Early Acquisition of TT Virus (TTV) in an Area Endemic for TTV Infection

F. Davidson, D. MacDonald, J. L. K. Mokili,†
L. E. Prescott, S. Graham, and P. Simmonds

TT virus (TTV) is widely distributed, with high frequencies of viremia in South America, Central Africa, and Papua New Guinea. The incidence and timing of infection in children born in a rural area of the Democratic Republic of Congo was investigated. TTV viremia was detected in 61 (58%) of 105 women attending an antenatal clinic and in 36 (54%) of 68 infants. Most infants acquired the infection at ≥3 months postpartum. Surprisingly, TTV infection was detected in a large proportion of children with TTV-negative mothers (13 [43%] of 30). Nucleotide sequences of TTV-infected children were frequently epidemiologically unlinked to variants detected in the mother. These three aspects contrast with the maternal transmission of hepatitis G virus/GB virus C in this cohort and suggest an environmental source of TTV infection comparable to hepatitis A virus and other enterically transmitted infections.

Like HGV/GBV-C, TTV infection is frequent in the general population; at least 1.9% of blood donors in the United Kingdom (UK) are viremic as are 12% in Japan. More remarkable are prevalences of TTV infection among nonurban populations in tropical countries (≤74% in Papua New Guinea and 83% in the Gambia [7]), findings that argue strongly for nonparenteral routes of transmission. In the current study, we compared frequencies of TTV and HGV/GBV-C infection in women of childbearing age in a rural area of the Democratic Republic of Congo (DRC, formerly Zaire) previously found to have a high population prevalence of TTV infection [7]. Plasma samples collected from children of HGV/GBV-C– and TTV-infected and uninfected women were tested by polymerase chain reaction (PCR) for TTV and HGV/GBV-C sequences to compare the frequency of vertical and alternative routes of acquisition of HGV/GBV-C and TTV infection.

Methods

Samples. Plasma samples were collected from a cohort of 134 women living in Kimpese and neighboring rural areas in Bas-Zaïre (now DRC) for a variety of prevalence and transmission studies of infection with human immunodeficiency virus (HIV) type 1 [20, 21]. Samples were separated and stored frozen on the day of col-
lection and shipped to Edinburgh without further freeze-thaw cycles. All study subjects were tested for HGV/GBV-C RNA, and a subset of 105 was also tested for TTV DNA sequences. Plasma samples were also collected at birth from the neonate or from cord blood and at intervals thereafter for $\approx$24 months from 68 children born to HIV-infected and -uninfected women.

Nucleic acid extraction and PCR. Viral RNA and DNA sequences were extracted as described [22]. In total, 100 $\mu$L of plasma was incubated at 37°C for 1.5 h with 1 mg/mL protease K in the presence of 40 $\mu$g/mL polyadenylic acid, 0.5% SDS, 0.1 M NaCl, 50 $\mu$M Tris HCl (pH 8.0), and 1 mM EDTA. RNA was extracted by adding phenol to the incubation mix; after centrifugation, the supernatant was reextracted with chloroform–isoamyl alcohol (50:1). Nucleic acid was precipitated overnight with the addition of 0.1 vol of sodium acetate (pH 5.2) and 2 vol of ethanol. The nucleic acid was pelleted and washed with 80% ethanol. The dried pellet was resuspended in 25 $\mu$L of diethylpyrocarbonate-treated water.

For detection of HGV/GBV-C, RNA was reverse transcribed and amplified using nested primers designed from sequences in the 5'-noncoding region (NCR) of the genome using primers and temperature cycles as described [23]. Amplified PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light. TTV sequences were amplified using previously described heminested primers [5] and fully nested inner antisense primer S4573 or an inner sense primer S4572. Amplified PCR products (primer set B [6]) in the N22 coding sequence [5]. Through a typographic error in the previous publication, the sequence of primer AS427 was incorrect and should be 5'-TCCATGTAGCT-CTCATTCTWA-3' (W = A or T; Y = T or C). In the current study, subjects were regarded as viremic if positive with either primer set or both.

