Fecal Excretion of a Novel Human Circovirus, TT Virus, in Healthy Children

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The role of TT virus (TTV) as a human pathogen is unclear, as is the mode of TTV transmission. To determine the prevalence of TTV infection and the possible fecal-oral route of transmission, we analyzed fecal specimens from 67 healthy, nontransfused children for TTV DNA sequences by heminested PCR, using the NG and T primer sets. The overall prevalence of TTV fecal excretion was 22.4% (15 of 67), with the T primer set (19.4%) being more sensitive than the NG primer set (10.4%). TTV prevalence based on gender or ethnicity showed no significant differences. None of seven children in the 0- to 6-month age group had detectable TTV in feces. Of three sets of siblings, two unrelated sets of twins, ages 33 and 37 months, were negative for fecal TTV DNA, while the third set of siblings, ages 99 and 35 months, was positive. The absence of TTV in the feces of children younger than 6 months and the high prevalence (40%) in children 7 to 12 months of age is consistent with age-specific acquisition of TTV infection by the nonparenteral route. TTV genotypes 1, 3, 4, and 5 were represented in our study population. TTV-positive siblings had TTV genotypes 1 and 4, suggesting unrelated environmental sources of TTV infection. This observation suggests a possible time frame for TTV acquisition in children which coincides with increased interaction with their environment and increased susceptibility to infectious agents.

Recent molecular studies have demonstrated that TT virus (TTV), the first known human circovirus, is a single-stranded, nonenveloped DNA virus with an approximately 3.8-kb circular viral genome (12, 13). TTV has been suggested as an etiologic agent of non-A to -E hepatitis, but its role in liver disease is still unclear (14, 25). TTV is widespread in the general population, with a reported prevalence of 1.9% in Scotland (22), 10% in the United States (2), 12% in Japan (17), 74% in Papua New Guinea, and 83% in Gambia (18).

We and others have previously demonstrated a lack of association between parenteral and sexual routes of TTV transmission (8, 10, 15). Recent detection of TTV DNA in non-blood products, such as saliva, breast milk, and feces, suggests a nonparenteral route of transmission (11, 16, 19, 21). Transmission by close physical contact would support the high prevalence of TTV infection observed in the general population. Moreover, the relatively common occurrence of TTV among children, ranging from 5.1% in Japan (3) to 54% in the Democratic Republic of Congo (1), suggests its early acquisition in children which coincides with increased interaction with their environment and increased susceptibility to infectious agents.

MATERIALS AND METHODS

In this study approved by the committees on human subjects of the Kapiolani Medical Center and the University at Manoa, 67 children (33 males and 34 females; age range, 1 to 133 months; median age, 21 months) were recruited from preschools and day-care centers within the local community.

Information regarding age, gender, ethnicity, and transfusion history were provided by parents. Fecal specimens, collected in diapers or urine cups, were kept on ice and processed within 24 h after collection. In rare situations, specimens were stored at room temperature and processed within 6 h after collection. Fecal specimens were initially suspended in phosphate-buffered saline (15%, v/v), using a glass rod to break up the solid particles, and were vortexed and centrifuged at 4,300 × g at 4°C for 10 min in an IEC Centra GFP centrifuge (Needham Heights, Mass.). Supernatants were then transferred to clean tubes and centrifuged for an additional 10 min at 4,300 × g at 4°C. Clarified supernatants were aliquoted and stored at −80°C until further use. DNA was extracted from 200 μl of the above supernatant using the QIAamp blood kit (Qiagen, Chatsworth, Calif.), following the manufacturer’s instructions, and was suspended in 200 μl of nucleic acid-free water.

To minimize PCR carryover, DNA extraction, use of the pre-PCR master mix, PCR cycling, and size fractionation of the amplicon on agarose gel were done in physically separated rooms. DNA extraction was conducted in biological safety cabinets, and outer and nested PCR were conducted in PCR cabinets equipped with UV light. All protocols were conducted using pipettes fitted with aerosol-resistant tips.

