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Mother-to-child transmission of TT virus: sequence analysis of non-coding region of TT virus in infected mother-infant pairs*

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Summary. To investigate vertical transmission of TT virus, TTV-DNA was looked for in serum samples taken from 22 mothers and their 22 infants at birth and during nine months of follow-up. Sixteen mothers at delivery and six infants within nine months of age had TTV-DNA detected by the amplification of the non coding (NC) region. Two of these newborns had positive viremia at birth. Sequence analysis of the NC region of five mother-infant pairs revealed that the TTV strains detected at three and six months of age in two of the infants were closely related to that of their mothers, whereas two that became TTV-DNA positive at three moths had a different nucleotide sequence from that of their mothers. One of the two infants with detectable viremia at birth also had a different nucleotide sequence from her mother. These findings suggest that both *in utero* and perinatal transmission of TT virus may occur, and that the strain detected in the infants was not invariably dominant in the mothers at delivery.

Introduction

TT virus has recently been recovered from patients with post-transfusion hepatitis of unknown etiology [10]. Its genome consists of a circular single-stranded DNA

*In accordance with Italian National Legislation, Ethics Committee permission was not required for taking blood samples, but written informed consent was necessary to participate in the study. The mothers taking part in the present study gave their written informed consent (in which it was specified that the study design was in accordance with the Helsinki Declaration) for themselves and their newborns. None of the authors has a commercial or other association that might pose a conflict of interest (e.g. pharmaceutical stock ownership).

with at least two open reading frames called ORF1 and ORF2. TT virus was first detected by PCR assay with primers from ORF1 (region N22) in blood donors and parenterally exposed individuals [11–18]. The development of a new PCR strategy with primers derived from the well conserved non-coding (NC) region has been used to demonstrate that TTV is widely distributed in the general population [23], but the analysis of ORF sequences has not revealed any geographical distribution of genetically characterised strains [1]. Further studies in infants and young adults have shown that TT virus is acquired early in life and that the prevalence of viral infection increases with age [2–6]. It has more recently been demonstrated that mother-to-child transmission of the virus is highly efficient [17–27], but its mechanism and timing is still little understood.

We studied TT virus vertical transmission using PCR assay with primers deduced from the ORF1 and NC regions. The TT virus genomic sequences were inferred by means of direct sequencing of the NC region in mother-infant pairs found to be TTV-DNA positive.

Patients and methods

Patients

The study involved 22 mothers (mean age 30 years, range 24–35) without HIV infection but with anti-HCV antibodies (seven of whom were HCV-RNA positive) and their 22 infants, all belonging to a prospective study of HCV vertical transmission [20]. Serum samples were taken from the babies at birth, and at three, six and nine months of age; the sera of two siblings were also analysed.

Only one woman had a parenteral risk factor for TT virus infection, being an ex-parenteral drug user. Eighteen of the women delivered vaginally and the other four by elective caesarean section. None of the mothers breastfed their children.

Sample collection

The serum samples were taken at birth from a peripheral vein in the infants to avoid contamination with maternal blood. Two separate aliquots of each specimen were stored and immediately frozen at −80 °C until use.

Detection of TTV-DNA in serum

TTV-DNA was detected by means of a PCR assay with two sets of primers: the first spanning the N22 region of ORF1, and the second in the NC region of TT virus.

Briefly, total DNA was extracted from 200 μl of serum using a nucleic acid extraction kit (Qiagen, Hilden, Germany) and resuspended in 60 μl AE buffer (Qiagen). The mix containing 10 μl DNA was pre-heated at 95 °C for 9 min to activate the AmpliTaq Gold DNA polymerase (Perkin Elmer, Corp., Foster City, CA, USA), and then underwent amplification with specific primers. TTV-DNA was first amplified by nested PCR with the primers derived from the ORF1.

