Visualization of TT Virus Particles Recovered from the Sera and Feces of Infected Humans

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TT virus (TTV) has not yet been cultured or visualized. We attempted to recover and visualize TTV-associated particles from the serum samples and feces of infected humans. Serum samples were obtained from 7 human immunodeficiency virus (HIV)-infected patients. Three patients had a high TTV DNA titer (10⁸ copies/ml), three had a low TTV DNA titer (10² copies/ml), and one was negative for TTV DNA. Fecal supernatant was obtained from a different TTV-infected subject. The serum samples were fractionated by high-performance liquid chromatography, and TTV DNA-rich fractions were subjected to floatation ultracentrifugation in cesium chloride. Virus-like particles, 30–32 nm in diameter, were found in the 1.31–1.33 g/cm³ fractions from each of the three serum samples with high TTV DNA titer, but not in any fraction from the four serum samples that either were negative for TTV DNA or had low TTV DNA titer. The TTV particles formed aggregates of various sizes, and immunogold electron microscopy showed that they were bound to human immunoglobulin G. Similar virus-like particles with a diameter of 30–32 nm banding at 1.34–1.35 g/cm³ were visualized in fecal supernatant with TTV genotype 1a by immune electron microscopy using human plasma containing TTV genotype 1a-specific antibody.

Key Words: TT virus; immune electron microscopy; buoyant density; immune complex; immunoprecipitation.

TT virus (TTV) is a recently discovered, unenveloped human virus originally isolated from a patient with transfusion-related hepatitis of unknown etiology (1, 2). TTV has a single-stranded, circular DNA genome of negative polarity, and is comprised of approximately 3.8 kilobases (kb) (3–5). It has been provisionally classified into the Circoviridae family, but differs considerably in nucleotide sequence and genomic size from known animal circoviruses including porcine circovirus (PCV) and beak and feather disease virus (BFDV) of parrots, and chicken anemia virus (CAV) (3, 5, 6). These animal circoviruses have been cultured and the recovered particles are known to be unenveloped and are 17–25 nm in diameter (7, 8).

To date, the natural properties of TTV virions have not been fully defined and there has been no report on the morphology of the TTV particle. Constituent proteins of TTV virions, such as the nucleocapsid protein, remain unknown mainly because TTV has not yet been cultured or purified from infected materials. Currently, the diagnosis of TTV infection is based on the detection of TTV DNA by polymerase chain reaction (PCR). TTV has an extremely wide range of sequence divergence. Thus far, phylogenetic analyses of TTV isolates have identified at least 16 genotypes (1 to 16), which differ from each other by more than 30% of the DNA sequence (9); some of the genotypes are further divided into subgroups such as 1a and 1b as well as 2a and 2b (2). Antibodies against TTV particle of genotype 1a are detectable in serum or plasma samples by the combined technique of immunoprecipitation and PCR using TTV particles of genotype 1a recovered from the feces of infected humans as an antigen probe (10).

In individuals with chronic TTV infection, the TTV particles in the serum can be precipitated with goat anti-human immunoglobulin G (IgG), which indicates that they form immune complexes in the circulation (11). Cesium chloride (CsCl) density gradient ultracen-
trifugation has shown TTV in serum bands at 1.31–1.33 g/cm³ and TTV in feces at a peak density of 1.35 g/cm³ (12). The particle size of TTV has been estimated to be 30–50 nm by filtration studies (3). The amount of TTV DNA in the plasma of TTV-infected individuals is generally low and has been estimated to be in the range of 50 to 50,000 (geometric mean: 620) DNA copies/ml (13), which have prevented direct visualization of this virus. However, among TTV-infected individuals, the titer of TTV viremia in patients infected with human immunodeficiency virus type 1 (HIV) is significantly higher than that in non-HIV-infected individuals (14). In the present study, using serum samples from HIV-infected patients with high TTV DNA titer, spherical, virus-like particles of 30–32 nm in diameter were visualized at a buoyant density of 1.31–1.33 g/cm³. Furthermore, similar virus-like particles in fecal supernatant were also visualized by immune electron microscopy using γ-globulins purified from human plasma containing TTV genotype-specific antibody.