Two sets of primers were used for TTV DNA detection. The first set consisted of the previously published heminested primers: NG059, NG061, and NG063 primers (17). First-round PCR primers were used at a concentration of 0.1 μM in a 25-μl reaction mixture consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.8 mM MgCl₂, 0.2 μM concentrations of each deoxynucleoside triphosphate (dNTP), 1.25 U of Thermus aquaticus DNA polymerase (Perkin-Elmer), and 5 μl of DNA (15). Using a 9700 DNA thermal cycler, the outer PCR mixtures were initially denatured at 94°C for 60 s and then cycled 10 times at 94°C for 15 s, 45°C for 30 s, and 72°C for 90 s, with an extension of 7 min at 72°C before being stored at 4°C. Subsequently, hot-start, heminested PCR was performed with the second-round PCR primers in a 20-μl reaction mixture. The difference between the outer and heminested PCR mixtures was the use of a higher concentration of inner primers (0.2 μM each). Each mixture was overlaid with a bead of PCRGem 50 Ampliwax (Perkin-Elmer), heated to 80°C for 5 min, and cooled at room temperature. A 30-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.25 U of Thermus aquaticus DNA polymerase (Perkin-Elmer), and 5 μl of the outer product was added on top of the solidified wax. The heminested PCR mixture was denatured at 94°C for 60 s and then cycled 45 times at 94°C for 15 s, 55°C for 30 s, and 72°C for 90 s, with an extension of 7 min at 72°C before being stored at 4°C. Enzymatically amplified DNA was size fractionated by electrophoresis on 2% agarose gels to ascertain the presence of the 271-bp product.

Since the titer of TTV in feces was presumed to be much lower than that in serum, a second independent set of primers, T801 and T935, reported to have
TABLE 1. Age-specific prevalence of TTV in feces of 67 healthy, nontransfused children

| Age (mo) | No. of specimens | No. (%) positive using primer: |
|----------|------------------|-----------------------------|
|          |                  | NG  | T  | NG or T |
| 0–6      | 7                | 0   | 0  | 0        |
| 7–12     | 15               | 1 (6.6) | 5 (33.3) | 6 (40.0) |
| 13–18    | 6                | 0   | 2 (33.3) | 2 (33.3) |
| 19–24    | 13               | 2 (15.4) | 1 (7.7)  | 2 (15.4) |
| 25–30    | 6                | 1 (16.7) | 2 (33.3) | 2 (33.3) |
| 31–36    | 10               | 2 (20.0) | 2 (20.0) | 2 (20.0) |
| ≥37      | 1                | 1 (10.0) | 1 (10.0) | 1 (10.0) |

higher sensitivity (16, 19, 26) was used to amplify all fecal specimens to confirm the results obtained with the NG primers. PCR primers were used at a concentration of 0.2 μM in a 50-μl reaction mixture consisting of 5-μl of 10× buffer (TaKaRa Shuzo Co., Otsu, Japan), 0.2 μM concentrations of each dNTP, 1.25 U of Takara Ex Taq, and 5 μl of DNA. Using a 9700 DNA thermal cycler, the outer PCR mixtures were initially denatured at 94°C for 60 s and then cycled 45 times at 92°C for 40 s, 60°C for 40 s, and 75°C for 90 s, followed by an extension of 5 min at 75°C before being stored at 4°C (4). PCR amplicons were then size fractionated by electrophoresis on 2% agarose gels to ascertain the presence of the 190-bp product.

Amplicons were purified using the QiAquick PCR purification kit (Qiagen) and were sequenced directly using the dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, Calif.) with inner PCR primers NG061 and NG063 on an automated sequencer (model 373A; Applied Biosystems). Additionally, to confirm two different TTV genotypes among the TTV-positive siblings, DNA was amplified and then cloned using the TA cloning kit (Invitrogen Corp./Novex, Carlsbad, Calif.), and three clones from each sibling were sequenced.

Sequence analysis was performed using programs available on the Vax computer system as part of the Genetics Computer Group (Madison, Wis.) and the Lasergene software (DNASTAR Inc., Madison, Wis.). TTV nucleotide sequences were aligned and compared with previously published and described sequences deposited in GenBank. Phylogenetic trees were created by the Clustal W method using the neighbor-joining program in SeqPup with 1,000 bootstraps. The phylogenetic positions of TTV strains from feces formed a tight cluster within their respective genotypes (genotypes 1 and 4), with 100% nucleotide sequence similarity among clones. Additionally, the three TTV clones from each sibling confirmed the presence of two different TTV genotypes (genotypes 1 and 4), with 100% nucleotide sequence similarity among clones. Additionally, the cloned sequences were 100% similar to the sequences derived from the TTV-positive siblings 99 and 35 months old are italicized. The phylogenetic positions of TTV genotypes 1 through 7 are depicted on the phylogenetic tree generated using the SeqPup program segregated per site. The scale represents nucleotide substitutions per position.

