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Mutation of Torque Teno Virus among Women with Urinary Tract Infection in Diyala Governorate

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ABSTRACT

Background: Torque Teno Virus (TTV) shows a high prevalence in infected and healthy individuals globally. The generation of many TTV variants is driven by the high mutation rate, which is closer to the RNA virus. The cause and mechanism of great genomic variation are unknown as ORF1 is the most extended encoded region in the genome of TTV with the highest rate of genetic mutations.**Objectives**:To determine the infection rateof Torque Teno Virus and types of mutations in women with urinary tract infections in Diyala Governorate.**Methods:**A cross-sectional study was performed between the 20thof September 2020 to the 20th of January 2021 for 100 women diagnosed with urinary tract infections (50 pregnant and 50 non-pregnant women). Their ages ranged from 17-77 years old of women admitted to Al-Batool Teaching Hospital for Maternity and Children, Women Emergency Unit and Unit of Urological Consultation at Baqubah Teaching Hospital in Diyala Governorate. The DNA extraction,was performed on the collected urine samples and followed with genes amplification with specific primers using a nested polymerase chain reaction and finally the phylogenic analysis was done to determine the genotype of TTV.**Results:**Torque TenoVirus DNA was detected in (8%). About 12.50% of positive women for TTV had UTI history during childhood, while 7(87.50%) without UTI history during childhood. Three of the positive samples (37.50%) who were positive to TTV had UTI before pregnancy compared to the rest 5(62.50%) of TTV positive infection in women without UTI infection before pregnancy. Statistical analysis revealed significant differences during childhood while non-significant before pregnancy.Genetically there are many substitution mutations were shown in local isolates such as isolate (1) in five positionsC\G,G\A,C\T,C\G, A\G, isolate (2)in three positionsG\A,T\A,A\G, isolate (3) did not show any mutation, isolate (4)in four positionsT\A,A\C,A\G,T\A, isolate (5) in three positions A\C,T\A,A\G, isolate (6)in three positions G\A,C\G,G\C, isolate (7) in two positions A\G,C\G and isolate (8) in three positions A\G,A\G,G\A.**Conclusion:**Transversion and transition mutation of amino acid occurred in most local isolates in two, three or five positions after alignment with some GenBank isolates, except isolate number three which was showed 100% similarity with Egyptian isolate.

Key Words: Torque Teno Virus,Urinary Tract Infections, PCR-Sequencing,Mutation, phylogenetic tree.

INTRODUCTION

Pregnancy can enhance the risk of urinary tract infections from the end of the first trimester through mechanical compression and progesterone release (1)The hormonal and mechanical changes in the urinary tract make women more vulnerable, starting from 6 weeks through 24 weeks (2). Higher maternal complications among teenage pregnant women represent an important health problem with social and medical impact (3). Very old patients with urinary tract infections had a higher risk of developing urosepsis shock than younger patients (4).

Risk factors for recurrent urinary tract infections include frequent sexual intercourse (three or more acts of intercourse per week), spermicide, and a new sex partner. A history of the first urinary tract infection before the age of 15 years increases one's risk of recurrent urinary tract infections(5).

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Torque Teno Viruses are small (~30 nm in diameter), icosahedral virions that lack an envelope with a circular, negative-sense, single-stranded DNA genome (6). Detection of Torque Teno Virus infection in 11% of healthy individuals suggests transmission through routes other than blood and injection (e.g., faecal-oral route), causing food and water contamination (7).

The virus is undergoing divergent evolution as very high sequence diversity was found in the ORF1 gene (8). A comprehensive study of codon usage analysis of the ORF1, which encodes the viral capsid protein, was undertaken for the first time to reveal its evolutionary history (9). The capsid is assembled from 60 copies of the capsid protein, viral protein (VP1 and VP2) is a phosphatase and VP3 (also called apoptin), which induces apoptosis only in cancer cells (10).

