TT Virus in Infants Sera and Mother Breast Milks

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ABSTRACT

The purpose of this study was to detect the route of transmission of TT virus infection of newborn infants via their mothers by detection of TTV DNA in breast milk and determination of the percentage of TTV carriers among women with no history of blood transfusion to evaluate the possibility of mother-child transmission. Breast milk samples were collected from 63 normal women with no history of blood transfusion and blood samples were collected at 3 months after birth from 12 infants and 8 infants born by vaginal delivery and abdominal cesarean section, respectively. TTV DNA was examined by semi-nested PCR using primers for the open reading frame (ORF-1). The expected amplification product of the first-round PCR was 286 bp and of the second-round was 271bp.The prevalence of TTV DNA in breast milk of mothers was found to be 31.75% (20/63) and TTV DNA was detected in 60% (12/20) of 20 infants who were borne from TTV positive mothers. Sequence analysis of the cDNA of the second-round of three random selected samples from both positive mother and infant samples showed that the TTV isolate was identified and related to TTV strain HN01 with the accession No. AF098278. TTV was detected in mother's breast milk and some of their infants indicated that breast milk could be a mother-child infection route. These findings suggest that TTV horizontal infection is more likely than vertical infection in mother-child transmission.

INTRODUCTION

Viral infections attracted little attention in the obstetric field until approximately 20 years ago. However, reports of teratogenicity due to the rubella virus and mother child transmission of cytomegalovirus and hepatitis B viruses highlighted the importance of viral infections in the obstetric field. In recent years, each time a new virus is discovered, studies on its effects on mothers and fetuses have received great attention (Iso et al., 2001).

Transfusion transmitted virus (TTV) was first isolated in 1997 from a patient with acute post-transfusion hepatitis (Nishizawa et al., 1997). This fact led to the conclusion that the virus was hepatotropic and could be one of the causative agents of acute hepatitis. Afterwards, the virus was found in other human tissues and serological studies revealed that it was widespread. Multiple tropisms of TTV and the fact of its high incidence in general population are considered to indicate no medical significance of TTV in human pathology (Tomasiewicz et al., 2005).

TT virus (TTV) is a recently described circular DNA virus of about 3.8 kb, which is related to the circoviridae viruses. It is commonly detected in healthy subjects and no association with any specific disease has been established. TTV is an unenveloped, single-stranded, with no external lipoprotein envelopes and is classified into either the Parvoviridae (Berns, 1996) or the Circoviridae family (Lukert et al., 1995), which consists of 3852 nucleotides and most closely resembles the members of the Circoviridae family (Zhong et al., 2001). Meanwhile, different studies suggested that TTV genotypes were so much and could vary frequently (Okamoto et al., 2000a and Toyoda et al., 2001).

The laboratory diagnosis of TTV infection is still rather primitive for many reasons:
1. No tissue culture system of sufficient sensitivity has been described that might be used for TTV isolation;
2. The immune responses elicited by the virus are poorly understood and, in particular, there are no easy to-use serological methods, nor it is known whether antiviral antibodies may be of practical utility in the clinical virology laboratory;
3. Although viremia can be quite substantial, no methods have been reported that detect viral antigen in plasma;
4. None of the many PCR formats assays used for demonstrating viral DNA have been validated for their

ability to detect the entire spectrum of TTV variants.

5. Uncertainties about the clinical relevance of TTV infection have discouraged commercial companies from developing and marketing adequate reagents and diagnostic kits *(Bendinelli et al., 2001).*

TTV infection in healthy blood donors as well as in patients with liver disease has been recently reported in many areas of the world *(Okamoto et al., 1998a, Berg et al., 1999 and Kao et al., 2000)*. These differences in prevalence between countries could be due to the different geographical distribution of TTV infections and the heterogeneity and variability of TTV isolates *(Okamoto, 1998b and Simmonds et al., 1998)*. Variation could also arise due to different experimental methods to determine TTV infection, such as the primers used and the sensitivity of the PCR methods employed *(Mizokami et al., 2000).*

The infection rate of TTV DNA varies largely in healthy population and patients with liver diseases, but generally, it is higher in patients than in healthy donors *(Abe et al., 2000 and Okamoto et al., 2000b)*. However, the role of these viruses in disease remains uncertain.