MATERIALS AND METHODS

Serum samples and fecal supernatant. Serum samples from three HIV-infected patients with TTV DNA titer of 10⁶ copies/ml and serum samples from four HIV-infected patients who either were negative for TTV DNA or had low TTV DNA titer (10³ copies/ml) (controls), were used in this study (Table 1). A 15% (wt/vol) fecal supernatant with TTV of DNA titer at 10⁶ copies/ml and of genotype 1a was added to 50 μl of ammonium sulfate to a final concentration of 50% (wt/vol) to precipitate the TTV particles. The precipitate and supernatant fractions by centrifugation was performed five times for each serum specimen, and the respective fractions were pooled. The indicated fractions were tested for TTV DNA by PCR as described below.

Precipitation of TTV particles in fecal supernatant by ammonium sulfate. To a volume of 440 ml of 15% fecal supernatant with TTV of genotype 1a was added ammonium sulfate to a final concentration of 30% (wt/vol). This was allowed to sit at room temperature for 2 h and then kept at 4°C overnight, followed by centrifugation at 11,816 × g at 4°C for 90 min to precipitate the TTV particles. The pellets were dissolved in 6.6 ml of saline with 0.1% (wt/vol) bovine serum albumin (BSA) and subjected to floatation centrifugation. TTV DNA was quantitated in both fractions.

Separation of TTV particles in serum specimens by size exclusion chromatography. The high-performance liquid chromatographic (HPLC) apparatus (SC8010 system) with TSKgel G4000SW (7.8 mm i.d. × 30 cm in length) and TSK guard column SW (7.8 mm i.d. × 4 cm in length) (TOSOH Co. Ltd., Tokyo, J apan) was equilibrated with phosphate buffered saline (PBS) containing 50 mM phosphate buffer (pH 7.0) and 0.15 M sodium chloride (NaCl) and 0.05% (wt/vol) sodium azide (NaNO₃). A 0.2-ml aliquot of serum from each HIV-infected patient with or without TTV DNA was applied to the column and eluted by PBS at a flow speed of 0.5 ml/min. This step was performed five times for each serum specimen, and the respective fractions were pooled. The indicated fractions were tested for TTV DNA by PCR as described below.

Separation of TTV particles in serum samples from four HIV-infected patients who either were negative for TTV DNA or had low TTV DNA titer (10³ copies/ml) (controls), were used in this study (Table 1). A 15% (wt/vol) fecal supernatant with TTV of DNA titer at 10⁶ copies/ml and of genotype 1a was added to 50 μl of ammonium sulfate to a final concentration of 50% (wt/vol) to precipitate the TTV particles. The precipitate and supernatant fractions by centrifugation was performed five times for each serum specimen, and the respective fractions were pooled. The indicated fractions were tested for TTV DNA by PCR as described below.

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Centrifugation in CsCl density gradient. Floatation centrifugation was performed by the method described previously (12) with slight modifications. In brief, a 6.6-ml volume of pooled TTV DNA-rich HPLC fractions or the corresponding fractions from TTV DNA-negative serum samples, or concentrated fecal specimen, was adjusted to 1.30 g/cm³ with solid CsCl, and this was layered onto a 1-ml cushion of 54% (wt/wt) CsCl in TEN buffer containing 0.01 M Tris-HCl (pH 7.5), 0.001 M EDTA and 0.1 M NaCl. They were then

