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TT Virus Infection in Intravenous Drug Users

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SUMMARY Our group has investigated 201 intravenous drug users for the presence of TTV DNA by means of polymerase chain reaction (PCR). The majority of the individuals tested were male, their age ranging from 16 to 63 years, and the duration of intravenous drug use from one to 40 years. TTV DNA was present in 62 of the 201 IVDUs (30.8%) with its prevalence on the ascent between the age groups below 20 and those between 21 and 30 years, as well as between the groups below 60 and between 60 to 120 months' duration of drug intake, respectively. When tested again after 9 months, nine IVDU (23.7%) were found TTV negative by PCR hinting at potential immunological clearance. Our control group comprised 200 healthy blood donors, 7% of whom were found to harbor TTV DNA in an age-dependent fashion, as observed with the IVDU. From the liver function tests performed we could not detect any statistically significant difference regarding ALT elevation observed in TTV-positive compared with TTV-negative individuals. To date, TTV does not appear to cause any serious liver disease in the majority of cases examined.

TT virus (TTV) represents a novel virus which had first been isolated by representational difference analysis (RDA)[1] as a clone of 500 nucleotides from the serum of a patient with post-transfusion hepatitis of unknown etiology, who exhibited elevated ALT levels indicating liver inflammation.[2] Subsequently, the same team of researchers have molecularly cloned and characterized the agent as a non-enveloped, single-stranded DNA virus. By now, approximately 3.7 kb of its genome harboring two potential open reading frames have been sequenced.[3]

In order to improve diagnostic efficacy and to elucidate the genetic characteristics of TTV, a group of Japanese researchers sequenced a ~2.4 kb segment of the TTV genome derived from eight Japanese isolates. The region sequenced was found to contain a long open reading frame (ORF-L) coding for a protein of 768-770 amino acids highly rich in arginine at its N-terminus and harboring three or four asparagine-linked glycosylation sites clustered in its central portion. Comparison of this long ORF encoded protein with those of known single-stranded DNA viruses suggested a possible phylogenetic similarity of TTV with chicken anemia virus, which belongs to the Circoviridae family rather than Parvoviridae.[4]

To date, five different genotypes of TTV have been isolated from serum of infected individuals in Japan where the virus has been shown to exhibit a high prevalence in patients at risk for parenteral exposure, such as hemophilia and hemodialysis patients, or intravenous drug users (IVDU). Likewise, TTV was detected among patients

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with non-A-to-G fulminant hepatitis and chronic liver disease at a frequency amounting to almost 50%.[3]

Phylogenetic analysis performed on TTV DNA isolated from patients with post-transfusion as well as acute and chronic hepatitis of unknown etiology in Thailand revealed three different genotypes of TTV with six distinct subtypes. Moreover, TTV was found to occur more frequently in patients with liver cirrhosis and hepatocellular carcinoma than in those with chronic hepatitis.[5] A group of researchers investigating the frequency of TTV viremia in UK blood donors and the extent to which this virus contaminates blood products, such as clotting factors, concluded from their findings that TTV viremia is rather widespread in the blood donor population and that its transmission through transfusion of blood components may have occurred extensively.[6] A study conducted in Japan on five patients with type B or C hepatocellular carcinoma reported TTV DNA to be present in sera from all, and in feces from three of the patients indicating the mode of transmission not to be restricted to the parenteral route.[7] Previously, we had conducted a study in order to elucidate the prevalence of TTV infection among the members of various groups at high risk of contracting blood-borne viruses in Thailand.[8] Upon integration of these preliminary results, the purpose of the present study was to establish the prevalence of TTV infection among intravenous drug users (IVDUs) in comparison with healthy blood donors in Thailand and to correlate the results with the medical history as well as the habits of intravenous drug users, thereby establishing the route of infection by blood borne viruses in members of high risk groups versus normal controls.