Nucleotide sequencing and phylogenetic analysis. Identified HGV/GBV-C–positive samples were reamplified for sequence analysis by a second-round amplification that used either a biotinylated inner antisense primer S4573 or an inner sense primer S4572. Amplicons were immobilized on streptavidin-coated magnetic beads (Dynabeads M280; Dynal, Merseyside, UK) as previously described [24]. Sequencing reactions were done using T7 DNA polymerase (Sequenase version 2.0; Amersham, Amersham, UK) according to the manufacturer’s instructions. TTV sequences amplified by PCR were sequenced by cycle sequencing using a thermostable DNA polymerase (Amersham) from both ends using the same primers as used for the PCR. Sequences obtained in this study were submitted to GenBank (accession numbers AF113958–AF113967 and AF113973–AF113994).

Phylogenetic analysis of sequences from the 5'-NCR was carried out by neighbor-joining of uncorrected (HGV/GBV-C) or Jukes-Cantor corrected (TTV) $P$ distances using the MEGA package [25]. Phylogenetic groupings were detected by bootstrap resampling.

Results

Prevalences of TTV and HGV/GBV-C infection. To investigate the frequency of TTV and HGV/GBV-C infections in women of childbearing age in rural Congo, plasma samples were obtained from 134 study subjects—79 from HIV-negative women attending an antenatal clinic and 55 from HIV-1–infected women enrolled in a study of vertical transmission of HIV-1 [20, 21]. TTV viremia was detected in 61 (58%) of 105 study subjects; 18 (13%) of 134 were infected with HGV/GBV-C. Among the 18 HGV/GBV-C–infected women, 14 were coinfected with HIV, a higher frequency of HIV infection than among the HGV/GBV-C–uninfected study subjects (41/116; $P = .001$; Fisher’s exact test; table 1). TTV viremia was also associated with HIV coinfection, although frequencies of infection were also high in HIV-negative women (43% vs. 72% in HIV-positive women; $P = .003$). There was no association between HGV/GBV-C and TTV coinfection either collectively or after separation of HIV-positive and -negative study subjects.

A history of prostitution was elicited from 28 of the 134 women interviewed. Prostitution was not associated with HIV infection in this study group (12 of 55 HIV-positive women were prostitutes compared with 16 of 63 HIV-negative women; $P = .832$). There was no association between TTV infection and a history of prostitution (table 1), although HGV/GBV-C coinfection was more frequent in HIV-negative prostitutes (3/4) than in 13 of 75 women without this risk factor ($P = .025$); larger numbers would be required to substantiate this association. Similarly, HGV/GBV-C infection was more frequent in women with a history of tribal scarification than in those without, but no association was found with TTV infection.

Mother-to-child transmission of TTV and HGV/GBV-C.

Table 1. Associated factors for TTV and hepatitis G virus/GB virus C (HGV/GBV-C) infection.

<table>
<thead>
<tr>
<th>Variable</th>
<th>TTV</th>
<th></th>
<th></th>
<th></th>
<th>HGV/GBV-C</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median)</td>
<td>105</td>
<td>27</td>
<td>26</td>
<td>.483</td>
<td>134</td>
<td>27.5</td>
<td>26</td>
<td>.590</td>
</tr>
<tr>
<td>HIV coinfection</td>
<td>105</td>
<td>39/22 b</td>
<td>15/29</td>
<td>.003</td>
<td>134</td>
<td>14/4</td>
<td>41/75</td>
<td>.001</td>
</tr>
<tr>
<td>HGV/GBV-C infection</td>
<td>105</td>
<td>12/49</td>
<td>5/39</td>
<td>.294</td>
<td>55</td>
<td>4/10</td>
<td>8/33</td>
<td>.447</td>
</tr>
<tr>
<td>Prostitution, HIV-positive</td>
<td>54</td>
<td>9/30</td>
<td>3/12</td>
<td>1.000</td>
<td>55</td>
<td>4/10</td>
<td>8/33</td>
<td>.447</td>
</tr>
<tr>
<td>Prostitution, HIV-negative</td>
<td>51</td>
<td>6/16</td>
<td>6/23</td>
<td>.741</td>
<td>79</td>
<td>3/1</td>
<td>13/62</td>
<td>.025</td>
</tr>
<tr>
<td>Transfusion in prior 2 years</td>
<td>101</td>
<td>2/57</td>
<td>2/40</td>
<td>1.000</td>
<td>127</td>
<td>1/17</td>
<td>4/105</td>
<td>.450</td>
</tr>
<tr>
<td>Tribal scarification</td>
<td>50</td>
<td>2/19</td>
<td>2/27</td>
<td>1.000</td>
<td>76</td>
<td>2/2</td>
<td>4/68</td>
<td>.029</td>
</tr>
</tbody>
</table>

NOTE. NA, not available.