**TABLE 1**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (years)/sex</th>
<th>AIDS</th>
<th>TTV DNA</th>
<th>HIV RNA</th>
<th>GBV-C/HGV RNA</th>
<th>TLMV DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49/male</td>
<td>No</td>
<td>10⁸</td>
<td>203</td>
<td>10⁷</td>
<td>10⁶</td>
</tr>
<tr>
<td>2</td>
<td>27/male</td>
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<td>10⁸</td>
<td>25,396</td>
<td>—</td>
<td>10⁷</td>
</tr>
<tr>
<td>3</td>
<td>39/male</td>
<td>Yes</td>
<td>10⁸</td>
<td>72,805</td>
<td>—</td>
<td>10⁷</td>
</tr>
<tr>
<td>4</td>
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<td>10²</td>
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<td>—</td>
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</tr>
<tr>
<td>5</td>
<td>45/male</td>
<td>Yes</td>
<td>—</td>
<td>13,785</td>
<td>—</td>
<td>10⁷</td>
</tr>
<tr>
<td>6</td>
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<td>10²</td>
<td>1,609</td>
<td>—</td>
<td>10⁷</td>
</tr>
<tr>
<td>7</td>
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<td>No</td>
<td>10²</td>
<td>2,250</td>
<td>—</td>
<td>10⁷</td>
</tr>
</tbody>
</table>

Note. All serum samples from the seven patients were negative for HBsAg, HBV DNA, anti-HCV and HCV RNA. AIDS: acquired immunodeficiency syndrome.
overlaid with 1.5 ml of TEN buffer. After centrifugation in a Beckman SW40 ultracentrifuge tube at 154,624 g at 10°C for 48 h, fractions (300 μl each) were collected from the surface of the tube, and the density of each fraction was measured by refractometry. Each fraction was tested for viral nucleic acids by the PCR procedures described below. The TTV DNA-rich fractions or the corresponding fractions from TTV DNA-negative serum samples were diluted with TEN buffer and spun down in a Beckman SW65 rotor at 262,483 g at 10°C for 2 h. The resulting pellets were suspended in 10 μl of saline containing 0.1% BSA.

Fifty μl of fecal supernatant was incubated with 250 μl of plasma SA1092 or plasma SA1010 diluted to 1:20 at 37°C for 1 h and then at 4°C for 8 h. Equilibrium density gradient ultracentrifugation was carried out in an SW65 rotor by the previously described method (12). Fractions (75 μl each) were recovered from the surface. The density of each fraction was determined; each fraction was also tested for the presence of TTV DNA.

Detection of TTV DNA. Nucleic acids were extracted from 5–50 μl of serum, fecal supernatant or fraction using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) and dissolved in 50 μl of ddH2O. A 10-μl aliquot of each, equivalent to 1- to 10-μl of the sample, was tested for TTV DNA by nested PCR using primers derived from the untranslated region (UTR) of the TTV genome and Perkin-Elmer AmpliTaq Gold (Roche Molecular Systems, Inc., Branchburg, NJ) (16).

Quantitation of TTV DNA was performed by real-time detection PCR, using 2-10 μl of the DNA solution as a template, primers NG133 and NG352 (16), a dual fluorophore-labeled probe [5′-(Fam)-AGT CAA GGG GCA ATT CGG GCT CGG GA-(Tamra)-3′, and a 35 cycles with primers derived from the well-conserved untranslated region (21). The first-round PCR (95°C for 10 s, with an additional 9 min in the first cycle; 55°C for 30 s; 72°C for 30 s, 30 cycles with primers NG352 (5′-GTA CAC TKM CCA ATG GCT GAG-3′ [K = G or T, M = A or C]) and NG381 (antisense 5′-AAG TTT CTT GCC CTT GCT ACG ACT-3′ [Y = T or C]), and the second-round PCR was performed under the same conditions for 25 cycles with primers NG381 (sense 5′-CAC TKM CCA ATG GCT GAG TTT-3′) and NG381 (5′-GTT TCT TGC CCG TCG YAG YAG YAG ACT-3′ [Y = T or C]). The amplification product from the first-round PCR measured 157 bp and that from the second-round PCR 152 bp. For semi-quantitating TTV DNA, serial ten-fold dilutions of nucleic acids extracted from the test serum or fecal supernatant were assayed, and the highest dilution testing positive was determined. The value was converted to relative titer (10^n) per 1 ml of serum or fecal supernatant.