Previous epidemiological studies suggest that TTV is mainly transmitted by blood *(Matsubara et al., 2000; Chan et al., 2000 and Luo et al., 2000)*, blood products *(Bjoro et al., 2001)* and body fluid routes *(Inami et al., 2000)*. It can also be transmitted by mother-to-child vertical *(Morrica et al. 2000, Schröter et al., 2000)* and horizontal route *(Iriyama et al., 1999 and Kazi et al., 2000)*, fecal-oral route *(Ukita et al., 1999 and Itoh et al., 2001)* and sexual route *(Krekulova et al., 2001).*

Okamoto et al. *(1998b)* found some TTV-positive patients among patients without any history of blood transfusion, although the percentage was lower than among those with a history of blood transfusion, suggesting that this virus may be transmitted through routes other than the blood and that further studies are necessary to identify non-blood transmission routes.

Breast-feeding is the recommended means of infant feeding worldwide, since it is associated with lower infant morbidity and mortality than formula feeding *(Lawrence, 1994).* However, breast milk has been demonstrated to be a route of transmission for a variety of viruses *(Hino et al., 1994 and Oxtoby, 1988)*. Furthermore, variation in the TTV prevalence in children from 5.1% in Japan *(Goto et al., 1999)*, to 54% in the Democratic Republic of Congo and there was a possible involvement of other specific environmental factors in the acquisition of TTV infection. To date, the routes of mother-to-infant transmission of TT virus (TTV) have not been fully elucidated. The Aim of the present study is to determine the

positive TTV response rate among women without a history of blood transfusion and determine percent transfer into breast milk and the presence or absence of mother- child transmission according to the type of delivery in women with positive TTV responses.

MATERIALS AND METHODS

Materials:
During of the period of March to December 2006, breast milk was collected from 63 women with no history of blood transfusion from pediatric follow up clinic of Al Galaa teaching hospital. Ten milliliters of breast milk was collected from the mothers at the 3rd. month after breath by vaginal delivery or abdominal cesarean section. Also, Three milliliters of venous blood was collected, 3 months after birth, from the back of the hand of 20 children (12 children born by vaginal delivery and 8 children born by abdominal cesarean section). All collected children's blood samples were breast-fed and were from TTV positive mothers in both cases. Informed consent was obtained from all women before sampling. Serum samples were separated from blood samples obtained to determine positive TTV response rates.

Detection of TTV DNA using polymerase chain reaction (PCR):
TTV DNA extraction:
Nucleic acids were extracted immediately after serum and breast milk samples were collected. Total viral DNA was extracted from 200µl of serum in a biosafety level 2 laboratories using DNA isolation kit (DNeasy, Qiagen®, cat. No.69504) according the manufacturer instructions. Briefly, the isolation method utilizes the ability of nucleic acids to absorb to silica membrane in the presence of a chaotropic salt. Serum sample was treated with buffer containing proteinase K and silica where nucleic acids are bound to silica surface of magnetic particles. Since the binding process is specific for nucleic acids, the bound nucleic acids are purified from salts, proteins and other impurities by washing. A low salt buffer is used to elute the DNA from silica membrane. The extracted nucleic acids from serum and breast milk samples were stored immediately at –80°C until examination.

TTV PCR:
Polymerase Chain Reaction (PCR) for TTV DNA was performed according to an already described method by *Okamoto et al. (1998b)*, with slight modifications. We used two set of primers in the present experiment; primers A (NG059 andNG061) and primers B (NG063 andNG061).

