Molecular Detection and Predominance of Human Torque Teno Virus in Children's with acute hepatitis and Environmental Waters

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Abstract: Introduction: Epidemiological studies have shown that TTV is described worldwide in various populations. The prevalence of TTV viremia in healthy adults of developed countries is in the range of 1-34%. Prevalence reported from third world countries was found to be higher, typically 40-70%. In people who have received multiple blood transfusion; the virus is almost universally present with more than one subtype in each individual. Objectives: This study was carried out to determine the incidence of TTV infection among children's, wastewater, and drinking water, also genotyping of detected TTV strains circulating in the studied samples collected from Cairo. Subjects and Methods: stool samples were collected from 152 Children's with age range from 6 months to 12 years. Also, 45 wastewater samples and 45 drinking water samples were collected monthly from inlet and outlet from wastewater treatment plant (Zenin) and from El-Giza drinking water treatment plant. Collected samples were submitted to semi- nested PCR for amplification of 3' non translated region of TTV. The expected fragment sizes of PCR products were 390 bp and 271 bp for the first and second round of PCR, respectively. PCR products, of 2nd round of some positive samples, were purified for nucleotide sequence analysis in both directions. Fragments nucleotide sequences were compared to sequences derived from the corresponding TTV genome of the same regions deposited in the Gene Bank. Results and Conclusion: Obtained data showed that the incidence of TTV in children's stool samples was 59.2% (90/152), and in sewage samples was 64.4% (29/45), while it was 17.8% (8/45) in drinking water samples. Statistical analysis indicated that there no significant difference in TTV infection between male and female in infant and early childhood age groups. The phylogenic tree of positive samples confirmed that the isolated virus sequencing was 100% of nucleotide identity to TTV isolate isolated in Germany in 2001 and deposited in the GeneBank with accession no. AF435014, and the sequence of TTV isolated in the present study belong to TTV genotype 28.

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Key words: TTV, incidence, PCR, sequencing, genotyping, children, phylogenic tree.

1. Introduction

The Torque teno virus (TTV) was originally isolated from a Japanese patient who had contracted, following a blood transfusion, hepatitis caused by an unknown etiologic agent (**Nishizawa *et al.*, 1997**). Although TTV was discovered relatively recently in 1997, it seems to be a well-adapted virus of human that has been a persistent source of infection since the distant past. However, TTV differs from all other known viruses in its ability to sustain lifelong viremia, where it actively replicate and continuously produce virus in the blood for decades, even in healthy individuals. Due to its global distribution and persistent viremia in human population, there is no definitive causal association of TTV infection with the diseases that have been investigated. It may be possible that TTVs do not cause any disease and do not have any adverse effect whatever on human health (**Griffiths 1999 and Simmonds *et al.*, 1999**).

TTV is a small, non-enveloped virus measuring 30–32nm with a 3.4–3.9 kb single-stranded, circular DNA genome of negative polarity (**Okamoto *et al.*, 2002**). Hepatitis TT virus, is a parenterally transmitted DNA virus which has high prevalence among healthy population and chronic hepatitis patients. The virus can be transmitted both parenterally and orally; its presence in feces and its remarkable environmental stability suggested the possibility of using it as an indicator of fecal contamination in the environment (**Verani *et al.*, 2006 and Griffin *et al.*, 2008**).

The prevalence of TTV DNA in Egypt did not differ among patients with chronic hepatitis B (46%), chronic hepatitis C (31%), schistosomal liver disease (36%) and blood donors (29%) (**Gad *et al.*, 2000**). It seems that the infection neither contribute to the severity of liver disease nor to the causation of hepatocellular carcinoma ;where TTV was

determined in the serum of 60 samples obtained from hepatocellular carcinoma patients and 30 healthy individuals, and the most prevalence TTV genotype in Egypt was genotype1**(Hafez *et al*., 2007)**.

