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Detection of TT Virus DNA and GB Virus Type C/Hepatitis G Virus RNA in Serum and Breast Milk: Determination of Mother-to-Child Transmission

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To investigate the vertical transmission of the newly described TT virus (TTV), serum and breast milk samples from 46 women as well as sera from their 47 newborns were examined for the presence of TTV DNA by PCR. TTV DNA was detected in 47.8% ($n = 22$) of the women. All but one child born to these women were also viremic for TTV from the first sample onward. TTV DNA was found in 73.9% ($n = 17$) of the breast milk samples derived from TTV viremic mothers. The one TTV-negative child born to a viremic mother remained negative during follow-up, although it was breast-fed. Our data show that TTV is highly effectively transmitted from mothers to their children during pregnancy. Although the majority of breast milk samples from viremic mothers are positive by TTV PCR, there is no need to discourage women from breast-feeding, because most children are TTV viremic even before breast-feeding begins.

A new virus has recently been detected by molecular biological methods in a serum sample derived from a patient suffering from non-A, non-G posttransfusion hepatitis and was named TT virus (TTV) (16, 19). TTV is presumed to be an unenveloped, circular, negative-stranded DNA virus containing a genome of 3,852 bases (15). Two possible open reading frames have been identified, and these open reading frames are capable of encoding 770 and 202 amino acids, respectively (19). Due to the structure of its genome and its banding by buoyant density gradient centrifugation, a relationship to the *Parvoviridae* family or *Circoviridae* family was first assumed (19). At present it is proposed that TTV is a member of a new virus family that infects humans, tentatively named the *Circinoviridae* (15).

The disease-inducing capacity of TTV is unclear. Although it was first isolated from patients with posttransfusion hepatitis (16, 19), other groups found no association between TTV and hepatitis (25, 26). To highlight the clinical impact of TTV it is necessary in a first step to examine the epidemiology of TTV. Until now, no serological assays for detection of antibodies against TTV have been described. As TTV was first isolated from a patient with posttransfusion hepatitis, the major route of transmission was assumed to be parenteral. However, the virus is detectable to a high extent in serum samples derived from healthy blood donors (19, 25). Therefore, other significant routes of transmission besides the parenteral route must be taken into account. Until now, besides blood, TTV DNA has been detected in stool specimens of infected patients (18).

Like TTV, GB virus type C/hepatitis G virus (GBV-C/HGV) was isolated from patients suffering from posttransfusion hepatitis of unknown origin (14, 27). However, it has been demonstrated that GBV-C/HGV is widespread in the general population (6), and its clinical impact is controversial (6, 28). Due to the high percentage of GBV-C/HGV PCR-positive healthy blood donors, transmission routes other than the parenteral route must be assumed.

We have examined serum samples from 46 hepatitis C virus (HCV)-infected women and their 47 newborns by PCR to evaluate whether TTV and GBV-C/HGV are transmitted during pregnancy. Additionally, breast milk samples were derived from all women shortly after delivery and were examined by PCR to investigate if breast-feeding contributes to transmission of these viruses.

MATERIALS AND METHODS

Patients. Serum samples from 46 women with known HCV infection as determined by confirmed antibody reactivity or a positive HCV PCR result were retrospectively examined for TTV infection by PCR. All sera were collected during pregnancy. At the time of delivery the women tested negative for hepatitis B virus and human immunodeficiency virus infection.

The 46 women gave birth to 47 children. Serum samples were drawn from the children within the first week of life. Follow-up samples were obtained for a period of 1 to 28 months (mean, 7.5 months).

All children were breast-fed. Breast milk was collected from the mothers within a range between the 1st and the 73rd days (mean, 6 days) after delivery and was also examined by TTV PCR. Nucleic acids were extracted immediately after serum and breast milk samples were collected. The extracted nucleic acids were stored by −80°C until examination.