* Ages compared by Kruskall-Wallis nonparametric test; other comparisons, Fisher’s exact test; significant differences in bold.

b Among 51 TTV-infected study subjects, 39 were coinfected with HIV-1; 22 were not.
TTV infection in 68 children was monitored by PCR testing of plasma samples collected 3 and 12 months after birth. In 29 children (43%), samples at both time points were negative and classified as TTV-uninfected. In 37 cases (54%), the 3-month samples were TTV-negative, but the 12-month samples were positive. Earlier evidence for TTV infection was found in the remaining 2 children: TTV viremia was detected in the 3-month samples and persisted until the 12-month sample in 1 and became negative in the other. Among children of TTV-infected mothers, 68% (26/38) became infected, although a high frequency was also found among children of TTV-negative mothers (57% of children of both HIV-positive and -negative mothers were infected with TTV. However, TTV infection was significantly more frequent in children who also acquired HIV infection (table 2). The frequency of vertical HIV transmission was 21% (6/29), and TTV cotransmission occurred in all 6 of the HIV-infected children but in fewer than half of those who were HIV-uninfected, consistent with shared risk factors for transmission of the 2 viruses or a greater susceptibility to TTV among those infected with HIV. The low number of mother-to-child transmissions of HGV/GBV-C (see below) did not allow its association with TTV transmission to be determined. All children investigated for vertical transmission were breast-fed. To investigate the possible role of breast-feeding as a source of TTV infection, we tested 29 breast milk samples collected within 1 year of birth. TTV DNA sequences were detected in 9 (41%) of 22 TTV-infected mothers and in 1 of 7 who was TTV-negative in plasma.

Nucleotide sequencing of amplified DNA from 24 maternal and 14 child samples revealed considerable sequence heterogeneity of TTV in the study population. Comparison of nucleotide sequences with those previously described allowed variants to be classified into genotypes 1 and 2 plus a novel genotype not previously described in UK or Japanese populations [4–6]. The most commonly detected genotype was type 2, although variants detected were highly diverse in sequence and did not correspond to the type 2a and 2b subtypes previously detected in Japan and the UK (table 3; figure 1A). Similarly, type 1 variants did not clearly group with the previously established a and b subtypes of type 1. Sequences from 3 subjects (m158, m144, p177) formed a separate diverse phylogenetic group equidistant from the previously classified TTV genotypes. There was no difference in the genotype distribution between mothers and children (table 3). Nucleotide sequencing was done directly on DNA amplified by PCR. In 4 samples (3 maternal, 1 child), approximately equal frequencies of 2 different genotypes were present.

To investigate whether TTV infection in children originated in the mother, nucleotide sequences amplified from samples of 10 children with TTV-infected mothers collected at 1 year were compared with those of maternal samples collected 6–12 months after birth (figure 1A). Identical sequences were observed for m07 and p07 (type 2), m08 and p08 (type 1), m10 and p10 (type 2), and m50 and p50 (type 1). Sequences from m20 and p20 (type 2) grouped together, but with marginal bootstrap support (67%), and showed 10 nt differences from each other, greater than between known epidemiologically unlinked infections [4, 6]. Sequences from the remaining paired samples were either unrelated variants of the same genotype (m02 and p02) or were of different genotypes (m51/p51, m120/p120, m155/p155, m158/p158).

The frequency and timing of TTV infection in children contrasted with that observed for HGV/GBV-C. First, there was a much stronger association between infection of the child and maternal status. Of the 6 HGV/GBV-C–infected children, 5 were born to HGV/GBV-C–infected mothers (P < .001). Infection was acquired earlier: 5 children with HGV/GBV-C–positive mothers became PCR-positive within 6 weeks on samples collected at varying intervals after birth (figure 2). In contrast, the 6th child (of an uninfected mother) became infected after 9 months, consistent with an alternative source of infection. All 29 breast milk samples collected within 1 year of birth were negative for HGV/GBV-C, including the 7 from women whose plasma samples were HGV/GBV-C PCR-positive. To identify the source of infection in the infected children, sequences amplified in the 5′-NCR were compared with those from maternal samples (figure 1B). All sequences obtained corresponded to the type 1 genotype, and in each case the sequence from the child grouped with that of his or her mother on phylogenetic analysis. Each grouping was supported by bootstrap resampling with values of 84%–98%. Nucleotide sequences from each mother-child pair were identical in 3 cases, while there was 1 nt difference between m08 and p08 and between m40 and p40. This degree of similarity is greater than between sequences from unlinked mothers and children (mean, 5.75 differences; range 3–9).