2. Subjects and Methods

A. Stool and environmental water samples:

1. Stool samples:

During December 2007 to November 2008, 152 fecal specimens were obtained from Children's age range ; one week to 14 years old, from the Pediatric Hepatology Unit of three hospitals (Abo El-Rish, El-Safe and Embaba) located in Cairo and involving 86 males and 66 females. All of them showed the typical symptoms of acute hepatitis including nausea, vomiting, abdominal pains, diarrhea, fever, hepatomegaly, jaundice, and/or dark urine. Specimens were stored at 4°C, transported and processed within 6 hours**.** Fecal specimens were initially suspended in phosphate-buffered saline (15% wt/vol) using a glass rod to break up the solid particle, and were dissociated by vortex , then centrifuged at 5000 rpm at 4°C for 10 min. Supernatants were transferred to fresh sterile tubes and centrifuged for an additional 5 min at 5000 rpm at 4°C. Clarified supernatants were a liquated and stored at −80°C until virus detection.

2. Wastewater samples:

During December 2007 to February 2009, 45 waste water samples were collected from Zenin wastewater treatment plant, where one sample was collected monthly, during a total study period of 15 months, from each wastewater type {15 sludge waste samples, 15 untreated wastewater samples (inlet) and 15 samples after treatment (outlet)}. TTV were concentrated from wastewater effluent samples by filtration of 5 liters through negatively charged nitrocellulose membranes according to **Rose *et al.*, (1984)**. Sample was then re-concentrated using an organic flocculation method according to **Katzenelson *et al.*, (1976).** Viruses were isolated from 100gm of sludge samples by using 10% beef extract and then the suspension was stirred at PH 9.5 for 30 min and centrifuged. The supernatant was re-concentrated using an organic flocculation method according to (**EPA, 1984**).

3. Drinking water samples:

Drinking water samples were collected monthly in the same period as in the wastewater. 45 drinking water samples were collected from El-Giza drinking water treatment plant (15 drinking water sludge, 15 raw inlet water and 15 treated outlet water samples). TTV Virus was concentrated from drinking water by filtration of 100 liters by using ultra-filtration system (Amersham pharmacia Bioscience, USA) through hollow fiber cartridge of 50k dalton pore size and surface area of 8400 cm2. The one hundred drinking water samples were concentrated to one liter each and viruses were re-concentrated using 3% beef extract by organic flocculation method according to **Katzenelson *et al*., (1976).**

B. DNA extraction and Polymerase Chain Reaction (PCR):

TT virus DNA was extracted from stool, wastewater, and drinking water samples using viral DNA extraction kit (Axygen, USA), according manufacturer instructions.

1. Oligonucleotide primers:

Two pairs of primers were used for first and second round semi nested PCR as shown in table (1).

Table (1): Primers sets used for 3'NTR region amplification of TTV DNA.

	Code of primer	Sequence of primer	polarity	Product size
1st round	TTVF1-P3B1	5'-GTGGGMSYTTCACTTGTCGGTGTC-3'	Sense	390bp
	TTVR1P3B3	5'-CMAATGGCRAGAAGATAAAGG-3'	Antisense	
2nd round	TTVF2-P3B2	5'-ARGTMRCYAAGCACTCCGAGCG-3'	Sense	271 bp
	TTVR1P3B3	5'-CMAATGGCRAGAAGATAAAGG-3'	Antisene	

Where Y represents C or T, R represent A or G, M represent A or C,
H represent A, T or C and D represent G, A or T.

2. Detection of TTV DNA by conventional semi-nested PCR:

For amplification at 3'non-translated region of TTV genome, conventional semi nested PCR was used in a reaction volume of 50µl according to the method described by Biagini *et al*. (2001). Briefly, 10µl of the extracted DNA, was added to 5µl (10x) PCR Buffer, 4µl (25mM) MgCl2, 0.5µl (5U/µl) Go Taq Flexi DNA Polymerase (Promega, Cat. No. M8305), 1µl (50 pmol/µl) of sense primer, 1µl (50 pmol/µl) of antisense primer, 4µl of dNTPs (10mM each, Promega, Cat. No. U122A) and 24.5µl of distilled water. For PCR amplification, an initial denaturation step of 5min at 95ºC followed by 35 cycles at 94ºC for 45sec., 52ºC for 30 sec. and 72ºC for 60 sec, and an additional final extension step at

72ºC for 10 min. the second round PCR was carried out in a reaction volume of 50µl using 1µl of the first-round PCR product, with 1µl (50 pmol/µl) of sense primer and 1µl (50 pmol/µl) of antisense primer of the 2nd round primers for 30 cycles at 50ºC annealing temperature and the same other temperature conditions as 1st round. Analysis of samples, for the presence of TTV DNA, were carried by mixing 10µl of 2nd round PCR products with 2µl of loading dye and analyzed by electrophoresis (General biosystem, Germany) using 2% agarose gel containing 10mg/ml ethidium bromide. The PCR products were analyzed by electrophorsis at 100V for 1hr, and bands were visualized under UV light compared by standard 100bp DNA ladder (SibEnzyme, Cat. No. M27, Russia), by using gel documentation system (Bio-doc analyzer, Biometra, Germany).