The first-round PCR was carried out in 50µl reaction mixture containing 7µl of the extracted TTV DNA solution,

as a template for N22 region of the open reading frame (ORF-1), 10µl 10x PCR Buffer, 4µl MgCl$_2$ (25mM), 0.5µl (5U/µl) GoTaq DNA Polymerase (Promega, Cat. No. M8305), 1µl (50 pmol/µl) of NG059 sense primer (5'-ACAGACAGAGGAGAAGGCAACATG-3'), 1µl (50 pmol/µl) of NG063 antisense primer (5'-CTGGCATTTTACCATTTCCAAAGTT-3'), 4µl of dNTPs (10mM each, Promega Cat. No. U1240) and 22.5µl DEPC treated water. For PCR amplification, an initial denaturation of 5min at 95°C and 35 cycles at 94°C for 30sec., 58°C for 60 sec. and 72°C for 60sec and an additional extension at 72°C for 4min was applied using using PCR system (Biorad®). Thereafter, the second round PCR was carried out using 1 µl of the first-round PCR product, using 1 µl (50 pmol/µl) of NG061 sense primer (5'-GGCAACATGYTRTGGGA TAGACTGG-3'), where Y=T or C; R=A or G) and 1µl (50 pmol/µl) of NG063 antisense primer for 25 cycles under the same aforementioned conditions. 10 µl of the PCR products was electrophoresis on 2% agarose gel containing ethidium bromide. Bands were visualized under UV light using UV transilluminator. Selected bands were cut and purified for direct sequencing.

Direct sequencing of cDNA fragments:

For sequencing, PCR products were purified from agarose gel using QIAquick Gel Extraction kit (Qiagene, Cat. No 28704) and 20-75 ng of the products was used for single-sided amplification with 1µl (3.2 pmol/µl), PCR primers using ABI Prism® BigDye® Terminator Cycle Sequencing Ready Reaction kit version 1.1, according to the instructions of the manufacturer. The total reaction volume, 20µl, of determinator products was purified by ethanol precipitation with 2µl (3M) sodium acetate and 3µl (5mg/ml) linear acrylamid and cold absolute ethanol. The nucleotide sequences were determined directly with an ABI Prism® 310 Genetic Analyzer (Applied Biosystems).

Analysis of nucleotide sequence:

The sequences obtained and extensive sequence information from the GenBank database, forty TTV sequences from different strains and isolates, were aligned by utilizing the Clustal X program *(Thompson et al. 1997)*. A phylogenetic tree was reconstructed with neighbour-joining analysis using TreeView version 1.6.6 (by Dr. Page R.D.M., Division of Environmental and Evolutionary Biology Institute of Biomedical and Life Sciences, University of Glasgow, UK).

RESULTS

The experiments presented here have checked the prevalence of TTV DNA in different breast milk from mothers and their infant sera to have an insight into the route of transmission

of these viruses. We used two sets of primers (Table 1) to increase the specificity and sensitivity of the detection of TTV DNA. The product of the first-round PCR was of 286bp and that of the second-round PCR was of 271 bp (Table 1 and Figure 1).

Table 1: Positivity of TTV DNA in serum from new born and mother's breast milk.

	Primers	Sequence of primer	PCR product
1st. round	NG059	sense: 5'ACAGACAGAGGAGAAGGCAAC ATG-3'	286 bp
	NG063	antisense: 5'CTGGCATT TTACCATTTCCAAAGTT-3'	
2nd. round	NG061	Sense: 5'-GGCAACATG YTRTGGGATAGACTGG-3',	271 bp
	NG063	antisense: 5'CTGGCATT TTACCATTTCCAAAGTT-3'	
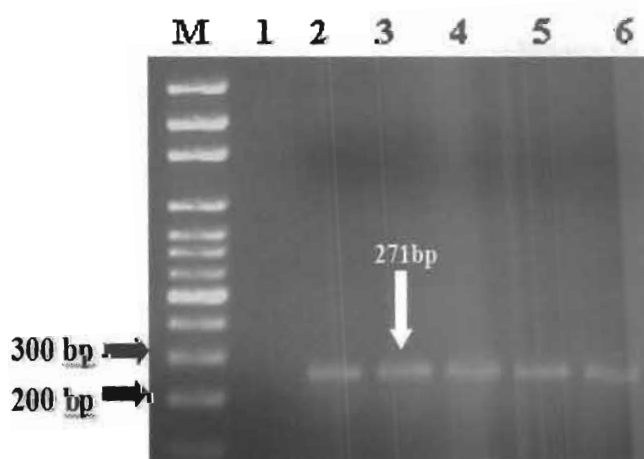
Figure 1: Semi-nested PCR Amplification product of 271bp for the region N22 of ORF-1 specific for the TTV DNA (lanes 26-) of extracted DNA from sera of newborn and milk samples of their lactating mothers.
Lane 1: Negative control sample to neglect the possibility of contamination.
Lane 2, 4, 6: Positive breast milk samples.
Lane 3, 5: Positive serum samples of newborn-breast feeding from same previous mothers.
Lane M: 100 bp DNA ladder.