### RESULTS

**Association of TTV Virions with Human IgG in the Circulation**

To characterize the association of TTV particles with human IgG in HIV-infected patients, the TTV virions in the serum samples of cases 1-3 who had high TTV viral load were precipitated with goat anti-human IgG, and TTV DNA was quantitated in the supernatant and precipitate fractions. Approximately 80–95% of the PCR signals were recovered from the precipitate fraction (Table 2), suggesting that most of the TTV virions circulate as immune complexes in these HIV-infected patients. In contrast, TTV recovered from the feces were detected predominantly in the supernatant fraction (Table 2), suggesting that the TTV particles shed into feces exist as free virions not complexed with IgG, IgA or IgM.

### Shift of Buoyant Density of TTV Particles in Feces after Incubation with Human Plasma Containing Antibody to TTV

TTV particles of genotype 1a recovered from the feces banded at a density of 1.34–1.35 g/cm³ in the gradient medium of CsCl. After incubation with human plasma (SA1092) containing antibodies against TTV particles of the same genotype, the density of TTV particles was shifted to lower buoyant density, consistent with the formation of antibody complexes. This result indicates that TTV particles shed into feces exist primarily as free virions, which are not complexed with antibodies.
shifted to 1.33 g/cm³, similar to that of TTV in the circulation (Fig. 1). Incubation with the negative control plasma (SA1010) did not affect the density of TTV from the feces, staying at 1.34–1.35 g/cm³. In agreement with these results, TTV particles in the fecal supernatant precipitated upon incubation with a dilution of 1:20, 1:200 or 1:2,000 of plasma SA1092 followed by goat anti-human IgG, but not upon incubation with a dilution of 1:20,000 of plasma SA1092 or any concentration of plasma SA1010.

Visualization of Virus-like Particles by Electron Microscopy in Serum Samples from Patients with High TTV DNA Titer

Spherical, virus-like particles with a diameter of 30–32 nm were identified by electron microscopy in the TTV DNA-rich fractions of 1.31–1.33 g/cm³ of the serum samples from each of the three HIV-infected patients with a TTV DNA titer of 10⁸ copies/ml (Figs. 2A–2C). The particles were aggregated to various extents, and were found to be bound to IgG by immunogold electron microscopy using gold-labeled goat anti-human IgG, but not upon incubation with a dilution of 1:20,000 of plasma SA1092 or any concentration of plasma SA1010.

DISCUSSION

The viremia level of TTV in infected individuals is generally low, which may have hampered large-scale purification and direct visualization of TTV particles. In the present study, we recovered TTV particles from serum samples of HIV-infected patients with TTV viral load of 10⁸ copies/ml, which is at least 10³-fold higher than the highest titer among the TTV-infected individuals without HIV infection. Patients infected with HIV constitute a relatively unique group with regard to immunosuppression and are at increased risk for infection with various bloodborne viruses that share the same route of transmission (22). HIV-infected patients with concurrent HCV have an elevated titer of HCV (23). Therefore, the serum samples of the HIV-infected patients in the present study or their fractions in CsCl density gradient ultracentrifugation were tested for the presence of known transfusion-transmitted viruses including HBV, HCV, GBV-C/HGV.
and TLMV by the respective PCR methods. All seven serum samples were negative for HBV DNA and HCV RNA, but only case 1 was positive for GBV-C/HGV RNA. Although all seven serum specimens were positive for HIV RNA and its viral load was in the range of $2.0 \times 10^2$ to $7.3 \times 10^5$ copies/ml, TTV-associated particles in the serum samples appeared to be morphologically distinguishable from HIV virions, since HIV is enveloped, is 80–100 nm in diameter, and has a buoyant density of 1.16–1.18 g/cm$^3$ in sucrose (22). Such HIV-like particles were not seen in any of the TTV DNA-rich fractions. The buoyant density of TLMV has been estimated to be similar to that of TTV (20). However, the size of TLMV virions estimated by filtration studies has been reported to be less than 30 nm in diameter, which is smaller than that of TTV virions (20). Furthermore, the viral load of TLMV was $10^2$- to $10^4$-fold lower than that of TTV in each serum sample used, and the TLMV DNA titer in CsCl fractions of 1.31–1.33 g/cm$^3$ was lower than the TTV titer, suggesting that the virus-like particles observed in the TTV DNA-rich fractions of 1.31–1.33 g/cm$^3$ are associated with TTV, and not with TLMV.