C. Direct sequencing of DNA fragments:

1. Sequencing reaction:

For sequencing, PCR products of random positive TTV DNA samples were sequenced to determine nucleotide identity among them and among TTV genomes deposited in the GeneBank. PCR products were cut and purified from agarose gel using Wizard® Sv Gel and PCR Clean-Up System (Promega, Cat. No. A1120) according the manufacturer instructions. Sequencing reactions on both strands of the obtained products were done as described by Biagini *et al.*, 2000, by using 20-75ng of the purified PCR products 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, 3µl (5mg/ml) linear acrylamide, and cold absolute ethanol. The nucleotide sequences were determined directly with an ABI Prism® 310 Genetic Analyzer (Applied Biosystems).

2. Computer-assisted sequence analysis:

Sequence data and changes analysis from both strands of the PCR products and extensive sequence information from the GeneBank database were aligned and compared by using the clustal X and blast programs (Thompson *et al.,* 1997). A phylogenetic tree was reconstructed with neighbor-joining analysis using Tree View version 1.6.6 (Page, 1996).

D. Statistical analysis:

Data were analyzed by Chi-Square test .

3. Results and Discussio

Data showed that the predominance of TTV DNA reach 59.2% (90/152) in stool samples of hospitalized children's with hepatitis, age range from 6 months to 12 years. Children's were grouped according to age into four groups. Data analysis showed that the percentage was 58% (11/19) in the age group 0.5-2 years; 62.9% (44/70), and 59.4% (19/32) in the age groups 3-5 years, and 9-12 years respectively. While the lowest prevalence rate of TTV was 51.6% (16/31) in the age group 6–8 years, Table (2). Prevalence of TTV DNA in children with hepatitis symptoms was considered as high incidence and it was slightly higher than previously reported in similar studies, where during a large outbreak in China it was 40% (45/112) Kangxian and Lian (2001), and in a similar study in Hawaii TTV was 22.4% (15/67) in stool samples of healthy children (Lin *et al.,* 2000). These results indicate that, TTV infection is common in children with symptoms of hepatitis in Cairo. The high prevalence of TTV DNA in infants and early childhood suggested that TTV may be transmitted in early life. The present study showed that the rate of transmission in female was higher than that in male in the age group from 0 to 2 years with no significant difference (Table 2), that may reflect that the hygienic care and behavior in male babies is much higher than that in female babies, or female babies are more susceptible to TTV infection than male babies in this age group. Prevalence of TTV in male and female was nearly equal within the same age group and also between all other studied age groups. Seasonal analysis of TTV in stool samples confirming that the TTV is endemic in the Egyptian children where the prevalence of virus was high and nearly equally distributed through the year, however summer season was higher than other seasons and the lowest prevalence of TTV was in autumn season (Fig. 1).

It was dependence between frequency of TTV infections and chronic diseases and mental-physical retardation of children (p=0.077; p=0.01). It was not confirming significant differences in depend on dwelling place, and children from worse social conditions were infected more often (p=0.05). It was not significant dependence between frequency of TTV infections and breastfeeding. It was confirmed that in children of parents with chronic diseases and children with brothers and sisters with chronic diseases are infected more often (for mothers p=0.054; fathers p=0.04, brothers or sisters p=0.062). Disturbances in pregnancy or delivery were not connecting to frequency of TTV infections (**Los-Rycharska**, *et al.,* 2008).