TTV-DNA in breast milk in TTV-positive women:

The results revealed that TTV DNA was detected in 31.75% (n: 20/63) of the breast milk samples from mothers with TTV viremia. Representative results of the TTV-DNA detection are shown in (Tables 2 and 3). The breast milk of the remaining 43 cases were TTV negative. These results showed that not all mothers with TTV viremia excrete TTV DNA in their breast milk.

Positive TTV response rates in children born from TTV positive mothers:

The prevalence of TTV DNA in different samples of the new born infants is summarized in (Table 2). Considerable number

of new born infants delivered by TTV DNA-positive mothers expressed TTV DNA in their serum when tested by PCR. Also, no TTV DNA was detected in the newborn babies born from TTV DNA negative mothers. Indeed, all TTV DNA-positive newborn infants were born from only TTV DNA-positive mothers. The results revealed that 60% (n: 12/20) of children, who breast feeding from mothers with TT viremia as shown in (Tables 2 and 3), were TTV positive at the 3rd month after birth (Seven children born by vaginal delivery and five children born by abdominal cesarean section). Furthermore; eight newborns from TTV positive women were TTV negative, at the 3rd month after birth, regardless of whether they were born by vaginal delivery or by abdominal cesarean section (Five children born by vaginal delivery and three children born by abdominal cesarean section).

## PCR product nucleotide sequence analysis:

For further analysis of the obtained TTV PCR product, nucleotide sequence of three random samples from both mother's breast milk and newborn serum were analyzed. The two nucleotide sequences (216 base pair), from both mother's breast milk and newborn serum were identical when aligned together. The nearest forty TTV sequences collected from GenBank database were aligned with our sequence to produce a phylogenetic tree (Figure 2). The sequence analysis of 216 bp of the purified PCR product revealed that our sequence is a novel isolate and closely related to the TTV strain HN01 detected and isolated from China with the accession No. AF098278.

Table 2: Prevalence of TTV DNA in serum from new born and breast milk mothers.

Source	No. of TT virus infected [a]	Percentage rate of TTV transmission
Mother's breast milk	20/63	31.75%
New born sera[b]	12/20	60%

Table 3: TTV DNA detection in breast milk of mothers and sera of children according to the type of delivery.