The first and second round PCRs were performed under the same conditions using a Perkin-Elmer 9600 thermal cycler, with denaturation at 94 °C for 30 sec, annealing 58 °C for 30 sec, extension 72 °C for 45 sec, for 40 cycles and an additional extension at 72 °C for 6 min and 30 sec. The sequences of the TT virus specific primers for ORF1-N22 for the first step of amplification were 5′-ACAGACAGAGGAGAAGGCAACATG-3′ NGO59, sense

primer, and 5′-TGACTGTGCTAAAGCCTCTA-3′ RDO38, antisense primer; the sequences for the second step were 5′-GGCAACATGTTATGGATAGACTGG-3′ NGO61, sense primer, and 5′-CTGGCATTTTACCATTTCCAAAGT-3′ NGO63, antisense primer, as designated by Okamoto et al. [11]. The single step PCR assay of the NC region was performed as follow: 95 °C for 9 min, 95 °C for 20 sec, 60 °C for 20 sec, 72 °C for 30 sec, for 55 cycles and an additional extension at 72 °C for 6 min and 30 sec.

The PCR assay of the NC region was performed in the same manner except that one set of primers was used and annealing took place at 60 °C.

The sequences of the TT virus specific primers for NC region were 5′-GCTACGTCACT AACCACGTG-3′ T801, sense primer, and 5′-CTBCGGTGTGTAAACTCACC-3′ T935, antisense primer; B = G, C or T as designated by Takahashi et al. [23]. The PCR products were revealed by means of ethidium bromide staining after electrophoresis on 1.5% agarose gel.

Direct sequencing of PCR products derived from the non-coding region

The PCR products derived from the NC region were purified using a microcolumn system (Amicon, Millipore, Milan, Italy), and directly sequenced by means of PRISM dye terminator cycle sequencing (Perkin-Elmer) using an ABI 373 automated sequencer according to the manufacturer's instructions. Both the sense and antisense strands were sequenced for each PCR sample, and the results were confirmed in two different experiments.

The sequences were analysed using the Sequencer 3.0 analysis program (Gene Codes Corp., Ann-Arbor, MI).

Phylogenetic analysis

The multiple alignment of the nucleotide sequence was inferred by Clustal_X version 164.b [24]. The nucleotide distances were calculated by generating a distance matrix using DNADIST program (PHYLIP 3.5c package) [3] maximum-likelihood model. A phylogenetic tree was then constructed using NEIGHBOR (PHYLIP) with random addition, and drawn using TreeViewPPC version 1.5.3 [15]. Bootstrap analysis was performed using SEQBOOT (100 resamplings) in order to place approximate confidence limits on individual branches.

Results

TTV-DNA was detected by amplifying the ORF1 in eight of the 22 mothers (36%) at delivery and one of the 22 infants (4.5%), who was infected at birth.

Amplification of the same samples with primers belonging to the NC region led to a higher rate of positive samples. Sixteen mothers (72%) at delivery and six babies (27%) during the 9-month follow-up were found to be TTV-DNA positive.

In particular, two of the babies had positive viremia at birth; three became TTV-DNA positive at three months of age, and one at six months (Table 1). All of these babies maintained TTV-DNA positivity throughout the follow-up period, except for the infant who was found to be positive at six months, who lost TTV DNA at nine months of age.

Surprisingly, the mother of one baby infected at birth was TTV-DNA negative at delivery. The remaining 16 babies were not infected at birth and did not acquire TT virus infection during the follow-up period.

The 4-year-old sibling of one infected baby had detectable TTV viremia, whereas the 2-year-old sibling of another baby with undetectable TTV viremia (born of a mother infected with TT virus) was also TTV-DNA negative.