The origin of this extreme diversity is unclear but may be due in part to the known high mutation rates of single-stranded DNA viruses (11). Multiple processes determine viral mutation rates, including polymerase intrinsic fidelity, replication mode, 3′ exonuclease activity, spontaneous nucleic acid damage, access to post-replicative repair, editing by host-encoded deaminases, imbalances in nucleotide pools, template sequence context, and template structure (12). Genetic variation is a necessity of all biological systems. Viruses use all known mechanisms of variation; mutation, several forms of recombination, and segment reassortment in the case of viruses with a segmented genome (13).

To our knowledge there is no molecular study about this virus in Diyala Governorate so the present study design to determine the infection rate of Torque Teno Virus and types of mutations in women with urinary tract infections, using the nested conventional polymerase reaction technique in Diyala Governorate.

MATERIAL AND METHODS

The study was performed between the 20thof September 2020 to the 20th of January 2021 for 100 women diagnosed with urinary tract infections (50 pregnant and 50 non-pregnant women). Their ages ranged from 17-77 years of women admitted to Al-Batool Teaching Hospital for Maternity and Children, Women's Emergency Unit and Unit of Urological Consultation at Baqubah Teaching Hospital in Diyala Governorate.

Microscopic Examination of Urine and Cultivation positive Samples

The general flow test of the urine samples in the present study was conducted according to the presence of purulent cells, blood cells, and other approved indicators that indicate inflammation and samples containing more than ten purulent cells (14).Routine urine culture microbiology was performed on Blood agar and MacConkey agar (15).

Molecular Detectionof Torque Teno Virus DNA

Extraction of Torque Teno Virus DNA

ZR viral DNA extraction kits (Cat. No. D3015 and D3016, Epigenetics - USA) are used to extract viral DNA directlyfrom a the collected urine samples.

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Torque Teno Virus Genome Amplification by Nested PCR.

Primers for Torque Teno Virus

Two pairs of primers were used to amplify a TTV gene fragment, NG059, NG061 (16), as summarized in Table 1 and 2.

Table 1: The Specific Primer NG059 of the Gene.

Primer *	Sequence	Tm (°C)	GC (%)	Product Size
Forward	5'- ACAGACAGAGGAGAAGGCAACATG- 3'	58.8	50	271 base pair
Reverse	5'-CTGGCATTTTACCATTTCCAAAGTT- 3'	54.7	36	

*Integrated DNA technology, Canada.

Table 2: The Specific Primer NG061 of the Gene.

Primer*	Sequence	Tm (°C)	GC (%)	Product Size
Forward	5'-GGCAACATGYTRTGGATAGACTGG-3'	56.1	45.8	271 base pair
Reverse	5'-CTGGCATTTTACCATTTCCAAAGTT-3'	51.8	36.4	

*Integrated DNA technology, Canada.

Principle of Nested PCR

A standard PCR has been performed to amplify the N-22 region (NG059, NG061 gene) (17). An amplified pitch (271bp) with the aid of reverse and forward primer and a PCR reaction mix was performed at (25µl) total volumes, as shown in Table 3.

Table 3: Components of Nested Polymerase Chain Reaction.

No.	Components	Final Concentration
1	Taq PCR PreMix	12.5µl
2	Forward primer	10 picomols/µl (1µl)
3	Reverse primer	10 picomols/µl (1µl)
4	PCR Product (PCR 1) external	1.5µl
5	Deionized water	9 µl
6	Final volume	25µl

Thermocycler was programmed to amplify the genome by MultiGeneOptiMax thermal cycler gradient, as shown in Tables 4.

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Table 4: The Thermal Cycling Condition for DNA Amplification Specific Primer NG059 and NG061 of the Gene (First and second Run).

No.	Steps	Temperature	Time	Cycles
1	Pre-Denaturation	94 ℃	5 Minutes	1 Cycle
2	Denaturation	94 ℃	1 Second	50 Cycles
3	Annealing	52 ℃	1 Second	
4	Extension	72 ℃	1 Second	
5	Final extension	72 ℃	7 Minutes	1 Cycle
6	Holding	4℃	-	

Agarose Gel

Electrophoresis

Agarose Gel was prepare according to (18). Three µl of the processor loading buffer have been mixed with 5 µl of the supposed DNA to be electrophoresis (loading dye). After the mixing process, the process of loadingis to the holes of the gel.