TTV PCR. TTV DNA was extracted from 140 μl of serum or breast milk with a nucleic acid extraction kit (Qiagen, Hilden, Germany), as recommended by the manufacturer. Nucleic acids were eluted in 50 μl of H_2O, and 3 μl was used for amplification of the TTV DNA in a buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 160 μM (each) deoxynucleotide triphosphate, 30 pmol of each sense or antisense primer, and 2 U of *Pfu* thermostable DNA polymerase (Stratagene, La Jolla, Calif.). For the first round of the nested PCR primers NGO59 (5′-ACAGACAGAGGAGAAGGCAACATG-3′; positions 1920 to 1943; numbering of nucleotide sequences is as described previously [19]) and NGO63 (5′-CTGGCATTTTACCATTTCCAAAGTT-3′; positions 2161 to 2185) were used, with denaturation for 30 s at 94°C, annealing for 60 s at 55°C, and extension for 60 s at 72°C. In a second amplification step 5 μl of the first-round product was used with the primers NGO61 (5′-GGCAACATGTTA TGGATAGACTGG-3′; positions 1935 to 1958) and NGO62 (5′-TAGTAGCC TGGCATTCAT-3′; positions 2085 to 2068). The amplification products were separated by agarose gel electrophoresis in 2% NuSieve 3:1 agarose (FMC BioProducts, Rockland, Maine), stained with ethidium bromide, and photographed under UV light. Two of the amplification products were automatically sequenced by Big Dye Terminator sequencing with the ABI Prism 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Foster City, Calif.). These isolates served as size standards when agarose gel electrophoresis was performed.

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TABLE 1. TTV DNA detection in sera and breast milk of mothers and sera of children[a]

PCR result	No. (%) of HCV-infected women (n = 46)		No. of children (n = 23) (rate [%] of TTV transmission) with TTV DNA in serum
	Serum	Breast milk	
HGV PCR positive	15 (32.6)	0	3 (20)
TTV PCR positive	22 (47.8)	17 (77.3)	22 (95.7)

[a] Serum and breast milk samples from 46 HCV-infected women as well as serum samples from their 47 newborns were tested by PCR for HGV and TTV. A positive result was observed for 23 of the children. Nucleic acids of both TTV and HGV were detectable in two of them. TTV was exclusively detectable in breast milk samples by PCR.

HCV PCR and GBV-C/HGV PCR were performed as described recently (6, 23). Relative titers of GBV-C/HGV RNA, HCV RNA, and TTV DNA were determined by serial dilution. To exclude interference of the breast milk with the PCR, the sensitivities of the TTV, GBV-C/HGV, and HCV PCRs were determined by mixing serum of PCR-positive individuals with breast milk. The PCR results for all mixtures of clinical samples were correct, and there was no significant difference in sensitivity between PCR with breast milk samples and PCR with serum samples.

Antibodies against HCV were detected by a screening assay (second-generation HCV EIA; Abbott Laboratories, North Chicago, Ill.) which was performed as recommended by the manufacturer. The results for reactive samples were confirmed by recombinant immunoblot assay as described previously (4).

RESULTS

Sera from 46 HCV-infected women were retrospectively tested for TTV DNA by PCR. Serum samples from their newborns, which had been drawn early after birth, were also tested. TTV DNA was detected in the sera of 47.8% (n = 22) of the women (Table 1). Serum samples from 95.7% (n = 22) of the 23 children of these women were positive by TTV PCR. During follow-up, all children with TTV viremia remained positive by PCR. However, no child developed clinical or biochemical signs of liver disease. The one initially TTV-negative child remained PCR negative within the follow-up period.

The 24 women without detectable TTV viremia gave birth to 24 children. All of these children were negative by TTV PCR from the first investigation onward during the entire observation period.

Breast milk samples were obtained from all mothers. The breast milk samples of all of those who had no detectable TTV in their serum samples were also PCR negative. However, TTV DNA was detected in 77.3% (n = 17) of the breast milk samples from mothers with TTV viremia. The one TTV-negative child who was born to a TTV PCR-reactive mother was breast-fed. Although TTV DNA was detected in breast milk samples from this woman, the child remained negative during follow-up.

GBV-C/HGV RNA was detected in 32.6% (n = 15) of the women. They gave birth to 15 children, and the first serum samples from 20% (n = 3) of these children were GBV-C/HGV PCR positive. All children remained positive by GBV-C/HGV PCR during follow-up. However, similar to the TTV-infected children, none of the GBV-C/HGV RNA-positive children showed clinical or biochemical signs of liver disease. The initially GBV-C/HGV-negative children remained PCR negative during follow-up.

All breast milk samples were screened for GBV-C/HGV by PCR. GBV-C/HGV RNA could not be detected in any of the breast milk samples, regardless of whether the women were viremic.

Of seven women who were positive for both GBV-C/HGV and TTV by PCR, two transmitted both viruses to their children. While TTV DNA was detected in the breast milk of one of these two women, GBV-C/HGV RNA was not. Only TTV DNA was detected in the sera of the children of the other five women.