Table (2): Age and sex-specific Torque teno virus DNA in children's stool in Cairo

Age (years)	Positive number of TTV		
	Male (%)	Female (%)	Total (%)
0–2	6/13 (46.1%)	5/6 (83%)	11/19 (58%)
3–5	21/31 (67.7%)	23/39 (59%)	44/70 (62.9%)
6–8	9/15 (60%)	7/16 (43.8%)	16/31 (51.6%)
9–12	16/27 (59.3%)	3/5 (60%)	19/32 (59.4%)
Total	52/86 (60.5%)	38/66 (57.6%)	90/152 (59.2%)

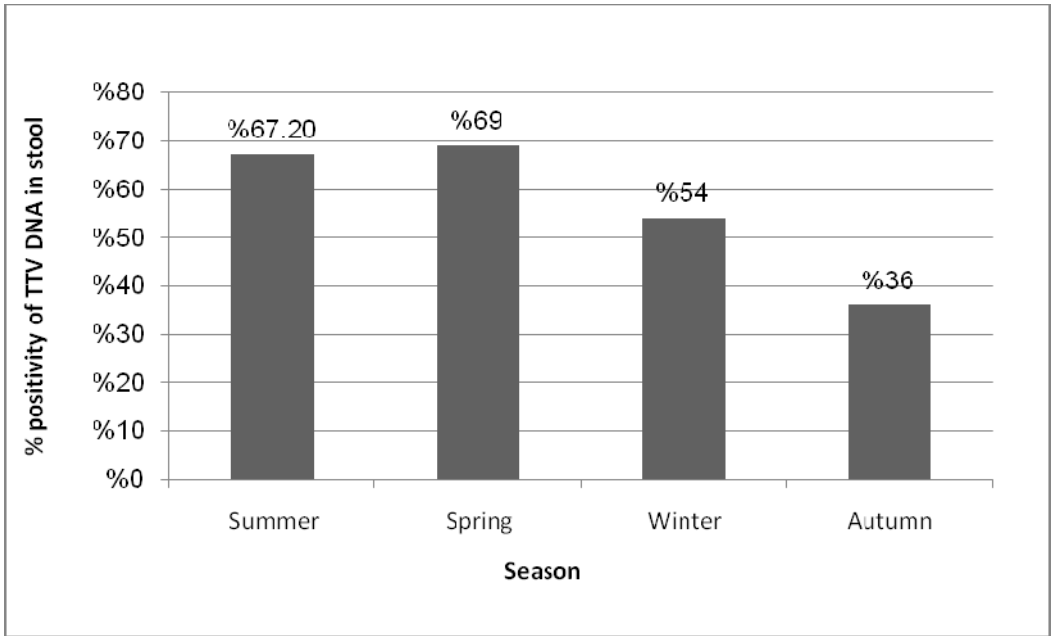

Fig. (1): Seasonal variation of TTV DNA in children's stool samples

In case of environmental water, generally the predominance of TTV DNA in Zenin wastewater treatment plant (WWTP) samples was 64.4% (29/45); where the percentage of TTV DNA in sludge samples was 26.7% (4/15) while it was 83% (25/30) in both treated and untreated wastewater effluent, represented as 93.3% (14/15) in raw untreated wastewater (inlet) and 73.3% (11/15) in treated wastewater (outlet) samples, Table (3). This result was slightly lower than a similar study of **Haramoto *et al.*, (2005)** in Japan who detected TTV DNA in 97% (93/96), 24% (23/95), of influents and final effluents wastewater, collected from WWTPs in Japan, respectively. This difference may be due to the difference in technology used for TTV detection. On the other hand, our result was greatly higher than that of **Vaidya *et al.*, (2002)** in India who found that, TTV was 12.7% (8/63) in wastewater samples, and higher than that of **Carducci *et al.*, (2009)** in Italy, who found that, TTV was 72% (21/29) and 62% (18/29) in inlet and outlet wastewater samples, respectively. These differences in incidence between our study and the studies of **Vaidya *et al.*, (2002)** and **Carducci *et al.*, (2009)**, may be due to lack of good hygienic knowledge and behavior in Egypt. However, all similar previously reported studies stated that TTV was distributed equally through the year (**Haramoto, *et al.*, 2005 and Hamza *et al.*, 2011**). Seasonal variation of TTV DNA frequency in Zenin wastewater treatment plant showed that the presence of TTV virus was higher in winter and spring than in summer and autumn (Fig. 2), these results may reflect the sensitivity of TTV to high temperature and tropical weather.