Table 1. Prevalence of TTV-DNA in mothers at delivery and newborns at birth and during the follow-up period

Subjects	Age	TTV-DNA positive	
		ORF1[a]	NC[b]
Mothers	30 years (mean age)	8/22 (36%)	16/22 (72%)
Infants			
	0 days	1/22 (4.5%)	2/22[c] (9%)
	3 months	1/22	5/22 (23%)
	6 months	1/22	6/22 (27%)
	9 months	1/22	5/22[d] (23%)

[a]ORF1 indicates the amplification of specimens with primers from the Open Reading Frame 1 of TTV coding region

[b]NC indicates amplification with primers selected within non coding region of the TT virus genome

[c]One of these two babies was found TTV DNA positive at birth, even though the mother had undetectable viremia at delivery

[d]A baby with positive viremia at 6 months of age became TTV-DNA negative at 9 months

Of the seven mothers who were HCV-RNA positive, two had TT virus sequences in their serum; none of them transmitted HCV (data not shown), but one of them possibly infected her newborn with TT virus.

Given that infection with TT virus can be related to liver disease, transaminase levels were measured in all of the mothers at delivery, and in their infants at birth and during the follow-up period. Alanine amino transferase (ALT) and aspartate amino transferase (AST) values were within the normal range (AST < 40 U/L, ALT < 50 U/L) in the mothers at delivery and newborns at birth and during nine months of follow-up.

Alignment of the partial NC sequence of five mother-infant pairs with 14 local controls (seven mothers infected with TTV-DNA at delivery, and seven healthy individuals taking part in a study of sexual transmission of TT virus) and ten isolates obtained from GenBank showed a wide distribution of sequence divergence within the region designated by the primers, thus indicating that nucleotide sequence variability can be shared within the conserved region in the TT virus genome (Fig. 1).

Comparison of the partial non-coding region revealed 99% and 97% identity between the sequences of two mothers and their babies, although the nucleotide sequence analysis of the TT virus strain in these two infants was performed when they were three and six months of age (the first sample obtained at birth was TTV-DNA negative in both babies). Analysis of the NC region in another mother-infant pair showed 88% identity (the child was TTV-DNA positive at birth). Divergent nucleotide sequences (12% and 17% divergence) were detected in two other mother-infant pairs (the specimens of the infants became TTV-DNA positive after three months).