RESULTS

All study participants were healthy with no history of blood transfusion. Of the 67 children, 13 were Caucasians, 26 were Asians or Pacific Islanders, and 26 were identified as others, which included children of mixed ethnicity. The median age of the study participants was 21 months (range, 1 to 133 months); 22 were 12 months of age or younger, of which 7 were in the 0- to 6-month age group. For 53 (79.1%) children, fecal specimens were collected from diapers, and for the remaining 14 (20.9%), feces were collected in sterile urine cups. Fecal specimens were processed within 12 to 24 h of defecation.

Based on use of the NG primers, TTV prevalence in feces was 10.4% (7 of 67). The more sensitive T801 and T935 primers detected TTV in 19.4% (13 of 67) of fecal samples. Of the seven TTV-positive specimens identified by the NG primers, five were positive with the T primers. An additional eight fecal samples were found to be positive for TTV using the T primers (Table 1). Overall, the TTV prevalence was 22.4% (15 of 67).

Six-month age-specific prevalence of TTV in feces ranged from 0 to 40.0% (Table 1). None of seven children in the 0- to 6-month age group had detectable TTV in feces. Of the three sets of siblings, two unrelated sets of twins, ages 33 and 37 months, were negative for fecal TTV DNA, while the third set of siblings, ages 99 and 35 months, was positive, as determined by both NG and T primers.

The 204-nucleotide sequences spanning the TTV open reading frame 1 (ORF1) from seven TTV strains from feces were aligned and compared with published TTV sequences representing genotypes 1 to 7 (5, 13, 27, 28). Phylogenetic analyses revealed that four of seven TTV strains from feces of healthy children belonged to genotype 1, and of the remaining three, one each belonged to TTV genotypes 3, 4, and 5 (Fig. 1). The intragenotype median divergence, calculated as the number of substitutions per 100 nucleotides, among the four TTV genotype 1 strains was 14.0 (range, 2.0 to 15.8). These data were similar to the median TTV intragenotype 1 divergence of 12.2 nucleotides (range, 3.0 to 18.3; n = 6) calculated using published TTV genotype 1 sequences (Fig. 1). The seven TTV strains from feces formed a tight cluster within their respective group, as depicted by the neighbor-joining tree. The phylogenetic tree generated using the SeqPup program segregated genotype 1 and 4 from the remaining genotypes with a bootstrap value of 75 (Fig. 1).

The nucleotide sequences and phylogenetic analyses of three TTV clones from each sibling confirmed the presence of two different TTV genotypes (genotypes 1 and 4), with 100% nucleotide sequence similarity among clones. Additionally, the cloned sequences were 100% similar to the sequences derived...
using the direct-sequencing technique. All TTV-positive children were Asians or of Asian descent with a known European Caucasian admixture (for more details refer to TTV sequences deposited in GenBank).

**DISCUSSION**

TTV, the first known human circovirus, initially identified in the blood of a patient with cryptogenic posttransfusion hepatitis, appears to be a ubiquitous virus, with prevalence rates ranging from 1 to 92% in different populations worldwide (9, 26). However, the role of TTV as a human pathogen is unclear, as is the mode of TTV transmission. Although the parenteral route of TTV transmission has been suggested, recent studies have demonstrated the presence of TTV in feces, saliva, and breast milk, implicating a nonparenteral route of transmission (11, 16, 19, 21).

The significance of TTV infection in children has been relatively overlooked. Prevalence data on childhood TTV infection has been reported to range from 5.1% in Japan (3) to 17% in Brazil (20), 25% in Taiwan (7), and 54% in the Democratic Republic of Congo (1). Although previous studies suggest TTV transmission through transfusion of contaminated blood and blood products, these data do not support the high prevalence of TTV infection observed in apparently healthy children with no history of such exposure (22). Furthermore, the endemicity of TTV in specific geographic locations, such as Gambia and Papua New Guinea (18), favors a nonparenteral route of transmission for this ubiquitous virus.