Type of delivery	No. and (%) of TT virus infected women (n= 20)	No. of children infected and (%) rate of TTV transmission
	Breast milk DNA	Serum
Cesarean Section	8/20 (40%)	5/8(62.5%)
Vaginal Delivery	12/20 (60%)	7/12 (58.3%)
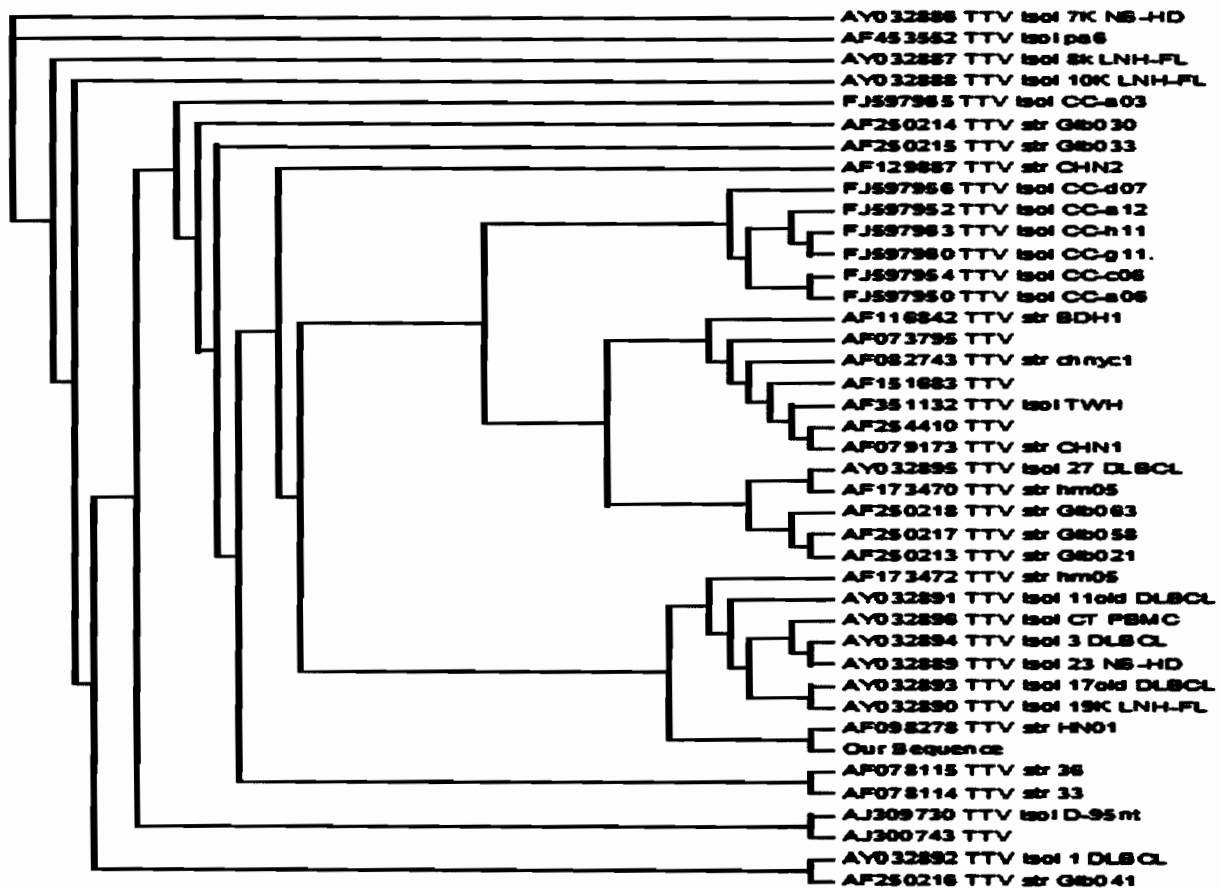
Figure 2: A neighbour-joining phylogenetic tree for forty reported TTV sequences with our obtained sequence.

DISCUSSION

TTV reproduces itself in the liver, entering not only the systemic circulation but the biliary tract and is eliminated into feces via the intestine *(Okamoto et al., 1998a)*. Recent studies have shown that it is also present in peripheral monocytes and marrow cells *(Simmonds et al., 1998)*. The fact that TTV is also detected in persons with no history of blood transfusion suggests that it can be transmitted not only via blood, but also by contaminated foods *(Okamoto et al. 1999; Davidson et al. 1999 and Ukita et al., 1999)*.

Since the discovery of TTV, the most important question concerning this new virus is its level of pathogenicity. There is no general consensus about this issue at present, although most investigators seem to suspect that it is not pathogenic. A clear answer to this question needs to be given as quickly as possible. In the meantime, obstetricians must pay close attention to clinical symptoms of mothers and newborns if this virus is detected. It is also important to obtain more information on routes of mother-child infections.

