&gt;12</td>
<td>1b</td>
<td>Periodontitis/BOP</td>
</tr>
<tr>
<td>64</td>
<td>M</td>
<td>115</td>
<td>103</td>
<td>&gt;12</td>
<td>1b</td>
<td>Periodontitis/BOP</td>
</tr>
<tr>
<td>71</td>
<td>F</td>
<td>14</td>
<td>23</td>
<td>12</td>
<td>2b</td>
<td>Periodontitis/BOP</td>
</tr>
<tr>
<td>71</td>
<td>M</td>
<td>124</td>
<td>71</td>
<td>12</td>
<td>1b</td>
<td>Periodontitis/BOP</td>
</tr>
<tr>
<td>71</td>
<td>M</td>
<td>47</td>
<td>55</td>
<td>12</td>
<td>1b</td>
<td>SCC</td>
</tr>
<tr>
<td>63</td>
<td>M</td>
<td>14</td>
<td>19</td>
<td>11</td>
<td>1b</td>
<td>OLP</td>
</tr>
<tr>
<td>66</td>
<td>F</td>
<td>59</td>
<td>67</td>
<td>11</td>
<td>1b</td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>F</td>
<td>61</td>
<td>35</td>
<td>11</td>
<td>1b</td>
<td></td>
</tr>
<tr>
<td>73</td>
<td>F</td>
<td>48</td>
<td>40</td>
<td>10</td>
<td>2a</td>
<td>Periodontitis/BOP</td>
</tr>
<tr>
<td>70</td>
<td>F</td>
<td>18</td>
<td>25</td>
<td>10</td>
<td>1b</td>
<td>Periodontitis/BOP</td>
</tr>
<tr>
<td>72</td>
<td>F</td>
<td>15</td>
<td>20</td>
<td>10</td>
<td>1b</td>
<td>Periodontitis/BOP</td>
</tr>
<tr>
<td>70</td>
<td>M</td>
<td>8</td>
<td>12</td>
<td>ND</td>
<td>Periodontitis</td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>M</td>
<td>12</td>
<td>30</td>
<td>8</td>
<td>1b</td>
<td>OLP</td>
</tr>
<tr>
<td>69</td>
<td>F</td>
<td>31</td>
<td>39</td>
<td>7</td>
<td>ND</td>
<td>SCC</td>
</tr>
<tr>
<td>73</td>
<td>M</td>
<td>16</td>
<td>24</td>
<td>6</td>
<td>ND</td>
<td>Periodontitis/BOP, SCC</td>
</tr>
<tr>
<td>72</td>
<td>F</td>
<td>20</td>
<td>22</td>
<td>6</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>M</td>
<td>12</td>
<td>18</td>
<td>ND</td>
<td>Periodontitis/BOP, SCC</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>F</td>
<td>5</td>
<td>17</td>
<td>ND</td>
<td>Periodontitis/BOP, SCC</td>
<td></td>
</tr>
<tr>
<td>69</td>
<td>M</td>
<td>13</td>
<td>20</td>
<td>ND</td>
<td>Periodontitis/BOP, SCC</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>M</td>
<td>22</td>
<td>23</td>
<td>ND</td>
<td>Periodontitis</td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>F</td>
<td>15</td>
<td>30</td>
<td>ND</td>
<td>SCC</td>
<td>Periodontitis/BOP, OLP</td>
</tr>
<tr>
<td>56</td>
<td>F</td>
<td>11</td>
<td>17</td>
<td>ND</td>
<td>Periodontitis/BOP, OLP</td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>F</td>
<td>12</td>
<td>21</td>
<td>ND</td>
<td>Periodontitis/BOP, OLP</td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>F</td>
<td>9</td>
<td>24</td>
<td>ND</td>
<td>Periodontitis/BOP, OLP</td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>M</td>
<td>26</td>
<td>25</td>
<td>ND</td>
<td>Periodontitis/BOP, OLP</td>
<td></td>
</tr>
<tr>
<td>79</td>
<td>F</td>
<td>22</td>
<td>21</td>
<td>ND</td>
<td>Periodontitis/BOP, OLP</td>
<td></td>
</tr>
</tbody>
</table>

a Abbreviations: F, female; M, male; ND, not detected; OLP, oral lichen planus; BOP, bleeding on probing; SCC, squamous cell carcinoma.

