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Prevalence and Phylogenetic Analysis of TT Virus DNA in Western India

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In western India, TT virus (TTV) DNA positivity varied from 6.7% (5 of 75) in chronic hepatitis patients to 24.4% (10 of 41) in hemophiliacs; 7.4% (4 of 54) of voluntary blood donors had circulating TTV DNA. Phylogenetic analysis revealed a predominance of genotype 1a. In India, TTV is transmitted mainly by nonparenteral routes and is not an important cause of chronic liver diseases.

Recently, a novel DNA virus termed TT virus (TTV) on the basis of the initials (T.T.) of one of the infected patients investigated was found to be associated with posttransfusion non-A to -G hepatitis cases in Japan (15). Although the virus shares some of the characteristics of parvoviruses, significant sequence similarity to the known parvoviruses has not been observed (17). TTV was found to be highly prevalent in Japan, with significantly higher proportions among patients with parenteral risk, patients with fulminant hepatic failure of non-A to -G etiology, and patients with chronic liver diseases (CLDs) of unknown etiology (17). A recent study from Japan (10) showed that rates of detection of TTV DNA did not differ statistically between patients with non-A to -E hepatitis and patients with hepatitis A, B, or C or controls. The clinical characteristics were comparable for patients with or without TTV DNA.

In order to investigate (i) whether TTV represents the causative agent for the residual non-A to -G hepatitis and (ii) the extent of transfusion-associated transmission of this virus, studies are being conducted in different parts of the world (4, 7, 8, 14, 18, 19, 21). The sequence data generated document the existence of several genotypes of TTV (7, 8, 14, 17, 21, 22). So far, no information is available from India. To ascertain the extent of TTV infection among Indian patients, we studied certain categories of individuals from western India. These included voluntary blood donors (n = 54), paid plasma donors from a commercial plasmapheresis unit (n = 31), hemophiliacs (n = 41), patients suffering from CLDs (n = 75) (including 54 who were hepatitis B virus [HBV] DNA positive and 21 who were HBV DNA as well as HCV RNA negative), patients undergoing hemodialysis (n = 24), and symptomless hepatitis B surface antigen (HBsAg) carriers with consistently normal serum alanine aminotransferase levels for a period of 3 years (n = 83). Aliquots of stored (−20°C) serum samples were utilized for TTV DNA screening.

All serum samples were screened for the presence of TTV DNA by nested PCR. DNA isolation was carried out using DNAZOL reagent (GIBCO-BRL Life Technologies) according to the manufacturer's instructions, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min for first- and second-round PCRs. Primers representing part of open reading frame 1 (ORF1), as described by Simmonds et al. (21), were used. Primers for the first round of PCR were A5430 (5′-CAG ACA GAG GAG AAG GCA ACA TG-3′) and A5427 (5′-TAC CAY TTA GCT CTC TAT TCT WA-3′). Primers for the second round of PCR were A8761 (5′-GGM AAY ATG YTR TGG ATA GAC TGG-3′) and A5432 (5′-CTA CCT CCT GGC ATT TTA CCA-3′).

Amplified DNA fragments (278 bases) were minicolumn purified (Wizard; Promega). Both strands of column-purified PCR products were sequenced using a *Taq* dye terminator cycle sequencing kit (Perkin-Elmer) and an automatic sequencer. Twenty TTV DNA-positive samples were sequenced. These included three samples each from voluntary blood donors, hemophiliacs, and HBsAg carriers; five from patients suffering from CLDs; four from paid plasma donors; and two from patients undergoing hemodialysis.

Phylogenetic analysis was based on the comparison of a 171-nucleotide fragment of ORF1. MEGA (11) and PHYLIP version 3.5c (6) software was employed to determine the phylogenetic status of different TTV isolates. For analysis with MEGA, the Jukes-Cantor algorithm was utilized, employing the neighbor-joining method. The reliability of different phylogenetic groupings was evaluated by using the bootstrap test (1,000 bootstrap replications) available in MEGA. For PHYLIP program-based analysis, the Jukes-Cantor algorithm was used, employing the neighbor-joining method with and without midpoint rooting. For evaluation of the results obtained, bootstrap analysis was performed (SEQBOOT; 1,000 bootstrap replications).