Transmission of HCV occurred in only 2.2% (n = 1) of the children. The child was negative by the first PCR but was positive from the following examination onward. In addition, no diminution of antibody reactivity in serological assays was observed.

Examination of breast milk samples from all mothers revealed that HCV RNA could not be detected in any of them by reverse transcription-PCR.

DISCUSSION

In the present study, vertical transmission of TTV was investigated in 46 women and their newborns. One of the most relevant findings is that TTV was transmitted from the carrier mothers to all but one of their children. Much lower rates of transmission have been reported for GBV-C/HGV and HCV (5, 13, 22). In our setting, we found transmission rates of 20 and 2.2% for GBV-C/HGV and HCV, respectively. The efficiency of mother-to-infant transmission of blood-borne viruses apparently correlates with the level of maternal viremia (11, 13, 17). The mean titer of TTV DNA in sera from infected mothers was determined by serial dilution to be 2×10^4 copies/ml. Since the mean titers of GBV-C/HGV RNA and HCV RNA were higher (9×10^4 and 5×10^5, respectively), the level of maternal viremia cannot explain the more efficient transmission of TTV.

The time point of transmission could also influence the efficiency of vertical transmission. Perinatal transmission of GBV-C/HGV was assumed earlier but could not be proven (13). In our study, positive GBV-C/HGV and TTV PCR results were always observed within the first days of life. Therefore, early transmission in utero rather than intrapartum transmission must be assumed (1). The prevalence of TTV viremia has been shown to increase during life (8), indicating that TTV is transmitted mainly via nonparenteral daily contact (3, 8, 25). However, the detection of TTV by PCR very shortly after birth is indicative of transmission in utero. Transmission of TTV by environmental sources was observed to occur mostly in children at an age of ≥3 months (3).

It has been reported earlier that HCV-infected newborns are negative for HCV RNA in the first week of life (21). However, in another study HCV-infected children were positive by PCR within the first week after birth (22). In our setting, PCR-positive results were obtained for the second sample drawn within the first month of life and thereafter. Therefore, transmission in utero must be assumed for HCV (1).

As mentioned above, TTV belongs to a different virus family than HCV and GBV-C/HGV, which are both members of the *Flaviviridae* family. The distinct viral construction and the difference in their sizes may be reasons for the different transmission rates. The structure of the viral surface may also contribute to the efficacy of vertical transmission. The organization of TTV needs to be investigated in greater detail to clarify the reason for the highly effective transmission during pregnancy.

Breast-feeding is the recommended means of infant feeding worldwide, since it is associated with lower infant morbidity and mortality than formula feeding (9). However, breast milk has been demonstrated to be a route of transmission for a variety of viruses (7, 10, 20). The question of whether HCV can be transmitted via breast milk has been addressed in many studies (12, 21, 22, 29). Although HCV RNA has sporadically

been found in breast milk (12), all studies have found no evidence of HCV transmission by breast-feeding. In the present study, neither HCV RNA nor GBV-C/HGV RNA was found in breast milk samples. To exclude a methodological problem, GBV-C/HGV- and HCV-positive serum samples were mixed with breast milk. There was no apparent interference between breast milk and the reverse transcription-PCR. The PCR results for all GBV-C/HGV- and HCV-positive samples mixed with breast milk were correct.

TTV DNA was found in nearly three-quarters of the milk samples. TTV DNA was not repeatedly found by PCR in samples of only five mothers. Nevertheless, the children of these women were TTV viremic, indicating that transmission of TTV occurs in utero rather than via breast-feeding. The one TTV-negative child born to a carrier mother was breast-fed. However, the child did not become viremic during follow-up, although TTV DNA was detectable in the mother's breast milk. This also indicates that breast-feeding does not significantly contribute to transmission of TTV. Due to our results we do not regard it as necessary to discourage mothers with TTV viremia from breast-feeding their children. In addition, the clinical impact of TTV is still unclear. It has been discussed as a candidate virus for the induction of fulminant, acute, or chronic hepatitis in humans (16, 19). However, none of the children with TTV viremia in the present study showed clinical or biochemical signs of liver disease. This fact and the high percentage of healthy blood donors with TTV viremia (24, 25, 26) challenge the pathogenic significance of TTV in humans. However, it cannot be excluded that the appearance of clinical signs of hepatitis due to TTV infection may be delayed for several years, as is well-known after vertical transmission of hepatitis B virus (2). Supposing that TTV had the potential to induce hepatitis in humans, the rate of manifestation must be very low considering the high rate of symptomless viremic individuals.

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