CCCA AGA CGT CTA GCC TAG TAG TGT TGG (6-carboxytetramethy- 
linodiamine)-3′, 200 μM concentrations of each deoxynucleoside triphosphate, 
3 mM Mn(OAc)2, 5 U of Thermus thermophilus DNA polymerase, 0.5 U of 
AmpErase uracil-N-glycosylase, and template RNA. The primers and probe 
were designed on the basis of the conserved sequences among HCV genotypes. The 
RT step was started with a 1-min incubation at 50°C, followed by 50 min at 56°C. 
Thermal cycling conditions were as follows: a preincubation period of 5 min at 
95°C followed by 50 cycles of denaturation at 94°C for 15 s and annealing at 55°C 
for 10 s and extension at 65°C for 1 min. All reactions and analyses of the 
amplification plots were performed on an Applied Biosystems PRISM 7700 
sequence detector (PE Applied Biosystems). Standard curves of the assays were 
obtained by plotting 10-fold serial dilutions of known concentrations of a syn-
thetic HCV genotype 1b transcript. HCV RNA copy numbers of the synthetic 
transcript were calculated from the quantity and its molecular weight. Using a 
standard curve, the Sequence Detector software calculated automatically the 
concentration of RNA copies in the experimental samples. We found that results 
obtained from our in-house real-time RT-PCR method were well correlated with 
those from the COBAS AMPLICOR HCV MONITOR Test, version 2.0 (Roche Diagnostics, 
Tokyo, Japan) (15), and that 1 HCV RNA copy/ml in our method corresponded 
to approximately 1 international unit/ml by the above-mentioned 
commercial assay (data not shown).

HCV genotyping. HCV genotype was determined by RT-PCR of the core 
region sequence with genotype-specific primers for determination of HCV ge-
notypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a, as described previously (24).

PCR amplification of β-globin DNA. Total DNA was extracted from saliva 
samples using a QIAamp DNA Mini kit (QIAGEN) according to the manufac-
turer’s instructions. To characterize the degree of cell contamination in saliva, 
isolated DNA was subsequently used as a template to amplify the human β-glo-
bin gene fragment of 268 bp with the following primers: 5′-GAA GAG CCA 
AGG ACA GGT AC-3′ and 5′-CAA CCT CAT CCA CGT TCA CC-3′ (21).

Statistical analysis. The spearman rank test was used for evaluating the 
correlation between variables: anti-HCV antibody levels and viral loads in serum, 
saliva, and GCF.

RESULTS

The clinical and virological characteristics of 26 patients are 
presented in Table 1. The study group consisted of 10 males 
(38%) and 16 females (62%) with a mean age of 69 years 
(range, 56 to 79 years). Their mean liver enzyme values were as 
follows: 30 IU/liter for alanine aminotransferase (ALT) and 33 
IU/liter for aspartic aminotransferase (AST). HCV RNA lev-
els were significantly correlated with anti-HCV antibody 
levels (r = 0.80, P < 0.0001) (Fig. 2A). In a number of 
cases (20 of 26; 77%), the viral load of the GCF was greater 
than that of the saliva. HCV RNA was detected in 31% of the 
saliva samples and 85% of the GCF specimens using real-time 
RT-PCR assay, which showed a detection limit of 102 
copies/ml and a linear range over 5 logs. Four of six serum 
samples whose HCV RNA levels were below the detection 
limit in this measurement were found to have detectable HCV 
RNA by the qualitative nested RT-PCR (4). We found no 
difference in efficiency and specificity of HCV cDNA amplifi-
cation among genotypes 1b, 2a, and 2b in the real-time RT-
PCR assay (data not shown).