Fisher's exact test and chi-square tests were used for comparison of two proportions. Odds ratios (ORs) were calculated for the assessment of risk of TTV infection in different categories in comparison with voluntary blood donors. The software EPI INFO (version 6.02) was used to carry out the computations.

Prevalence of TTV DNA. Table 1 documents the TTV DNA positivity among different groups screened in nested PCR. Voluntary blood donors exhibited 7.4% (4 of 54) positivity. None of these 54 voluntary blood donors were positive for HBsAg or antibodies to HCV (data not shown). The prevalence of TTV DNA among voluntary blood donors has been shown to vary from 1% in the United States (3) and 1.9% in the United Kingdom (21) to 10.7% in the United States (5) and 12% in Japan (17). Considering recent reports (5, 12) of underreporting of TTV on the basis of PCR assays utilizing the primers used by Simmonds et al. (21), higher exposure rates may be found among Indian populations following the use of more efficient primers. All the same, this first report from India documents circulation of TTV for the last 10 years at least.

Of the 83 symptomless HBsAg carriers (a subset of the voluntary blood donors screened earlier), 7 were found to be

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TABLE Frequency of TTV DNA positivity

Category	No. positive/no. tested (%; 95% confidence interval)	OR (95% confidence interval for OR
Voluntary blood donors	4/54 (7.4; 0.4–14.4%)	1 (reference value
Paid plasma donors	4/31 (12.9; 1.1–24.7%)	1.85 (0.35–10.7)
Hemophiliacs	10/41 (24.4; 11.2–37.4%	4.03 (1.03–18.9)
Patients undergoing hemodialysis	2/24 (8.3; 0.0–12.3%)	1.14 (0.13–8.6)
CLD patients	5/75 (6.7; 1.0–12.3%)	0.89 (0.19–4.74)
HBV DNA-positive CLD patients	5/54 (9.3; 1.5–17%)	1.28 (0.27–6.8)
HBV DNA- HCV RNA-negative CLD patients	0/21	0 (0–3.9)
HBsAg carriers	7/83 (8.4; 2.5–14.4%)	1.15 (0.28–5.6)

TTV DNA positive (8.4%). Thus, HBsAg-positive or -negative voluntary blood donors were not significantly different ($P > 0.9$) with respect to TTV DNA positivity. A significantly higher proportion ($P < 0.05$) of hemophiliacs (10 of 41; 24.4%) were found to have circulating TTV DNA when compared to voluntary blood donors, patients suffering from CLDs, and HBsAg carriers. Based on the estimation of ORs, hemophiliacs appeared to be at a fourfold-higher risk of TTV infection (Table 1).

The TTV DNA positivity among CLD patients (5 of 75; 6.7%) and voluntary blood donors was not significantly different ($P > 0.5$). Fifty-four of 75 patients suffering from CLDs were HBV DNA positive. All five TTV DNA-positive CLD patients belonged to the HBV DNA-positive subset of CLD patients examined. The comparable prevalence rates among CLD patients and voluntary blood donors suggest that TTV may not be pathogenic in most infected individuals, confirming observations from the United Kingdom (14), Taiwan (8), and recently, Japan (10). However, these observations are in contrast to other reports from Japan (17), the United States (3), and Thailand (19). Interestingly, none of the 21 HBV DNA- and HCV RNA-negative CLD patients were TTV DNA positive. However, TTV DNA positivity among HBV DNA-positive and HBV DNA- and HCV RNA-negative CLD patients was not significantly different ($P > 0.5$). Thus, TTV may not represent the major etiologic agent for non-B, non-C chronic hepatitis in western India. In fact, all five TTV DNA-positive patients suffering from CLDs were also HBV DNA positive. No clinical follow-up of these patients is available to address disease severity and outcome among patients positive for both HBV and TTV or for HBV DNA alone.