DNA Sequencing and Phylogenetic Analysis

The PCR products sequencing was carried out by sending the PCR DNA products with their specific primers in a freezer bag to Macrogen company in Korea, https://dna.macrogen.com. The sequencing study was designed between the standard genes and local isolates in the basic local alignment search tool (BLAST), available at the National Center of Biotechnology Information (NCBI) online http://www.ncbi.nlm.nih.gov. The evolutionary analysis was conducted using MEGA6 (Molecular Evolutionary Genetics Analysis software version 6 software).

RESULTS

Detection of TTV

In this study only 8% of TTV virus infections was detected among (50 pregnant and 50 non-pregnant women) with urinary tract infection from different regions in Diyala Governorate. Whaere as the rest samples show negativeresult (92%), as shown in Figure 1.

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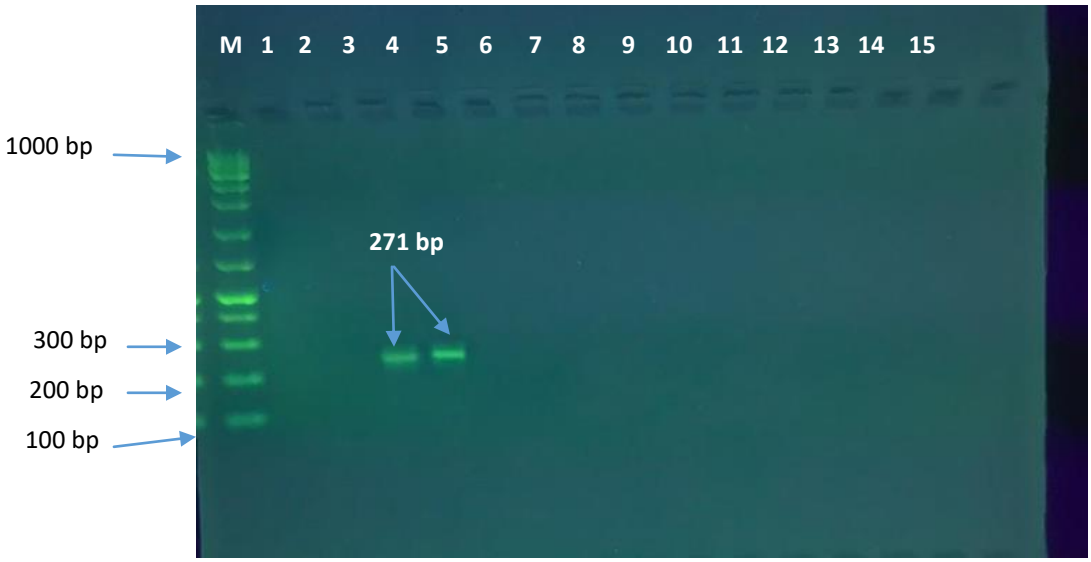

Figure 1: Gel Electrophoresis of The Second-Round PCR, Amplification for (NG061). M: DNA ladder (100-1000 plus), 3 and 4 samples were positive while other samples were negative. The product (271 bp) was electrophoresis on 1% agarose at 7 volt/cm^2. TBE buffer 1X for 1:30 hours, stained by red safe stain and illustrated under UV light.

Distribution of Torque Teno Virus Infection According to the History of Urinary Tract Infection among the Study Population

Concerning the history of urinary tract infection among the study population, Table (6) showed one case (12.50%) positive to TTV that UTI history during childhood, while 7(87.50%) without UTI history during childhood. Three (37.50%) who were positive to TTV had UTI before pregnancy compared to 5(62.50%) of TTV positive infection in women without UTI infection before pregnancy. Statistical analysis revealed significant differences during childhood while non-significant before pregnancy.