Figure 1 summarizes viral loads in the serum, saliva, and 
GCF specimens of the patients. A mean serum HCV RNA 
level of 5.1 × 105 copies/ml was observed among samples with 
viral loads greater than 102 copies/ml. As expected, serum viral 
RNA levels were significantly correlated with anti-HCV antibody 
levels (r = 0.80, P < 0.0001) (Fig. 2A). In a number of 
cases (20 of 26; 77%), the viral load of the GCF was greater 
than that of the saliva. HCV RNA was detected in 31% of the 
saliva samples and 85% of the GCF specimens using real-time 
RT-PCR. Mean viral RNA levels were 1.9 × 103 (saliva) and 
3.1 × 104 (GCF) copies/ml in these samples. It should be noted that most (seven out of eight) of the saliva samples contained 
1.4 × 102 to 8.2 × 103 copies/ml of HCV RNA, with a mean 
value of 2.0 × 103 copies/ml among these seven samples (Fig. 1).

Among the 18 patients with HCV RNA-negative saliva, 102 to 
103 copies/ml of viral RNA were detected in the GCF of 3 
patients, 103 to 104 copies/ml of viral RNA were detected in 
the GCF of 2 patients, and >104 copies/ml were detected in
the GCF of 9 patients. No significant association was observed between viral RNA levels in the serum and viral RNA levels in the saliva (Fig. 2B) or GCF (Fig. 2C). However, relatively high serum viral loads (>10^5 copies/ml) were observed in five out of eight patients with HCV RNA-positive saliva, while serum viral loads were 1.5 x 10^3 copies/ml or less in most of the patients whose saliva specimens were negative (13 out of 18). Four patients with HCV RNA-positive saliva and/or GCF had no detectable serum HCV RNA by real-time RT-PCR (Fig. 2B and C); however, viral RNA was detectable in their sera by qualitative nested RT-PCR. Although no visible contamination of the saliva and GCF with blood was observed, there may be a small amount of cells or lysed cells in the fluids. To determine the degree of cell content in samples, total DNA was extracted from three saliva specimens, which contained >10^3 copies/ml of HCV RNA (Fig. 2B), and tested for the presence of cellular DNA by amplifying a human β-globin gene. A certain amount of cellular DNA was detectable in the saliva specimens (data not shown), suggesting some salivary HCV RNA may be derived from HCV-infected cells, such as BMC and mucosal epithelial cells, as discussed below. Various amounts of HCV-infected cells in the saliva and GCF may, in part, account for differences in the viral loads.

HCV RNA was detectable in most GCF and/or saliva specimens obtained from patients with clinical evidence of oral diseases: HCV RNA was detected in all 14 (100%) patients with periodontitis, 6 of 7 (85%) patients with squamous cell carcinoma, and 3 of 4 (75%) patients with lichen planus. Three out of four patients with HCV RNA-negative GCF, however, also had some oral epithelial lesions. On the other hand, among seven patients without oral diseases, HCV RNA was detected in the GCF and saliva of six and three patients, respectively. There was a trend toward increased viral loads in the oral fluids, especially GCF, among patients with bleeding on probing compared to those without the bleeding. The viral RNA loads in the GCF and saliva had no correlation with age, gender, or serum levels of ALT or AST. It also seems that their viral RNA levels were not correlated with HCV genotype, although the viral genotypes in 12 of 26 patients were not determined.

**DISCUSSION**

Identification of HCV in body fluids other than blood is important in order to evaluate possible nonparenteral routes of transmission. The role of oral fluids in HCV transmission remains controversial. Although the presence of HCV RNA in saliva has been reported by several research groups (6–11, 14, 17, 23, 25, 27, 29–33, 36–38), only one study has attempted to quantify HCV RNA in saliva, in which patients coinfected with HCV and human immunodeficiency virus were examined using a branched DNA assay (27). Moreover, limited information exists regarding the prevalence of HCV in the GCF of patients with hepatitis C, apart from one study in which a qualitative RT-PCR method was used to detect HCV in 59% of GCF and 35% of saliva specimens from patients with HCV viremia (20).

To the best of our knowledge, this study is the first to quantitate HCV loads within the saliva and GCF of anti-HCV antibody-positive patients using real-time RT-PCR. To search for a possible oral route of HCV transmission, whole saliva and GCF containing cell fractions were used to determine the viral loads.
ACKNOWLEDGMENTS

We thank Yasushi Inoue and Ryosuke Suzuki for technical advice and helpful discussion on data analysis. We also thank Makiko Yahata for technical assistance and Tomoko Mizoguchi for manuscript preparation.

This work was partly supported by grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan to T.S. and T.S.

REFERENCES