To assess the extent of transfusion-associated transmission of the virus, TTV DNA positivity was evaluated for three risk groups, i.e., patients undergoing hemodialysis, hemophiliacs, and paid plasma donors from a blood products manufacturing organization (who have a very high prevalence of anti-HIV [2], anti-HCV [9], and anti-HEV [1] antibodies). Compared to that among voluntary blood donors, TTV DNA positivity among paid plasma donors (4 of 31; 12.9%) and patients undergoing hemodialysis (2 of 24; 8.3%) was not significantly different ($P > 0.5$), indicating that the risk of TTV infection in these categories was not high. The fourfold-higher risk of TTV infection in hemophiliacs could be associated with intermittent use of imported blood products.

The data also emphasize that the parenteral route may not be an important mode of transmission of TTV in the Indian population and that other modes may be operative to an appreciable extent. In this connection it is important to note that TTV DNA has been detected in feces and bile samples and a possibility of fecal-oral transmission was suspected (16, 23). A recent study attributing an outbreak of enterically transmitted non-A, non-E viral hepatitis to TTV is noteworthy (13). Among the rural populations of Nigeria, Gambia, Brazil, and Ecuador (20), TTV DNA positivity was found to be 7 to 74%. An environmental source of TTV infection, comparable to those for other enteric infections, has also been suggested (4). The predominant mode of TTV transmission among Indian populations remains to be elucidated.

Based on the limited data, infection of the population under study with dual or multiple variants of TTV does not appear to be frequent.

Phylogenetic analysis. A wide variation among different TTV sequences from Japan led to classification of different isolates of the virus (17), with those separated by an evolutionary distance of 0.3 constituting types (1 and 2) and those separated by a distance of >0.15 representing subtypes (1a, 1b, 2a, 2b, and 2c). Subsequently, additional genotypes (types 3 and 4) were discovered (22, 23).

As evidenced by the dendrogram shown in Fig. 1, the 59 isolates analyzed in the present study fall into six major genotypes. The majority of the isolates (36 of 59; 61%), including 19 of 20 Indian isolates, belonged to genotype 1. This genotype was further divided into two branches. Branch 1a (28 isolates) included all 19 Indian isolates, 2 German isolates, 1 isolate each from China and Thailand, and 5 isolates from Japan. Both of the isolates from Germany, one isolate each from Thailand and the United Kingdom, and four isolates from Japan constituted genotype 1b.

Genotype 2 included eight isolates and was subdivided into types 2a, 2b, and 2c. One Indian isolate grouped along with genotype 2c sequences represented mainly by the German isolates, whereas genotype 2a consisted of one isolate from Japan. Isolates from Japan and Germany belonged to genotype 2b.

FIG. 1. Phylogenetic analysis based on a 171-nucleotide fragment of the ORF1 genes of 59 TTV isolates. These included one isolate from China (TTVCHN1 [accession no. AB079173]), 13 isolates from Germany (C2, H3, A3, D3, F5, F4, D5, A4, H1, H4, A1, C1, and Be7 [AF108946 to AF108958]), 20 isolates from India (present study, indicated in boldface [AF177443 to AF177462]), 20 isolates from Japan (TA278, G104901, TY96117, N22, TX011, TS003, NA004, CMR12, MONG969, JAM18, JAM21, JAM28, JAM29, JAM53, JAM59, MONG268, MONG301, TC1, CK7, and EN3 [AB008394, AB011489, AB011494, AB017767, AB017769 to AB017771, AB017877, AB017881, AB017886 to AB017893, AB018850, AB018961, and AB018963]), 2 isolates from Thailand (TTV33 and TTV36 [AF078114 and AF078115]), and 3 isolates from the United Kingdom (BLDN9, BLDN20, and BLDN17 [AF072749, AF079541, AF079543]). Percent bootstrap supports (1,000 replicates) are shown by numbers at respective nodes. Genotypes (in boxes) and subgenotypes are marked in boldface. Each dash represents 0.004833 substitution per nucleotide.