### Table 2. Associated factors for TTV mother-to-child transmission.

<table>
<thead>
<tr>
<th>Variable</th>
<th>No.</th>
<th>TTV Positive</th>
<th>TTV Negative</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median)</td>
<td>68</td>
<td>28</td>
<td>28</td>
<td>.760</td>
</tr>
<tr>
<td>TTV-infected mother</td>
<td>68</td>
<td>26:12</td>
<td>13:17</td>
<td>.050</td>
</tr>
<tr>
<td>Maternal HIV coinfectiona</td>
<td>68</td>
<td>20:15</td>
<td>19:14</td>
<td>1.000</td>
</tr>
</tbody>
</table>

* Fisher’s exact test.

### Table 3. TTV genotype distribution in study subjects.

<table>
<thead>
<tr>
<th>Study subject</th>
<th>No.</th>
<th>Type 1a</th>
<th>Type 2a</th>
<th>New</th>
<th>Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mothers</td>
<td>24</td>
<td>7</td>
<td>12</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Children</td>
<td>14</td>
<td>4</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

a Type 1 and type 2 sequences were distinct from those previously described as 1a, 1b, 2a, and 2b and have not been classified into subtypes.
Figure 1. Phylogenetic analysis. (A) N22 sequences amplified from mothers (○, m) and children (●, p) infected with TTV (bases 93–323 in N22 clone [4]) and (B) 5'-noncoding region of hepatitis G virus/GB virus C (HGV/GBV-C) (bases 161–370, numbered as in [3]) amplified from mothers and children infected with HGV/GBV-C. Branches show % of trees supporting phylogeny on bootstrap resampling (values >75%). P distance scale shown under tree.
Figure 2. Detection of hepatitis G virus/GB virus C (HGV/GBV-C) RNA sequences at different postpartum times of 12 children born to infected mothers and 1 child of HGV/GBV-C–uninfected mother (p36). ●, HGV/GBV-C polymerase chain reaction (PCR)–positive; ×, PCR-negative; *HIV-coinfected.

Discussion

Epidemiology of TTV infection. The main observation in the current study was the extremely high incidence of TTV infection in children during the first year of life (54%). Although samples from children 1 year old were not tested, it is possible that TTV infection acquired at this time could persist and therefore underlie the extremely high population prevalences observed in adults in central African countries (e.g., DRC, Nigeria, and Gambia), Central America, and tropical Southeast Asia [7]. Unlike HGV/GBV-C, there was little association between TTV infection and a history of sexual or parenteral exposure among the maternal study group, although TTV infections were more frequent in HIV-infected subjects. This may either indicate that the 2 viruses share a common undetermined risk factor or that HIV coinfection predisposes to greater susceptibility to infection, reinfection, or frequency of persistence into adulthood.

In contrast with a study of UK blood donors, there was no association between maternal age and TTV infection. The increasing prevalence of TTV infection with age in the UK was interpreted as indicating a falling incidence of infection in the sampled population [6], while the absence of an association in the current study subjects is consistent with the extremely high overall prevalence and endemic pattern of infection in the DRC.

In these aspects, the epidemiology of TTV appears comparable to that of enterically transmitted viral infections, such as HAV, where frequent infection in thefirst year of life and evidence for universal past exposure in adults is common in developing countries, while Western countries, such as the UK, have experienced a marked decline in incidence in this century (and an age stratification of past infection in blood donors similar to that observed for TTV).

Mechanism of TTV and HGV/GBV-C transmission to children. Evidence for the source of TTV infection in children was sought through investigation of the association of TTV infection in mothers and their children, the timing of TTV infection after birth, and by comparison of TTV variants amplified from mother and child paired samples. Findings in all of these investigations indicated that TTV infection may be acquired through nonmaternal routes, although the experimental data limit the strength of the conclusions reached.