Table 6: Distribution of Torque Teno virus Infection According to the History of Urinary Tract Infection among the Study Population.

History of UTI		PositiveNo%	NegativeNo%
During Childhood	Yes	1(12.50%)	25(27.17%)
	No	7(87.50%)	67(72.83%)
	Total	8(100%)	92(100%)
Chi-Square (χ^2)		4.5*	19.174 **
Before pregnancy	Yes	3(37.50%)	53(57.61%)
	No	5(62.50%)	39(42.39%)
Chi-Square (χ^2)		0.500 NS	2.130 NS
* (P≤0.05), ** (P≤0.01).			

NS= Non-significant

Alignment of TTV Sequenceswith a Reference Sequence

The DNA sequences of result of eight local isolates of the TTV displayed a significant alignment with Torque Teno Virus strain NA-MU 15 ORF1 gene, partial CDS (ID: KY750543.1) length: 269Number of Matches: 1. Pairwise sequences alignment of all eight isolates were performed on the amplicon of ORF1 region (271 bp) compared to the reference isolates. The nucleotide sequence on ORF1 was produced from the amplification of

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two genes that were put on Blast tool, translated to a specific amino acid and produced a significant alignment with ORF1 partial Torque Teno Virus sequence (ID: AVV62254.1) length: 89 Number of matches: 1, using the GenBank library in NCBI site (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All local isolates were registered in GenBank under ID (MW513364.1, MW513365.1, MW513366.1, MW513367.1, MW513368.1, MW513369.1, MW513370.1 and MW513371.1) as shown in (Table 7).

Table 7: Variation in Nucleotides and amino Acid in TTV ORF Genes (Capsid Proteins) (NG059) and (NG061) in Local Isolates.

Source: TorqueTeno Virus \ ORF Gene						
No. of isolate	**Variation**	**position**	**Nucleotide change**	**Diversity in Amino Acid**	**Sequence ID with compare**	**Identities**
1	Transversion	66	TGC\TGG	Cysteine\ Tryptophan	ID: KY750543.1	98%
	Transition	114	GAG\GAA	Glutamic acid\ Glutamic acid		
	Transition	153	CAC\CAT	Histidine\ Histidine		
	Transversion	219	CTC\CTG	Leucine\ Leucine		
	Transition	231	GTA\GTG	Valine\ Valine		
2	Transition	198	CTG\CTA	Leucine\ Leucine	ID: KY750543.1	99%
	Transversion	199	ATA\TTA	Leucine\ Isoleucine		
	Transition	231	GTA\GTG	Valine\ Valine		
3	----------				ID: KY750543.1	100%
4	Transversion	102	GGT\GGA	Glycine\ Glycine	ID: KY750543.1	98%
	Transversion	196	ATA\CTA	Isoleucine\ Asparagine		
	Transition	208	AAC\GAC	Asparagine\ Aspartic acid		
	Transversion	255	GGT\GGA	Glycine\ Glycine		
5	Transversion	150	GAA\GAC	Glutamic acid\ Aspartic acid	ID: KY750543.1	99%
	Transversion	199	TTA\ATA	Leucine\ Isoleucine		
	Transition	231	GTA\GTG	Valine\ Valine		
6	Transition	114	GAG\GAA	Glutamic acid\ Glutamic acid	ID: KY750543.1	99%
	Transversion	219	CTC\CTG	Leucine\ Leucine		
	Transversion	264	ATG\ATC	Methionine\ Isoleucine		
7	Transition	147	ATA\ATG	Isoleucine\ Methionine	ID: KY750543.1	99%
	Transversion	214	CCC\GCC	Proline\ Alanine		
8	Transition	144	ATA\ATG	Isoleucine\ Methionine	ID: KY750543.1	99%
	Transition	208	AAC\GAC	Asparagine\ Aspartic acid		
	Transition	261	ATG\ATA	Methionine\ Isoleucine		