MATERIALS & METHODS

Population study

IVDU: From December 1997 until mid January 1998, samples of venous blood were collected from a total of 201 IVDUs, all of whom had previously also been examined for the presence of GBV-C virus as published elsewhere.[9] All of them had been using drugs intravenously for varying periods of time, and for the purpose of routine check-up, attended the outpatient service at the Drug Addict Centre, Health Department, Bangkok Metropolitan, for methadone therapy. Before drawing blood, each individual was interviewed by means of a standard questionnaire. In October 1998, in order to investigate the potential of immunological clearance of the virus, the patients found positive for TTV DNA were invited for follow-up and again, blood was drawn and serum was subsequently separated for a repeated PCR-based test for the presence of TTV DNA. At this juncture, it turned out that of the 62 initially TTV-positive IVDU only 38 appeared for the follow-up study and were available for the second PCR-based test.

Voluntary blood donors: Two hundred sera collected consecutively and at random from voluntary blood donors at the National Blood Centre, Thai Red Cross, between November 25 and December 2, 1996 were examined for the presence of hepatitis viruses. These 200 specimens, which served as control samples, had been obtained from 150 men and 50 women, respectively, their ages ranging from 19 to 60 years (mean age 32.7 ± 10.5 years).

All patients were informed as to the objective of the study to elucidate the prevalence of various hepatitis viruses and subsequently provided their consent. Blood was obtained during examinations; sera were separated by centrifugation and stored at -70° C until subjected to the respective test

Laboratory methods

TTV DNA extraction

DNA was extracted from 50 µl of serum, twice per each sample, with proteinase K/SDS in Tris buffer, followed by phenol-chloroform extraction and ethanol precipitation. The pellet was dissolved in 20 µl of sterile water and directly subjected to polymerase chain reaction.

TTV DNA detection

TTV DNA was detected by polymerase chain reaction using semi-nested primers as published elsewhere.[8] Briefly, the amplification reaction was performed in a 50 µl reaction volume containing 1 U of *Taq* polymerase (Perkin Elmer Cetus), and each of four deoxynucleotide triphosphates at a concentration of 200 µM, primer pairs NG 059 and NG 063 for the first round and NG 061 and NG 063 for the second round, at a concentration of 1 µM each, 10 mM Tris, 1.5 mM $MgCl_2$ and 5 µl of each DNA sample. According to Okamoto *et al.*[3] the nucleotide sequences of the TTV

primers derived from the N-22 region, which represents the most conserved sequence of the 5 genotypes described to date, were: NG 059 (5'-CAG ACA GAG GAG AAG GCA ACA TG-3'), NG 061 (5'-GGC AAC ATG TTA TGG ATA GAC TGG-3') and NG 063 (5'-CTG GCA TTT TAC CAT TTC CAA AGT T-3'). The first round amplification reaction using primer pair NG 059 and NG 063 was performed for 30 cycles (denaturation at 94°C for 36 seconds, annealing at 55°C for 42 seconds, and extension at 72°C for 90 seconds, final extension at 72°C for 10 minutes). The second round of amplification was performed using 2 µl of the PCR product along with primer pair NG 061 and NG 063 for 30 cycles under identical conditions in a final reaction volume of 20 µl. Upon conclusion of the PCR, the reaction mixture was centrifuged for 1 minute at 10,000 rpm, and 10 µl each of the amplified DNA were fractionated by electrophoresis in a 2% agarose gel stained with ethidium bromide and visualized under UV light. The product band shows at 271 base pairs. The gels were photographed on a UV light box. Sera obtained from IVDU and known to be positive for TTV-DNA and sterile water were used as positive and negative controls, respectively.

ALT determination

Serum ALT was determined by automated chemical analyser (Hitachi 911) at the Central Laboratory, Chulalongkorn Hospital. In our laboratory, normal values for healthy individuals are defined as ranging between 0 and 38 U/l, whereas values above 38 U/l are considered elevated.