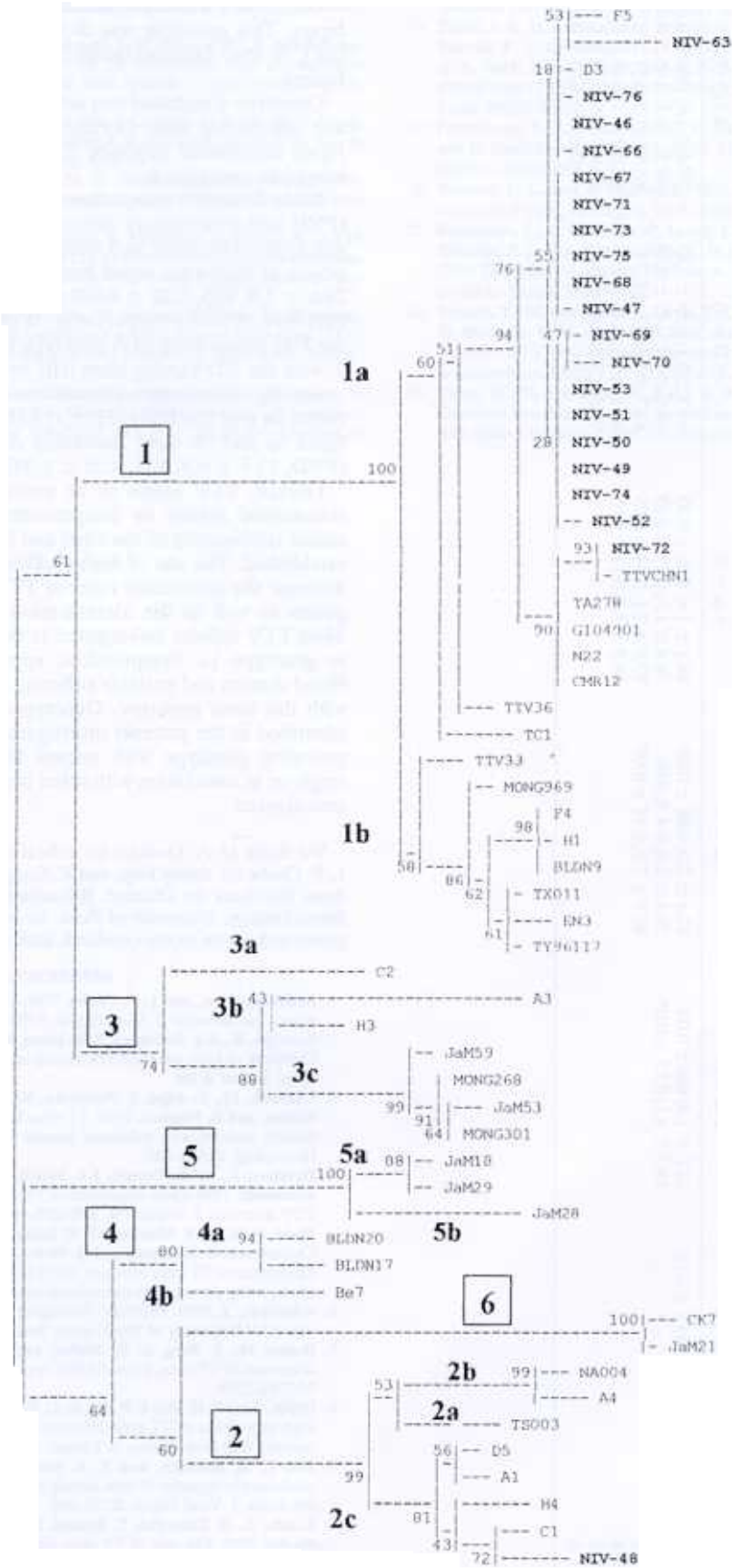
F5
NIV-63
D3
NIV-76
NIV-46
NIV-66
NIV-67
NIV-71
NIV-73
NIV-75
NIV-68
NIV-47
NIV-69
NIV-70
NIV-53
NIV-51
NIV-50
NIV-49
NIV-74
NIV-52
NIV-72
TTVCHN1
YA27H
G104901
N22
CMR12
TTV36
TC1
TTV33
MONG969
F4
H1
BLDN9
TX011
EN3
TY96117
C2
A3
H3
JaM59
MONG268
JaM53
MONG301
JaM18
JaM29
JaM28
BLDN20
BLDN17
Be7
CK7
JaM21
NA004
A4
TS003
D5
A1
H4
C1
NIV-48
1
1a
1b
2
2a
2b
2c
3
3a
3b
3c
4
4a
4b
5
5a
5b
6