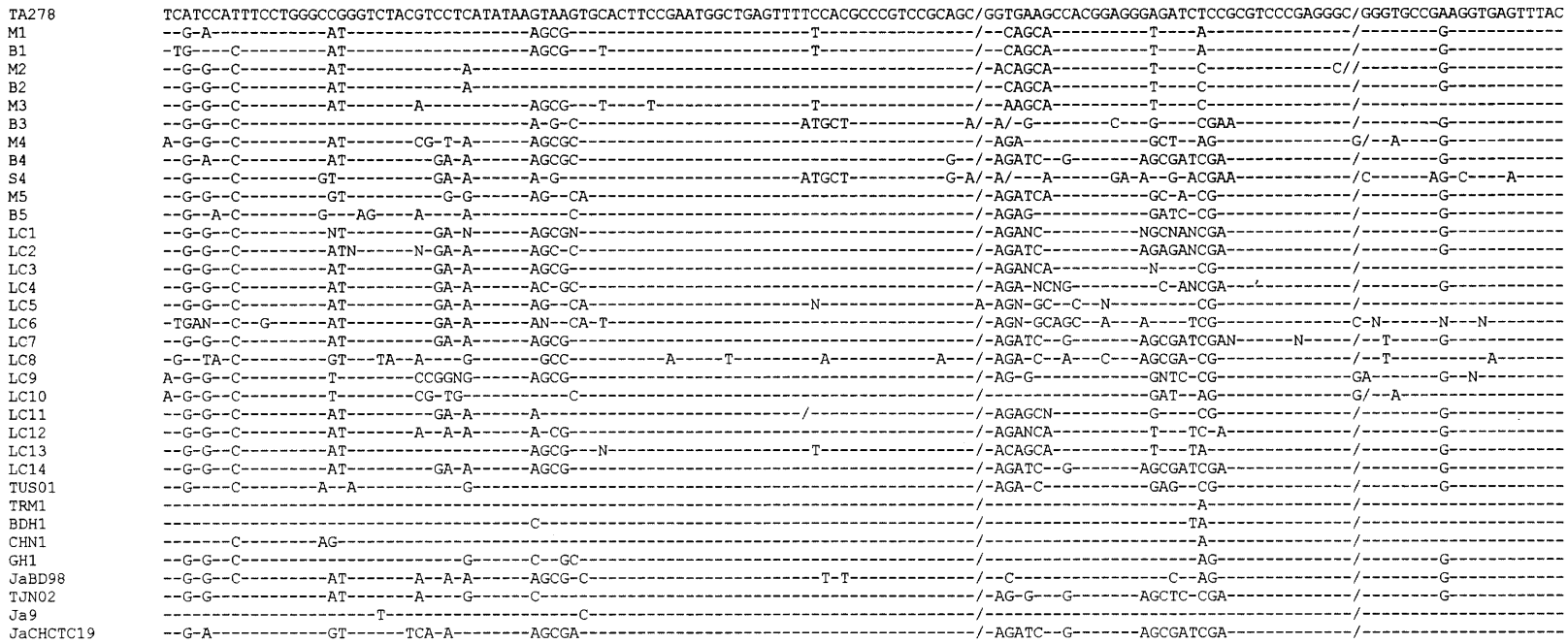
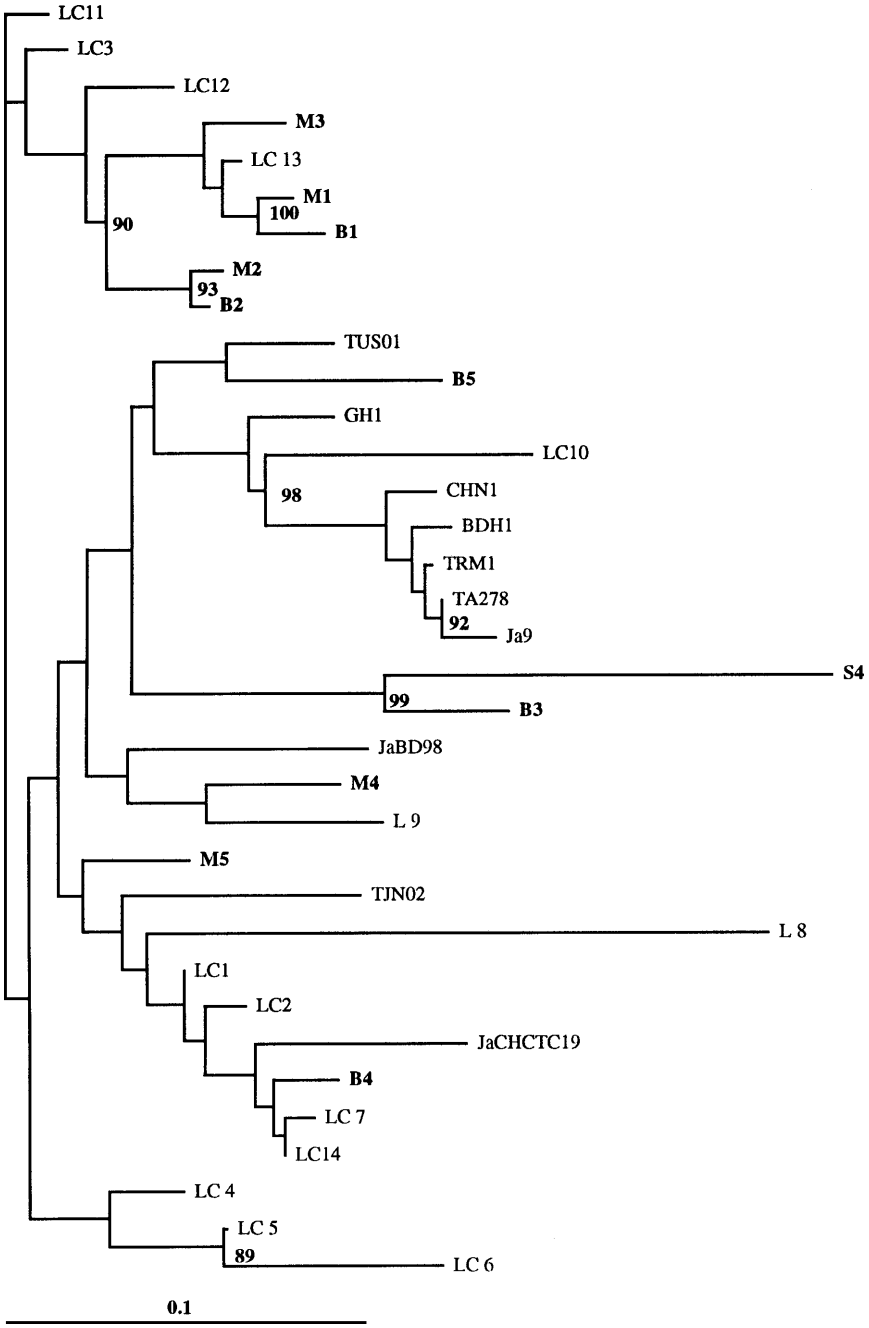