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Phylogenetic Tree

The most common approaches that constructed the phylogenetic tree were constructed based onthe neighbour-joining method using NCBI and MEGA 6 software for eight local isolates and 17 reference isolates (Table 8). In the current study, the phylogenetic tree results analysis according to the closest revealed that the local isolate No. 6 showed clustered with the reference isolates (ID: KY750543.1 Egypt, ID: AF397741.1 USA, ID: AJ402241.1, England, ID: AF146809.1 Australia and ID: AY256672.1Saudi Arabia). Isolate No.1 showed very closed to ID: AF212332.1Italian and ID: DQ665287.1 Brazilian isolates. The isolates number (3 and 7), (2 and 5) and (4 and 8) were very closely related to each other and closed to ID: GQ179967.1Iran isolates. Finally,These results confirmed that they are in P-distance, as shownin Figure (2).

Table 8: Compatibility Local Isolates with Reference Isolate from GenBank.

No.	Accession number	Country	Source	Compatibility
1.	ID: KY750543.1	Egypt	Torque teno virus	98%
2.	ID: AY256672.1	Saudi Arabia	Torque teno virus	98%
3.	ID: AF397741.1	USA	Torque teno virus	98%
4.	ID: AJ402241.1	Italy	Torque teno virus	98%
5.	ID: AF185129.1	United Kingdom	Torque teno virus	97%
6.	ID: AF146809.1	England	Torque teno virus	97%
7.	ID: AF268450.1	Turkey: Istanbul	Torque teno virus	97%
8.	ID: AF212332.1	Australia	Torque teno virus	97%
9.	ID: DQ665287.1	Brazil	Torque teno virus	97%
10.	ID: AF108853.1	Spain	Torque teno virus	96%
11.	ID: AF241474.1	Argentina	Torque teno virus	96%
12.	ID: GQ179963.1	Iran	Torque teno virus	94%
13.	ID: AJ571653.1	Portugal	Torque teno virus	94%
14.	ID: AF123969.1	Japan	Torque teno virus	92%
15.	ID: AF222052.1	France	Torque teno virus	92%
16.	ID: GQ179967.1	Iran: Isfahan	Torque teno virus	93%
17.	ID: AJ309728.1	Poland	Torque teno virus	91%

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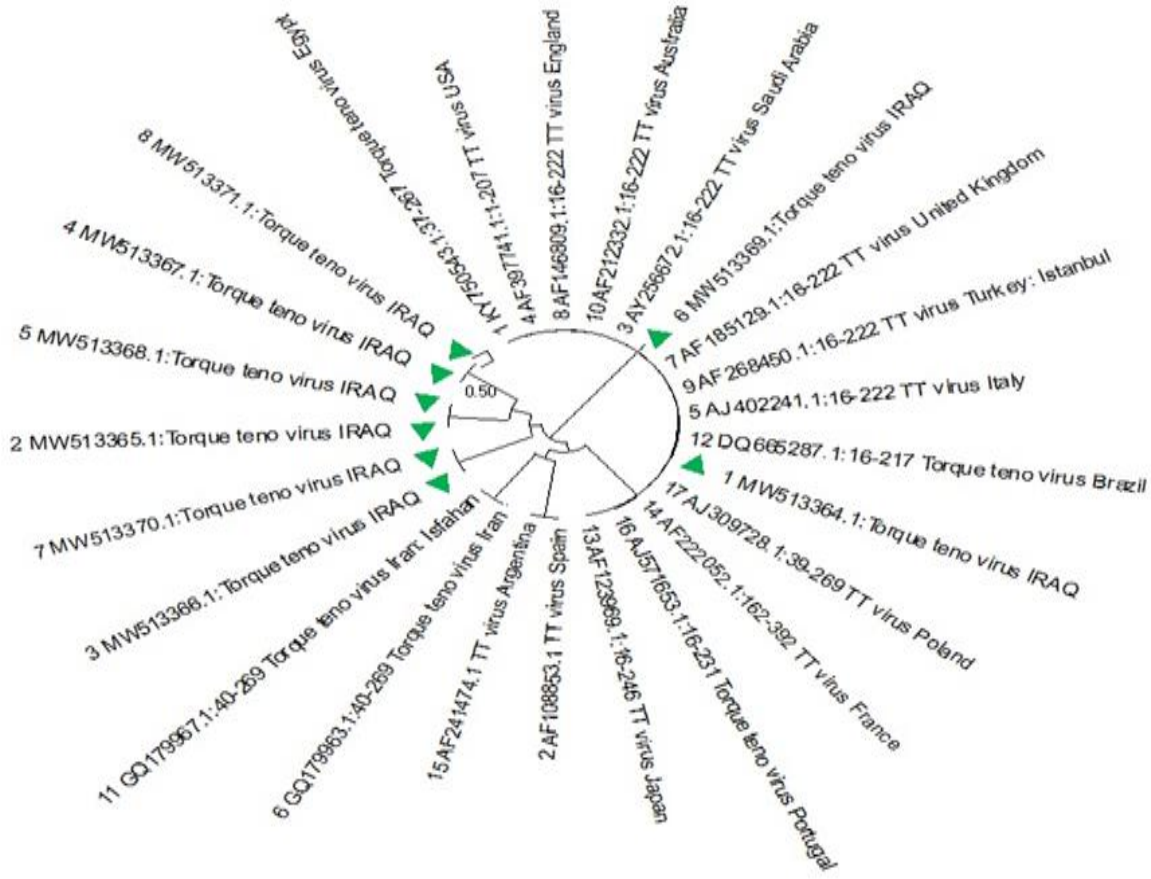