Data analysis

We expressed the data by determining their respective arithmetic mean values along with the standard deviation (SD), using the unpaired *t*-test, Mann-Whitney U test and Chi-square test for statistical analysis.

RESULTS

We obtained venous blood from 201 IVDUs in all, 11 of whom were female, the remaining 190 male, their ages ranging from 16 to 63 years with a mean age of between 28 and 33 years, and the duration of intravenous drug use ranging from one to forty years with a mean duration of between 150 and 143 months in the TTV-positive and TTV-negative subjects. The drug injected by all of them had been heroin, between one and ten times per day. Approximately 50% smoked marijuana in addition and about 20% took various tranquillisers, in a few cases opium (four) and morphine (two). The overall prevalence of TTV DNA was established at 30.8%, in that 62 of the 201 IVDUs tested were found positive, with the frequency of infection being on the ascent between the age groups of below 20 years and between 21 and 30 years. Thenceforward it remained rather steady until undergoing a sharp decline in the age group over 50 years (Table 1, Fig. 1). Likewise, the duration of drug use appeared to lead to an increase in TTV-positive sera attaining a steady state between approximately 120 and 240 months (Table 1, Fig. 2). No statistically significant difference regarding age, duration, ALT level and ALT elevation was observed between the IVDU groups with and without detectable TTV DNA. The ALT levels determined for TTV-positive and -negative subjects alike were elevated, though in a slightly higher percentage among the TTV-positive ones (32.2 vs. 25.2 %) (Table 1). Of the 201 IVDUs 121 were also tested for anti-HIV antibody, 33 (27.3%) were found positive versus 88 (72.7%) negative.

In those healthy blood donors found to harbor TTV DNA, the viremia was clearly age-dependent in that its prevalence steadily increased with ascending age groups until reaching a plateau with that of between 41 to 50 years (Fig. 1).

The second PCR-based test performed on samples obtained from identical sources, but approximately nine months later, showed that 29 out of 38 of the 62 formerly TTV- positive IVDUs, who were still available for follow-up (76.3 % of 38) still carried the virus.

DISCUSSION

The present study was performed with 201 IVDUs all of whom, due to their habit of intravenous drug use accompanied by needle sharing, are at an increased risk of exposure to parenterally transmitted viruses, such as HIV, HBV, and HCV. Accordingly, our results also show a high prevalence of TT virus infection, one more parenterally transmissible agent to be added to the already existing list, among the members of this particular high-risk group. The results obtained in the present study furthermore show a rather strong similarity to those garnered in thalassemia children having undergone multiple blood transfusions. In their case the prevalence of GBV-C in-