Fig. 1. Alignment of nucleotide sequences of the partial non-coding region of five mother-infant pairs infected with TT virus (M1–M5, mothers at delivery), (B1–B5, infants found to be TTV-DNA positive during the follow-up), the sibling of an infected infant (S4), 14 local controls and ten sequences obtained from GenBank. Alignment was performed on the basis of the TA278 sequence. The dashes (-) represent nucleotides identical to the sequence of TA278; the slashes (/) represent deletion of nucleotides; N represents two equal or near equal peaks at a particular base position using automated DNA sequencing

Analysis of the NC region in the 4-year-old sibling revealed 75% and 80% identity with his mother and the newborn.

Phylogenetic tree analysis confirmed that the strains of two infants were more closely related to those of their mothers than to the other isolates (including 14 local controls), but no such correlation was found in three other mother-infant pairs (Fig. 2), even though one baby was TTV-DNA positive at birth. Furthermore, the



sibling of one baby with a different sequence from the mother did not show any genetic correlation with either the mother or the newborn (Fig. 2).

Discussion

Previous studies [13–21] have shown that the detection of TTV by amplifying the NC region is more sensitive than using ORF1 because of the inability of the primers selected in ORF1 to detect all of the genotypes.

In this study, we found a low prevalence of TT virus infection in mothers and their infants using a PCR assay from ORF1 for TTV-DNA detection, and a higher rate of TTV-DNA positivity when we used primers derived from the NC region, thus confirming the greater sensitivity of this region in detecting TT virus infection in our geographical area. However, comparison of the NC sequences in isolates obtained from GenBank, the mother-infant pairs and local controls indicated that the NC region spanning the primers designated by Takahashi et al. [23] are not so well conserved among individuals of different geographical areas.

The timing of TT virus transmission is uncertain: a number of reports have suggested that transmission may occur early in life, but vertical transmission seems unlikely [16–19], whereas others indicate the efficiency of both *in utero* and perinatal transmission [5–9].

However, these studies investigated vertical transmission of TT virus by using PCR assay on cord blood samples. In the present study, the serum sample taken at birth was not obtained from cord blood but was drawn from a peripheral vein of the infant, thus avoiding any contamination with maternal blood.

We found a high prevalence of TT virus infection in infants born of mothers who were anti-HCV positive, which could influence the positivity rate of TTV-DNA in serum of the newborns. However, a number of studies [8–18] have not found any correlation between HCV infection and an increased prevalence of TT virus viremia.