Table (3): Detection of Torque teno virus in Zenin WWTP by semi- nested PCR.

No. of samples	Percentage of positive TTV semi RT-PCR			
	Inlet samples	Outlet samples	Total	Sludge samples
45	13.3% (2/15)	6.7% (1/15)	10% (3/30)	33.3% (5/15)

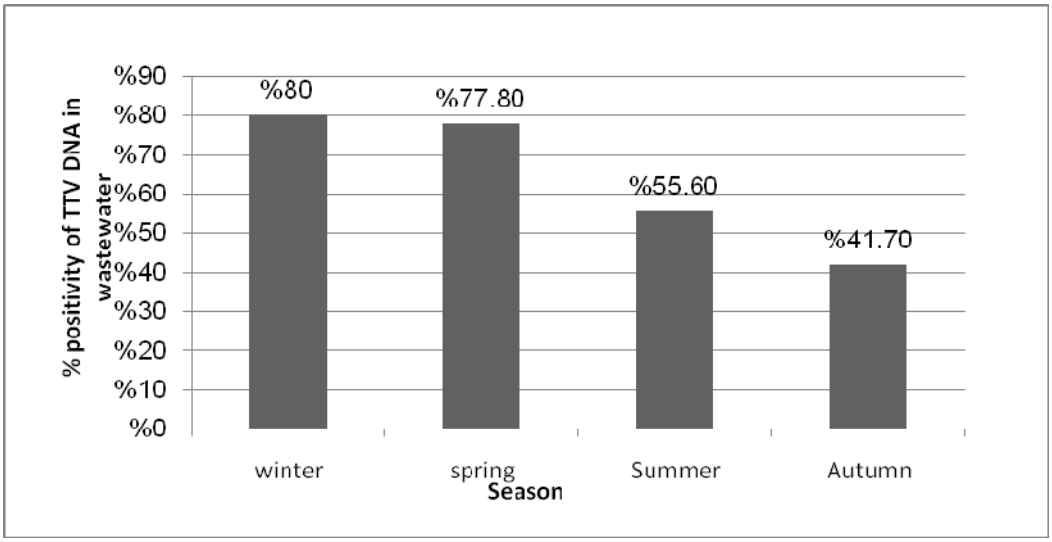

Fig. (2): Seasonal variation of TTV DNA prevalence in Zenin wastewatertreatment plant.

Predominance of TTV in drinking water samples was 17.8% (8/45) where the percentage of TTV was 33.3% (5/15) in collected drinking sludge samples, while it was 10% (3/30) in drinking water effluent samples represented as 13.3% (2/15), and 6.7% (1/15) in raw Nile river water (inlet) and in treated drinking water (outlet) samples, respectively, Table (4).

Table (4): Detection of Torque teno virus (TTV) in EL-Giza DWTP by semi nested PCR.

No. of samples	Predominance of TTV DNA			
	Raw Inlet samples	Treated outlet samples	Sludge samples	Total
45	93.3% (14/15)	73.3% (11/15)	26.7% (4/15)	64.4% (29/45)

TTV DNA recorded percentage in drinking water samples of the present study was lower than that of **Mendes *et al.*, (2008)** in Brazil who detected TTV in Brazilian Amazon using two molecular methods; conventional PCR, where TTV DNA was detected in a percentage of 37% (19/52) in the samples, and real-time PCR where TTV DNA could be detected in a percentage of 92% (48/52) in the samples. These results showed the role of difference in technology for the TT virus detection, where the sensitivity of viral genome detection differs from technique to other according to the technology used. Also **Haramoto *et al.*, (2010)** in Japan detected TTV in Tamagawa River water samples with a percentage of 5.6% (1/18). Japanese's author data is not far different than the present study data reflecting the wide distribution and diversity of TTV all over the world.

Result for the presence of TTV in drinking water showed also that the distribution of TTV in sludge was higher than that in raw Nile river water that was higher than treated drinking water, (Table 4).

Seasonal variation of TTV DNA revealed that the viral incidence was 26.7% (4/15) during winter and 22.2% (2/9) during summer but it reached to 16.7% (2/12) in autumn season, while in spring season the virus was not detected (Fig. 3).