The fecal-oral route of TTV transmission has been suggested by several investigators (16, 20, 25). However, no data have been reported on TTV in the feces of healthy children. Previously, Okamoto and colleagues demonstrated the presence of TTV DNA in paired serum and feces from three of five adult patients with hepatocellular carcinoma (16). In comparison to the serum hepatitis C virus titer, the TTV titer in serum was logarithmically lower, and a correlation was found between low TTV titer in serum and failure to detect TTV in feces (16). More recently, Ross and colleagues examined secretions and excretions from eight TTV-seropositive individuals and detected TTV DNA in four sera, four fecal samples, and no urine samples (19). The finding that the TTV titer in bile secretions from patients with cholestasis is 10 to 100 times higher than in serum may further explain its shedding in feces and support its fecal-oral route of infection (29). It is important to note that although our detection of TTV DNA in the feces of healthy children suggests fecal-oral transmission, this specific route of infection may merely be one of the many potential nonparenteral routes for TTV transmission.

Previous studies on serum or plasma have demonstrated an age-associated acquisition of TTV in children (7, 24). In 90 children under the age of 15 years from Taiwan, Hsieh and colleagues demonstrated a TTV prevalence of 17% (4 of 23) among children younger than 1 year of age but no evidence of infection (0 of 30) among newborn infants (7). Using T primers, Yokozaki and colleagues found TTV DNA in the blood of 94.1% (32 of 34) children under 6 years of age but failed to detect TTV in cord blood (n = 48) and in serum from 2- to 3-day-old infants (n = 48) (30). Similarly, in a longitudinal study of 68 children from the Democratic Republic of Congo, TTV viremia was found at 12 months of age among 37 children (54%) who had been TTV negative at 3 months (1). With nine longitudinally monitored children from Japan, Sugiyama and colleagues demonstrated TTV positivity between the ages of 6 and 14 months after birth (24). In a study conducted among 101 pregnant women in Italy, TTV infection was found in 8.9% (9 cases), but none of the neonates born to TTV-infected women had evidence of TTV infection. In contrast, Schroter and colleagues demonstrated a 47.8, 95.4, and 73.9% prevalence of TTV DNA in the sera of pregnant women and their newborn infants and in their breast milk, respectively (21). Our data on the failure to detect TTV among children under 6 months of age and the high prevalence (40%) in children 7 to 12 months of age (P = 0.1) is consistent with published data on age-specific TTV infection in children, which coincides with increased interaction with their environment and increased susceptibility to infectious agents.

In this study, TTV genotypes 1, 3, 4, and 5 were found in the feces of healthy children, with genotype 1 occurring most frequently (57.1%). A higher prevalence of TTV genotype 1 has been reported worldwide (3, 7, 13, 15, 22, 28). That said, data are available on the predominance of TTV genotype 2 among certain populations (1, 6, 14). Two different genotypes of TTV among TTV-positive siblings suggest independent sources of virus infection within the same family. Sugiyama and coworkers have similarly demonstrated two different genotypes of TTV in a mother and child within the same family (24).

Commercial serodiagnostic assays for estimating the prevalence of TTV infection are unavailable. Currently available data on TTV prevalence are based on PCR amplification of TTV gene sequences from a variety of bodily fluids, such as blood, breast milk, saliva, and feces (1, 2, 11, 19, 23). The sensitivity of detecting TTV DNA by PCR is variable and is dependent on the primer pair (25, 30). The first described and later modified primer pair, NG, is the most widely employed primer pair for amplification of TTV (17). Using these primers, Prescott and Simmonds demonstrated TTV prevalence as high as 83% in blood samples (18). A TTV prevalence of 92% has been demonstrated in blood samples by several investigators using T primers (2, 23, 25, 26, 30). Although our data revealed an overall TTV prevalence of 22.4%, T primers were more sensitive than NG primers (19.4 versus 10.5%). While the frequency of TTV detection in feces was comparable to that previously reported in serum, it must be noted that low viral titers in the feces and limitations in detection methodology may have underestimated the actual rate of infection in our study population.

Further studies on the development of sensitive detection methods and serodiagnostic assays are necessary to understand the epidemiology and natural history of TTV infection, as well as to establish any association with human disease. Finally, although no disease has been etiologically linked with TTV, its genomic similarity to an avian circovirus, chicken anemia virus, may provide insights into possible human diseases.

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