Figure 2: Phylogenetic Tree of TTV (ORF1 Region) The tree was built using Mega 6 software with the neighbor-joining method of the sequences of ORF1 in eight TTV isolates. the phylogenic tree includes reference isolates isolatesobtained from GenBank. The green triangle demonstrates recent isolates.

DISCUSSION

The infection rate of TTV in the present study was 8% depending on urine samples taken from women with urinary tract infection based on microscopic examination and culture who were admitted to Al-Batool Teaching Hospital for Maternity and Children, Women's Emergency Unit and Urological Consultation Unit at Baqubah Teaching Hospital in Diyala Governorate. There is a limit information available and few studies that conducted to investigate TTV in Iraq. This rate is relatively low compared to several Iraqi studies that revealed a variation in TTV infection rates in different populations, such as15% among hemodialysis patients in Kirkuk Governorate (19), 23.3% among healthy blood donors versus 30.8% of the HCV-positive patients and 89.2% of the HBV-positive patients in Baghdad city (20),29.2% among thalassemia patientsin Baghdad city (21),and recently high rates in oral carcinoma patients (43.33% in saliva and 40% in tumor biopsy) than in controls (11% in saliva and 18.33% in tumor biopsy) in Baghdad city (22).

This study's rate is relatively high compared to other Iraqi studies, such as the study by (23) who found only2% TTV infection among patients with hemoglobinopathies and hematological malignancies in Basrah Governorate. Also, (24) detected the virus of the 150 patients, 2 (1 male and 1 female) were TTV positive in Iraqi women suffering of failure kidney disease in Baghdad city.

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The results showed a significant correlation between TTV and UTI history prevalence during childhood while non-significant before pregnancy in the current study. The result showed that 7(87.50%) women had no UTI history during childhood while 1(12.5%) woman had a UTI history during childhood. Also, 5(62.50%) women were positive to TTV infection. However, they had no history of UTI before pregnancy and 3(37.50%) of women had a history of UTI before pregnancy and were positive to TTV infection. Little is known about the urinary tract's unknown viruses that potentially result in transmission (25). The study has been suggested that TTV infection is associated with many diseases. However, there is no direct evidence of links between infection and specific clinical diseases or possible candidates for holistic monitoring (26). The explanation for this variation may be due to the notion that the TTV virus is present in the healthy and diseased population (27).