Generally the distribution of the TT virus in the present study was high in drinking water that should be free of virus contamination; however TTV is slightly less stable in the environment and less resistant to changes of environmental conditions. The high distribution of TT virus in raw drinking water reflecting that the probability of pollution of raw Nile river (the source of raw water in the studied drinking water treatment plant), with contaminated wastewater by TTV. The data showed also that the TTV is endemic in the Egyptian environment and the high prevalence of TTV in both stool and environmental water samples reflecting that the mode of transmission of virus in Egypt may be mainly by consumption of contaminated drinking water (fecal-oral route) rather than by blood transfusion specially because none of the children in the present study was

transfused blood in their live. The presence of TTV in treated drinking water showed also a defect in drinking water treatment technology because the treatment failed in complete removal of TTV from treated water and the percentage of removal was considered as low removal efficiency because it was 6.6% (Table 4).

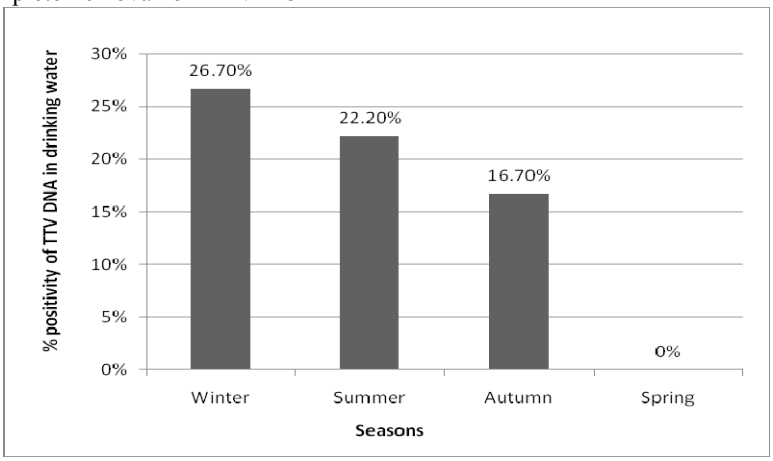

Fig. (3): Seasonal variation of TTV DNA prevalence in El-Giza drinking water treatment plant.

The present study has checked the prevalence of TTV DNA in three different types of samples (stool, wastewater, and drinking water samples), using semi-nested PCR by two sets of primers from the 3'NTR to increase the specificity and sensitivity of TTV detection. TTV DNA was detected in collected positive samples where amplified PCR product of 2nd round was 271 bp, the amplified PCR product appeared as a dense band in the most of sludge and wastewater samples than in the amplified drinking water samples as shown in Fig. (4), reflecting that the number of viral particles detected in these samples more than that in drinking water samples**.**

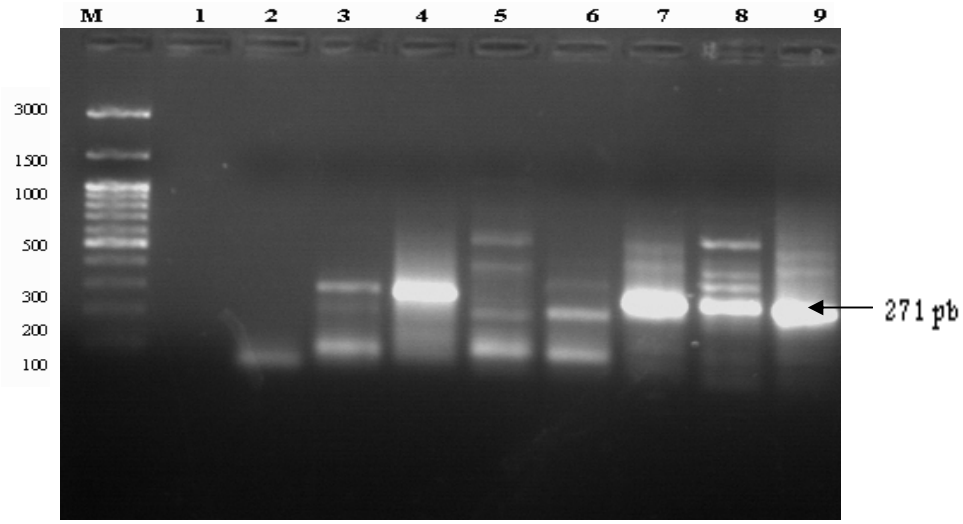

Fig. (4). Agarose gel electrophoresis of 2nd round semi- nested PCR for TTV DNA. Amplification product of 271 bp at 3'NTR. Lane M: 100 bp DNA ladder. Lane 1: Negative control, Lane 3, 4, 7, 8 and 9 were positive samples but 2, 5 and 6 were