TABLE Relationships among different genotypes

Genotype	(PNI; ED) with genotype:					
	1 (n = 36)	2 (n = 8)	3 (n = 7)	4 (n = 3)	5 (n = 3)	6 (n = 2)
1	90.9 ± 8.4; 0.11 ± 0.13	62.0 ± 1.8; 0.6 ± 0.04	67.4 ± 2.0; 0.46 ± 0.04	68.1 ± 1.4; 0.45 ± 0.03	64.7 ± 5.1; 0.55 ± 0.09	59.4 ± 1.6; 0.66 ± 0.04
2		88.7 ± 4.1; 0.12 ± 0.05	70.3 ± 2.8; 0.4 ± 0.06	76.4 ± 1.8; 0.29 ± 0.03	68.0 ± 1.7; 0.45 ± 0.04	68.1 ± 1.1; 0.46 ± 0.03
3			86.8 ± 7.5; 0.15 ± 0.09	73.5 ± 3.1; 0.34 ± 0.05	64.7 ± 1.4; 0.5 ± 0.03	65.4 ± 1.3; 0.51 ± 0.02
4				89.3 ± 3.4; 0.11 ± 0.05	69.1 ± 1.2; 0.43 ± 0.03	68.5 ± 1.1; 0.44 ± 0.03
5					91.3 ± 4.8; 0.09 ± 0.05	63.0 ± 0.6; 0.59 ± 0.01
6						98.0 ± 0.0; 0.01 ± 0.0

Genotype 3 was characterized by isolates from Germany and Japan. This genotype was divided into 3a (one German isolate), 3b (two German isolates), and 3c (all four isolates from Japan).

Genotype 4 included two isolates from the United Kingdom and one isolate from Germany, whereas three isolates from Japan constituted genotype 5. Two isolates from Japan belonged to genotype 6.

Table 2 shows a comparison of percent nucleotide identities (PNI) and evolutionary distances (ED) among the six major genotypes. Minimum and maximum percent nucleotide divergences (PND) were noted between genotypes 2 and 4 (PND, 23.6 ± 1.8; ED, 0.29 ± 0.03) and 1 and 6 (PND, 40.6 ± 1.6; ED, 0.66 ± 0.04), respectively. Within individual genotypes, the PNI varied from 98 in genotype 6 to 86.8 ± 7.5 in genotype 3, with the ED varying from 0.01 to 0.15 ± 0.09, respectively.

Among subgenotypes, minimum divergence was noted between 5a and 5b (PND, 12 ± 1; ED, 0.12 ± 0.001). Subgenotypes 3a and 3b were maximally divergent from each other (PND, 21.5 ± 0.5; ED, 0.25 ± 0.003).

Overall, TTV seems to be prevalent in India and to be transmitted mainly by nonparenteral routes. However, the causal relationship of the virus and liver disease remains to be established. The use of highly efficient primers for PCR may increase the prevalence rates of TTV DNA in different categories as well as the identification of additional genotypes. Most TTV isolates investigated in the present series belonged to genotype 1a. Symptomless, apparently healthy voluntary blood donors and patients suffering from CLDs were infected with the same genotype. Genotypes 3, 4, 5, and 6 were not identified in the patients investigated. The significance of the prevalent genotype with respect to the severity of disease, singly or in association with other hepatitis viruses, needs to be investigated.

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