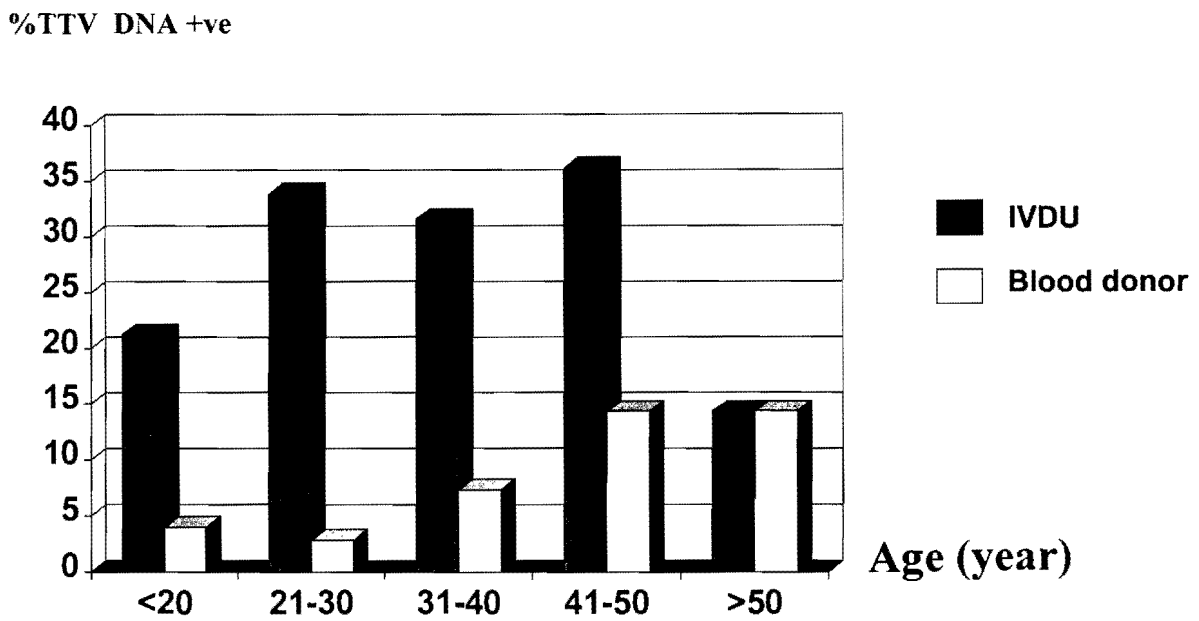

Fig. 1 Percentage of TTV DNA in relation to the respective age of the IVDUs and blood donors.

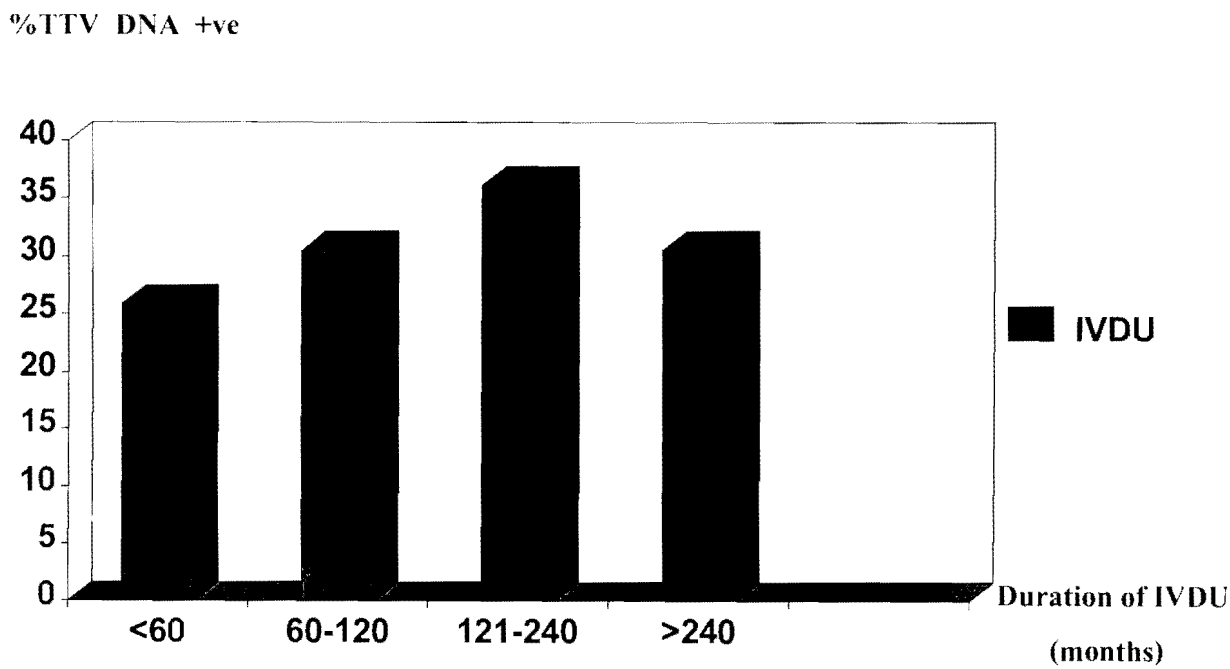

Fig. 2 Percentage of TTV DNA in relation to the duration of intravenous drug use.