Most of amplified PCR product fragments of positive samples were sequenced, compared to each other and to sequences derived from the corresponding TTV genome region deposited in GeneBank, (Fig. 5).

CTTCTTCGGAGCCGCGCGCTACGCCTTCGGCTGCGCGCGGCACCTCAGACCCCCGCTCGTGCTG ACACGCTTGCGCGTGTCAGACCACTTCGGGCTCCCGGGGGTCGGGAAATTTGCTAAACAGACTC CAAGTTGCCATTGGACACTGGAGCTGTGAATCAGTAACA

Fig. (5) Nucleotide sequence of amplified fragment at 3'NTR of TTV DNA.

For further analysis of the obtained TTV partial genome detected from wastewater, drinking water and stool samples of hospitalized children, the complete sequence fragment was compared with the all highly similar partial TTV genome sequences deposited in the GeneBank to produce a phylogenetic tree. The phylogenetic tree was constructed by alignment of the obtained partial sequence with 32 highly similar TTV nucleotide sequences **(Fig. 6)**. This analysis confirmed that the isolated virus nucleotide sequence was most to the novel TTV KAV isolated that isolated from Germany in 2001.

Nucleotide sequence analysis showed that there is only one TTV genotype circulating in the studied Egyptian environment in the period from 2007 to 2009 and was closely related to the novel isolate named KAV that isolated from Germany in 2001 and deposited in the GenBank with accession no. AF435014, and considered as a new genotype of the TTV family and provisionally designated as genotype 28 (Heller, *et al.*, 2001).

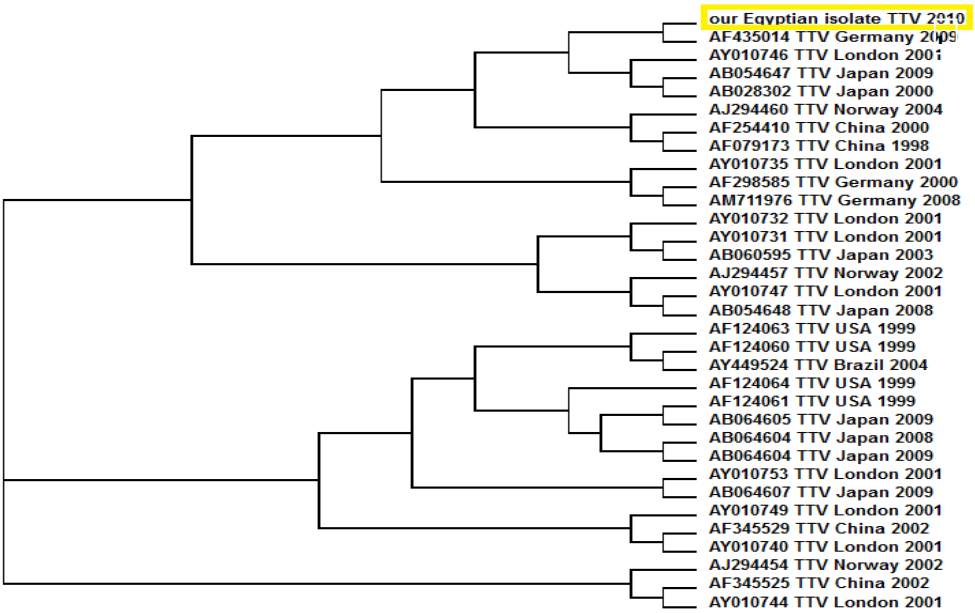

Fig. (6): A Neighbour-joining Phylogenetic Tree for highly similar sequences of TTV with the present study TTV sequence (Egyptian isolate 2010). Data showed that the obtained sequence is closely related to isolate KAV isolated from Germany in 2001 with Accession No. AF435014.

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