Numerous studies have suggested that the parenteral transmission via transfusion of contaminated blood and blood products is the most common route of TTV infection *(Simmonds et al., 1998)*, the detection of TTV in many individuals with no history of blood transfusion indicates that other routes of transmission of TTV may exist. This assumption has been further supported by the detection of TTV in saliva *(Ross et al., 1999)*, breast milk *(Schröter et al., 2000)*, semen *(Okamoto et al., 1998a)* and suggested a possible fecal-oral transmission. Although this high prevalence of TTV DNA in these body fluids may be responsible for the maintenance of global reservoirs of TTV, the route of transmission of this virus remains elusive *(Matsubara et al., 2001)*.

Breast-feeding is the recommended means of infant feeding worldwide, since it is associated with lower infant morbidity and mortality than formula feeding *(Lawrence, 1994)*. However, breast milk has been demonstrated to be a route of transmission for a variety of viruses *(Hino et al., 1994 and Oxtoby, 1988)*.

One of the most relevant findings is that TTV was transmitted from the carrier mothers to 60 % of children. However, no child developed clinical or biochemical signs of liver disease. All the children of the 43 women without detectable TTV virus were also during the entire observation period. However, TTV DNA was detected in 31.75% (20/63) of the breast milk samples from mothers with TTV viremia. This indicates that breast-feeding significantly contribute to transmission of TTV. Due to previous results they regard it as not necessary to

discourage mothers with TTV viremia from breast-feeding their children because the clinical impact of TTV is still unclear. It has been discussed as a candidate virus for the induction of fulminate, acute, or chronic hepatitis in humans *(Nishizawa et al., 1997 and Okamoto et al., 1998a)*. However, none of the children with TTV viremia in the present study showed clinical or biochemical signs of liver disease.

TTV DNA was not found in any newborn infants which born from negative TTV DNA mothers. As well as negative TT virus showed in eight infants from TTV DNA-positive Mothers. This data is consistent with the reported publications in this regard *(Yokozaki et al., 1999; Hsieh et al., 1999; Sugiyama et al., 1999 and Toyoda et al., 1999)*. However, the routes of mother-to infant transmission of TT virus (TTV) have not been elucidated, our data indicate that the detection of genotype of TTV DNA may not been induce intrauterine transmission. Therefore, more studies will be needed with larger sample size to elucidate the routes of mother-to-infant transmission of TT virus. In fact, there have been few studies about the prevalence of TTV in the infants and about the mother-to-child transmission of this virus *(Yokozaki et al., 1999; Prati et al., 1999; Hsieh et al., 1999; Sugiyama et al., 1999; Goto et al., 1999 and Toyoda et al., 1999)*. Moreover, it is not known whether this virus can be transmitted through intrauterine route.

TTV was detected in children's serum in 58.3% (7/12) of cases 3 months after birth by vaginal delivery and the breast milk of the mothers of all these children contained the same type of TTV, suggesting that horizontal TTV infection is more likely than vertical infection. Because vaginal secretions contain TTV virus infection in the parturient canal has also been suspected *(Iso et al., 2001 and Matsubara et al., 2001)*. Our findings support this hypothesis because TTV was detected in children at 3 months after birth. However, infection may not be detected for several months after birth due to antibodies transferred from mothers. TTV may have been detected for the first time 6 months after birth for this reason.

The prevalence of TTV viremia has been shown to increase during life *(Desai et al., 1999)*, indicating that TTV is transmitted mainly via nonparenteral daily contact *(Mizokami et al., 2000)*. However, the detection of TTV by PCR very shortly after birth is indicative of transmission in uterus. Transmission of TTV by environmental sources was observed to occur mostly in children age of 3≥ months *(Okamoto et al., 1998a)*.

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 *(Nishizawa et al., 1997, Okamoto et al., 1998a)*. However, none of the children with TTV viremia in the present study showed clinical or biochemical signs of liver disease.

CONCLUSION

because TTV was detected in breast milk from TTV-positive women and some of their infants were TTV-positive, suggests that this may also act as a permanent source of this virus i.e. breast milk was thought to be a mother-child infection route. These findings suggest that horizontal infection is more likely than vertical infection in mother-child transmission of TTV.

construction:

As well as 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.

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