Table 1 Demographic data of IVDUs separated into groups with and without detectable TTV DNA.

	TTV DNA	
	Positive	**Negative**
Number	62	139
Sex (M:F)	62:0	130:9
Age in years		
≤ 20	7	26
21 - 30	23	45
31 - 40	18	39
41 - 50	13	23
> 50	1	6
Mean age in years and S.D. (range)	32.34 ± 9.84 (16 - 59)	31.82 ± 10.60[b] (16 - 63)
Duration of IVDU in months*		
< 60	14	40
60 - 120	18	41
121 - 240	13	23
> 240	15	34
Mean duration of IVDU in months and S.D. (range)	150 ± 111 (12 - 480)	143 ± 111[a] (12 - 396)
Mean ALT in U/l and S.D. (range)	40.16 ± 47.51 (5 - 249)	38.58 ± 59.36[b] (2 - 373)
Number of IVDUs with ALT elevation (> 38 U/l)	20	35[c]

[a]Unpaired *t*-test ($p > 0.05$)
[b]Mann-Whitney U test ($p > 0.05$)
[c]Chi-square test ($p > 0.05$)
*No data available in 3 patients

fection amounted to 32.6% compared to that determined for healthy subjects supposed to be at low risk regarding infection by blood borne viruses, e.g. 1.2% in adolescents, 5% in voluntary blood donors and pregnant women.[10] Among the 201 IVDUs tested in this study 62 (30.8%) were positive for TTV DNA, a finding correlating well with the subjects comprising the members of a high risk group.

With regard to TTV infection, a rather steep ascent can be noted between the age groups below 20 and those between 21 and 30 years, after which the percentage of positive sera remains rather steady until sharply declining again with the age group above 50 years. Yet, due to the small size of the patient group above 50 this result ought to be viewed with caution, in that it can be surmised that had the sample size been more representative, the percentage of TTV-positive sera might well have been found within the same range as that determined for the previous three age groups. Hence, the prevalence of TTV infection apparently levels out at approximately 35% within the age group of between 21 and 30 years, from whenceforward it remains quite steady save for some random decrease which might prove intermediate.

This assumption is corroborated by the results obtained with healthy blood donors, in whom the prevalence of TTV viremia was clearly age-dependent, attaining a saturation level with the age group above 40 years.

Very similar results were obtained upon plotting the percentage of TTV-positive sera against the duration of intravenous drug use. Here, an unmistakable increase in TTV DNA positive sera could be found, beginning with the group be-

low a 60-month duration, ascending past the group of between 60 and 120 months, until reaching a peak with the group of a 121-to-240-month duration. As with the results obtained with the different age groups, the data presented here also indicate long lasting chronicity of TTV infection prior to eventual immune clearance which might or might not be complete. Alternatively it can be speculated that due to the novelty of TTV, infections detected in individuals with a history of more than 20 years intravenous drug use might well have been acquired very recently, thereby rendering any theories regarding a potential immune response invalid.

On the other hand, if we go along with Nishizawa *et al.*[2] and presume antibodies to TTV to be raised by the host, irrespective of TTV being a non-enveloped virus, and if we further presume that these antibodies account for the transient infections described by the above group of researchers in two cases, as well as in the nine cases of IVDU observed by us, then it would still be premature to speak of immune clearance in the true sense of the word. Hence, the apparent clearance of TTV in 9 of the 38 TTV-positive IVDU might be deceptive in that these people might have been examined at the time when they had turned negative, yet might have been re-infected by a different strain of TTV by now. In other words, with the small number of persons tested, who furthermore belong to high-risk groups, the results obtained cannot be interpreted conclusively.

In addition, those patients infected with TTV showed normal ALT, and of those with enzyme elevation, about half had levels raised just above the upper limit of normal. Therefore, future studies for determining the clinical significance of novel viruses, the tropism of which first of all needs to be elucidated, are certainly required.

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