Although hitherto undetected viruses may be lurking in the tested blood of the immunosuppressed HIV-infected patients, the observed virus-like particles seem to be associated with TTV based on the following results obtained in the present study. First, the size of the TTV-associated particles was determined to be approximately 30–32 nm in diameter, which is similar to the previous estimation by filtration studies utilizing human plasma (3), although the estimated size of TTV particles is larger.
than that of known animal circoviruses including PCV, BFDV and CAV (which are 17–25 nm in diameter) (7, 8). This may reflect the remarkable differences in genomic length between TTV and the known animal circoviruses. Second, consistent with the observation that most TTV particles in the circulation were precipitated with goat anti-human IgG, naturally occurring clusters or aggregations of virus-like particles were recovered from the serum samples of patients infected with high-titer TTV. The involvement of human IgG in the aggregates was confirmed by immunogold electron microscopy using gold-labeled goat anti-human IgG, gold-labeled goat anti-mouse IgG, serving as a control, did not bind with the aggregations of virus-like particles. This finding illustrates the difficulty in identifying individual TTV particles in serum. In contrast, most of the TTV virions in fecal supernatant were not precipitable by goat anti-human IgG, IgA or IgM, suggesting that fecal TTV exists as free virions. Therefore, we performed immune electron microscopy on fecal supernatant with high TTV DNA titer by a specific antigen-antibody reaction. Third, TTV-associated particles were observed in the TTV DNA-rich fraction of 1.31–1.33 g/cm\(^3\) recovered from each of the three HIV-infected patients with high TTV viral load (10\(^8\) copies/ml), but not in that recovered from any of the four patients who either were negative for TTV DNA or had low-titer TTV DNA of 10\(^2\) copies/ml.

Fourth, similar virus-like particles with a diameter of 30–32 nm were recovered from 440 ml of fecal supernatant with TTV DNA of 10\(^6\) copies/ml obtained from an infant who was not infected with HIV and who was immunologically competent, and visualized by immune electron microscopy. For immune electron microscopy, \(\gamma\)-globulins that had been purified from the SA1092 plasma containing antibodies to TTV particles of genotype 1a with a titer of 1:1000, were added to purified fecal TTV particles, and aggregates with halos were observed. In contrast, when \(\gamma\)-globulins that had been purified from the SA1010 plasma which did not contain antibodies to TTV were used as a control, no aggregates were seen, supporting the specificity of the formation of TTV-associated aggregates. The specificity of this antigen-antibody reaction is further supported by the evidence that the buoyant density of TTV virions in fecal supernatant shifted from 1.34–1.35 g/cm\(^3\) to 1.33 g/cm\(^3\) upon incubation with human plasma containing antibodies to TTV genotype 1a (SA1092).

The fecal supernatant was positive for TLMV DNA, but the viral load was 10\(^7\) copies/ml, being 10\(^2\)-fold lower than that of TTV DNA. Therefore, probable TTV particles of 30–32 nm in diameter observed in the fecal supernatant are considered to be different from TLMV with an estimated size of <30 nm in diameter (20). Although it is true that numerous, different, small, round, virus-like particles are present in human fecal materials (24), the virus-like particles recovered from the fecal supernatant of an apparently healthy infant negative for the genomic RNAs of hepatitis A virus and hepatitis E virus (15) and visualized by the above-mentioned immune electron microscopy, seem to be associated with TTV.

In the current study, we were able to visualize probable TTV particles in serum samples and fecal supernatant obtained from TTV-infected individuals, although large-scale preparation of TTV particles was not feasible due to limited volume and viral load of infected materials. An appropriate culture system capable of supporting productive infection of TTV, which has been developed for known animal circoviruses (25, 26), should be established for extended virological studies of this unique, circular, single-stranded DNA virus with a remarkably high degree of genetic heterogeneity (9) and with possible hepatitis-inducing capacity (1, 2, 15).
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REFERENCES