There was only a loose association between maternal TTV status and infection before age 1 year in the child: TTV infection occurred in 12 (41%) of 29 children with noninfected mothers. While the large number of TTV infections among children of PCR-negative mothers indicates a nonmaternal source of infection, this conclusion depends on the ability of the PCR methods used to accurately diagnose maternal infection. Problems
with interpreting PCR results in this and other studies include the observation that levels of circulating viremia are extremely low [4, 6], and it is possible that some TTV-infected persons have plasma virus levels that are undetectable by current methods. Using the analogy of HCV [26], it is possible that mothers with low circulating levels of TTV may be less likely to transmit TTV vertically, which would explain the lower frequency of TTV infection in children of “TTV-negative” mothers (43% vs. 68%). Underdiagnosis of TTV infection in mothers is suggested by the finding of TTV in breast milk of 1 mother who was PCR-negative in plasma. There was also a measurable frequency of samples in this and previous studies [6] that were positive with one set of primers but negative with the other, indicating that the PCR may perform suboptimally. Finally, because it is difficult to predict the full extent of genetic heterogeneity of TTV, it is unclear whether the fully nested or heminested primers used in the current study can amplify all TTV genetic variants. It is possible that other TTV transmissions to children were not detected.

A second argument for a nonmaternal source of infection among the children is the timing of infection. TTV viremia became detectable in all but 2 cases between ages 3 and 12 months, making perinatal infection or in utero infection unlikely, as the incubation period for TTV in a previous transmission study was 6 weeks [4]. However, incubation periods may vary with the amount of exposure and route of transmission. In the current study, the delayed acquisition of TTV perinatally could possibly be attributed to a longer incubation period than among persons transfused with whole units of TTV-contaminated blood.

Finally, the observation that many of the variants recovered from children were dissimilar in sequence or genotype from maternal variants suggested that acquisition of infection in these cases was from an epidemiologically unrelated environmental source. A possible complicating factor in the interpretation of the sequence information is the possibility of multiple infections with different TTV strains in the mother. Given the high general population prevalence of TTV in DRC, multiple exposures to TTV is likely, and reinfection or coinfection with heterologous strain(s) is a possibility given the failure of the immune system to prevent persistent infection. Sequences in the current study were derived from direct sequencing of PCR product, and at least 4 samples showed evidence of mixed infection (table 3). The existence of genetically distinct minor populations of TTV in infected mothers who transmitted either preferentially or by chance to the child could explain the failure of 6 of the 10 mother-child paired sequences to group together in the phylogenetic tree (figure 1A). Further work is required to examine the population complexity of circulating TTV in persistently infected persons.

Despite these necessary qualifications, each of the observations for TTV contrasts with this and previous investigations of HGV/GBV-C transmission. The association between HGV/GBV-C maternal infection and transmission to the infant was clearer: Only 1 of the 6 infected children had an uninfected mother, and the infection in this child occurred ≥9 months after birth. In marked contrast to TTV, sequence comparisons of HGV/GBV-C sequences always demonstrated a close relationship between variants recovered from each mother and child pair, implicating the former as the immediate source of infection. These findings combined with evidence for an early onset of infection in this and other studies [27–30] are consistent with infection being acquired perinatally or in the immediate postnatal period.

**Enteric transmission of TTV.** Low titers of TTV DNA have been detected in feces of some infected persons [31], potentially providing a route of transmission comparable to that of enteroviruses, such as HAV, and of animal paroviruses. This route of transmission is consistent with the epidemiology of TTV infection in adults and would resolve the contrasting features associated with infant infection by TTV and HGV/GBV-C found in the current study. While it is possible that contaminated water or food was the source of infection acquired orally (as for HAV and other enterically transmitted viruses), the frequent detection of TTV in breast milk provides an alternative source of infection. However, accepting the limitations of the experimental data (see above), transmission by this route would not readily explain the sequence differences between certain mother-child sample pairs, the frequent infection of children of TTV-negative mothers, or the delay in infection to ≥3 months after birth.

TTV differs from enterically transmitted viruses in that it can establish persistent infection, which facilitates its transmission through transfusion and other parenteral routes [4–6] and contributes to a frequency of active infection in the general population that rivals that of herpesviruses and polyomaviruses. The finding of a high prevalence of active infection after age 1 year and its likely long-term persistence into adulthood is inconsistent with TTV being a highly pathogenic infectious agent, although further work is needed to establish its in vivo cellular tropism, its possible disease associations, and the effect of cellular and humoral responses to acute and chronic infection.

**References**