The finding that TTV-DNA was undetectable at birth but detectable after three months in two infants suggests perinatal TT virus transmission, also because the strain detected in the babies was closely related to that of their mothers

Fig. 2. Phylogenetic tree analysis of the NC region in five mother-infant pairs (M1–M5 indicate the nucleotide sequences of the infected mothers; accession numbers AF378229, AY035348, AY035347, AY035356 and AY035368; B1–B5 indicate the sequences of the corresponding babies; accession numbers AY035345, AY035349, AY035366, AY035361 and AY035367), one sibling (S4; accession number AY035365), 14 local controls (LC-1 to LC-14; accession numbers AY035362, AY035363, AYAY035350, AY035355, AY035353, AY035354, AY035359, AY035364, AY035358, AY035357, AY035352, AY035351, AY035346 and AY035360) and ten sequences obtained from GenBank (JaCHCTC19, TJN02, JaBD98, GH1, TTVCHN1, BDH1, TRM1, JA9, TA278, TUS01; accession numbers AB030487, AB028669, AB030489, AF122913, AF079173, AF116842, AB038340, AF122915, AB017610 and AB017613). Bootstrap values of 85% or more (100 replicates) are indicated. Evolutionary distance units are indicated in the scale

as confirmed by phylogenetic tree analysis. Furthermore, none of the mothers breastfed their babies, thus excluding this route of transmission.

However, given that the non-parenteral route of transmission has been described [4–12] and TTV virus became positive in these two infants at three months, post-birth contamination with the biological fluids of the mothers cannot be completely excluded.

Two other babies found to be TTV-DNA positive at three months of age had low sequence homology with their mothers; hence, another source of infection (such as faeces or saliva drops) may have been involved in these cases. Recent studies [13–26] indicated that individuals could be infected simultaneously by different TT virus types. As a more recent report [22] showed different TT virus strains in mothers and their newborns suggesting that the babies became infected with only one of some of the TT virus genomes detected in their mothers, one other explanation for this finding could be that these two infants acquired a variant from their mothers who were infected with a mixed viral population. This latter hypothesis is possibly true in the baby with a low sequence identity (88% homology) with her mother, because she was TTV-DNA positive at birth and another source of infection is therefore unlikely. Moreover, single-strand conformational polymorphism (SSCP) analysis, which can distinguish DNA fragments as different electrophoretic migrations of single-stranded DNA [14], showed the presence of a single strain in all of the infected babies and two mothers who shared a strain strictly related to that of their infants, whereas at least two genomes were detected exclusively in the three mothers with different sequences from their newborns (data not shown), suggesting that the infants acquired only one of the TTV strains present in their mothers infected with a mixed viral population.

One 4-year-old sibling shared divergent nucleotide sequences from his mother and the newborn. He probably acquired a single strain from the mother infected with a mixed viral population, but contamination from other sources was not completely excluded.

One baby was TTV-DNA positive at birth and remained so during the 9-month follow-up, whereas the mother had no detectable viremia at delivery. In this case, it is possible that the transmission occurred *in utero* and that the mother had very low levels of TTV viremia at delivery. It is unlikely that the baby acquired the infection from an alternative source as he was TTV-DNA positive at birth. Furthermore, contamination of the specimen with PCR products was excluded because protocols to avoid contamination were strictly followed [7], all of the experiments were performed on different aliquots of the same sample, and the results were confirmed in at least two different experiments. Furthermore, this baby was persistently TTV DNA positive.

It has been suggested that TT virus is related to liver disease [10–25]. We found no alterations in transaminase levels in the babies at birth or during the follow-up, and so liver disease due to TTV infection seems unlikely; however, no definitive conclusions could be drawn because the follow-up in the babies was too short.

In conclusion, this is the first report analysing TTV-sequences in serum obtained from a peripheral vein at birth. The fact that TT virus was detectable in

two infants at birth indicates *in utero* virus transmission in these cases. Sequence divergence between the specimen taken from a mother at delivery and that of the infant at birth suggests that the mother was infected with a mixed viral population and transmitted a variant transplacentally that became dominant in the infant. Perinatal transmission was likely in two infants found to be TTV-DNA positive after birth, who were infected with a strain closely related to that of their mothers. Vertical transmission may therefore account for a considerable portion of childhood infection in our geographical area, although the possible pathogenic implications of chronic TT virus infection by this route needs to be established over a longer period of observation.

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