After nucleotides sequence, a significant alignment with Torque Teno Virus strain NA-MU 15 ORF1 gene, partial cds with accession number (ID: KY750543.1) length: 269 were produced by using NCBI and MEGA 6 software. The sequences analysis results showed many mutations (transition and transversion) at TTV genome-related byORF1-N22 region except the local isolate (No. 3) that was 100% identical with reference isolates, such as isolate (1) in five positions C\G, G\A, C\T, C\G, A\G, isolate (2) in three positions G\A, T\A, A\G, isolate (4) in four positions T\A, A\C, A\G, T\A, isolate (5) in three positions A\C, T\A, A\G, isolate (6) in three positions G\A, C\G, G\C, isolate (7) in two positions A\G, C\G and isolate (8) in three positions A\G, A\G, G\A. Except isolate number three, which was showing 100% similarity with Egyptian isolate. The explanation is that all eight isolates were nucleotides sequence analysis highly identities with this reference isolate ranging between 98% to 100%. Also, high identition ranged between 96% to 100% with amino acids sequence analysis of all local isolates with this reference isolate.

The neighbour-joining method was used to construct the phylogenetic tree according to (28) using MEGA 6 and NCBI software. According to the closestisolate (No. 6) closed to Egyptian isolate ID: KY750543.1 and American isolate ID: AF397741.1. Also, England isolates ID: AF146809.1, Australian isolates ID: AF212332.1 and Saudi Arabian isolate ID: AY256672.1. Local isolate (No. 1) clustered with Italian isolate ID: AJ402241.1and Brazilian isolate ID: DQ665287.1, while local isolates (No.3) showed very closely to isolate (No. 7), isolate (No.2) and isolate (No.5) are shown very closely and isolate (No.4) clustered with isolate (No.8) and all these six isolates closed to Iranian isolates with accession number ID: GQ179967.1. All isolates high compatibility with isolates revealed by (29) who reported TTV a high prevalence among children with thalassemia and non-thalassemic with genotypes 1 and 2 being the most prevalent in Egypt.This related with phylogenetic, ordination, and evolutionary diversity analyses indicated that TTV is transmitted readily between humans across the country's geography and between various species of animal domesticates (30).

Likewise, the current study agreed with Iraqi studies interested in the phylogenic study to TTV, such as (22), (23)and (24) found that sequence analysis showed a mutation in the genome for TTV corresponding to ORF1 (N22). While unlike Brazilian study, they found that the prevalence of TTV in healthy individuals was 69.0% using ORF (N22) primers(31). The N22 regions sequence used to detect TTV DNA and classify various genotypes (32). The local isolates may have multiple recombination and mutations in the Iraqi population. This causes the generation of a large number of TTV variants driven

by the high mutation rate, which is closer to the RNA virus (33), as well as, ORF1 is the longest encoded region in the genome of TTV with the highest rate of genetic mutations (27), and intragenomic rearrangement generating open reading frames which could not be deducted from the genomic sequence (34).

The virus is undergoing divergent evolution as very high sequence diversity was found in the ORF1 gene(35). Amplification ORF1(N22 region) give high diversity strains when used in phylogenetic analysis (36). Some causes suggested a higher than expected rate of TTV mutation. It may be due to the presence of a hypervariable in ORF1 (37). Therefore, variation of the great genomic is occurring due to hypermutation or recombination (38).Results of the study done by (39) showed that hypermutated TTV exists in healthy individuals and HBV carriers and that TTV genomes were susceptible to immune reaction directed to HBV by interacting with APOBEC3 proteins. The variation of TTV may be due to the virus having a single strand genome and the small size make it more tend to mutate, adding to many immunity reactions to facilitate immune evasion.

CONCLUSION

Transversion and transition mutation of amino acid occurred in most local isolates in two, three or five positions after alignment with some GenBank isolates, except isolate number three which was showed 100% similarity with Egyptian isolate.

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