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

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ABSRACT

Due to anatomy and physiological functions, the Torque Teno Virus can cause urinary tract infections in all ages and women more than men.
***Objectives:** Determine TTV infection rates and genotypes in women with UTIs and correlate them with other parameters.*
***Methods:** Cross-sectional data from women with UTIs was used. Bacterial culture, DNA extraction, microscopic investigation, and phylogenic analysis with specific primers by nested polymerase chain reaction were performed on urine samples.*
***Results:** Eight of 100 samples were TTV-positive. TTV infection was 75% in the statistically significant age group (31-47). Three Staphylococcus aureus (37.5%), two Escherichia coli and Proteus mirabilis (25%), and one Enterococcus fecalis (12.5%) co-infected with positive TTV. Phylogenetic analysis showed that all eight urine-derived TTV DNA isolates were type 1 and 2. According to the closest phylogenetic tree analysis, local isolate No. 6 clustered with reference isolates. One resembled Italian and Brazilian isolates, while numbers 2-5, 3-7, and 4-8 resembled each other and Iranian isolates.*
***Conclusion:** All UTI isolates from Diyala Governorate women were TTV genotypes 1 and 2. Local isolate #6 was similar to Egypt, USA, England, Australia, and Saudi Arabia, while #1 was similar to Italian and Brazilian isolates. Six more isolates resembled Iranians.*

Keywords: Urinary tract infections, Torque Teno virus, Molecular detection, Phylogenic analysis

INTRODUCTION

Over half of all women and almost one in ten men will get a urinary tract infection at some point. Despite the prevalence of lower urinary tract infections, persistent scarring, and kidney damage can result from repeated pyelonephritis. UTIs account for 25% of all infections; women are more prone to them than men are, and 50% of women will get one at some point in their lives. Up to 75% of those who receive kidney transplants will eventually develop urinary tract infections. Independent risk factors for recurrent urinary tract infections in renal transplant recipients include recipient age, female gender, and graft function delay [1-3].

Environmental factors such as pH, oxygen tension, osmolarity, food availability, adhesion sites, and immunological interaction play a role in microbial colonization. Bacteria such as *Escherichia coli* and *Klebsiella* species, *Staphylococcus aureus* and coagulase-negative Staphylococci, *Proteus mirabilis* and Enterococcus species, *Pseudomonas aeruginosa* and *Enterobacter* species, non-hemolytic streptococci and *Citrobacter* species, and others are the etiological agents of various diseases [4].

In the immuno-compromised host, human cytomegalovirus, polyomaviruses, John Cunningham virus (JC), and BK virus can proliferate in the kidney and cause systemic illness.

Torque teno viruses are small, common viruses that infect various hosts and have a varied, single-stranded, negative-sense DNA genome. They comprise a sizable fraction of the mammalian virome and have been found at high levels in healthy and sick people. The genome can be broken down into two major categories: Coding and non-coding sections. The replication process is controlled by a conserved non-coding region roughly 1.2 kb in length. There are several Open Reading Frames (ORFs) in the coding area. Torque Teno Virus (TTV) infection can spread worldwide due to its ease of transmission between humans and across different species. Almost one hundred percent prevalence of TTV has been documented, including detections in a wide variety of human tissues and body fluids. The transplacental transmission of TTV is still unknown, even though several studies have reported mother-to-child postnatal transmission of TTV in infancy. Several research have shown a correlation between elevated TTV DNA levels and impaired immunity due to aging, chronic infections, and cancer [5-9].

While 2% of patients with hemoglobinopathies and hematological malignancies in Basrah Governorate have TTV DNA, 15% of patients on hemodialysis in Kirkuk did. 30.8 percent of HCV-positive patients and 89.2 percent of HBV-positive patients in Baghdad Governorate; 29.2 percent of Iraqi thalassemia patients in Baghdad Governorate and most recently, a significantly higher rate (43.33 percent in saliva and 40 percent in tumor biopsy) in oral carcinoma patients compared to controls (11 percent in saliva. However, no research has been conducted among Iraqi women with UTIs in Diyala Governorate [10-13].

MATERIALS AND METHODS

Study design

A cross-sectional study surveyed one hundred women with UTIs between September 20 and January 20, 2021 (50 pregnant and 50 non-pregnant women). The urine samples were selected based on the presence of pus cells and other signs characteristic of UTIs. Women hospitalized at Baqubah teaching hospital's unit of urological consultation, women's emergency unit, and Al-Batool teaching hospital for maternity and children in Diyala Governorate ranged in age from 17 to 77.

Microscopic and molecular analysis

The 60-20 ml of urine was collected in sterile cups while wearing disposable gloves and the samples were then labeled. Afterward, 5 ml was extracted to immediately analyze the macroscopic and microscopic. The pus-containing samples were grown directly in MacConkey and blood agar. In order to directly extract viral DNA, all positive specimens were ultra-centrifuged at 60000 rpm for 20 minutes, carefully removing the supernatant, and then collecting the sediment in sterile Eppendorf tubes 1.5 ml. ZR viral DNA extraction kits were used. The samples were labeled and kept at -20°C until analysis.

Primers for TTV genes

Tables 1 and 2 summarize the two primer pairs utilized to amplify the TTV gene fragment: NG059 and NG061.

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	
Note: *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	
Note: *Integrated DNA technology, Canada				

Primers preparation

The primers were lyophilized, and after that, they were dissolved in free ddH_2O to produce a stock solution with a final concentration of 100 pmol/l. The stock was held at -20°C to prepare a ten pmol/l concentration as a working primer suspended 10 ml of the stock solution in 90 ml of free ddH_2O water to reach a final volume of 100 ml, which was evaluated by IDT (Integrated DNA technologies company, Canada).

Nested PCR principle

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

Table 3. Components of nested polymerase chain reaction

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

The thermocycler was programmed to amplify the genome by MultiGene OptiMax thermal cycler gradient, as shown in Tables 4 and 5.

Table 4. The thermal cycling condition for DNA amplification specific primer (NG059) of the gene (first run)

No.	Steps	Temperature	Time	Cycles
1	Pre-Denaturation	94°C	5 minutes	1 cycle
2	Denaturation	94°C	1 second	50 cycles
3	Annealing	52°C	1 second	
4	Extension	72°C	1 second	
5	Final extension	72°C	7 minutes	1 cycle
6	Holding	4°C	-	

Table 5. Thermal cycling requirement for the gene's specific primer (NG061) during DNA amplification (second run)

No	Steps	Temperature	Time	Cycles
1	Pre-denaturation	94°C	5 minutes	1 cycle
2	Denaturation	94°C	1 second	50 cycles
3	Annealing	52°C	1 second	
4	Extension	72°C	1 second	
5	Final extension	72°C	7 minutes	1 cycle
6	Holding	4°C	-	

Phylogenetic and DNA sequencing

The PCR results were sequenced by mailing the PCR DNA products and their unique primers in a freezer bag to a Korean company called Macrogen. The Basic Local Alignment Search Tool (BLAST), which is accessible online at the National Center for Biotechnology Information (NCBI), was used to create the sequencing study between the sequences of standard genes. Using MEGA6 for the evolutionary analysis.

Statistical analysis

Statistical Analysis System-2012 (SAS) was used to identify the effects of various study parameters on various aspects. In this study, a significant comparison between percentages (0.05 and 0.01 probability) was made using the *chi-square* test.

RESULTS

Molecular detection of TTV

Eight percent (8 out of 100) of women with UTI in Diyala Governorate (50 pregnant, 50 not pregnant) tested positive for TTV. As indicated in Figure 1, the nested polymerase chain reaction results revealed that 92% were negative.

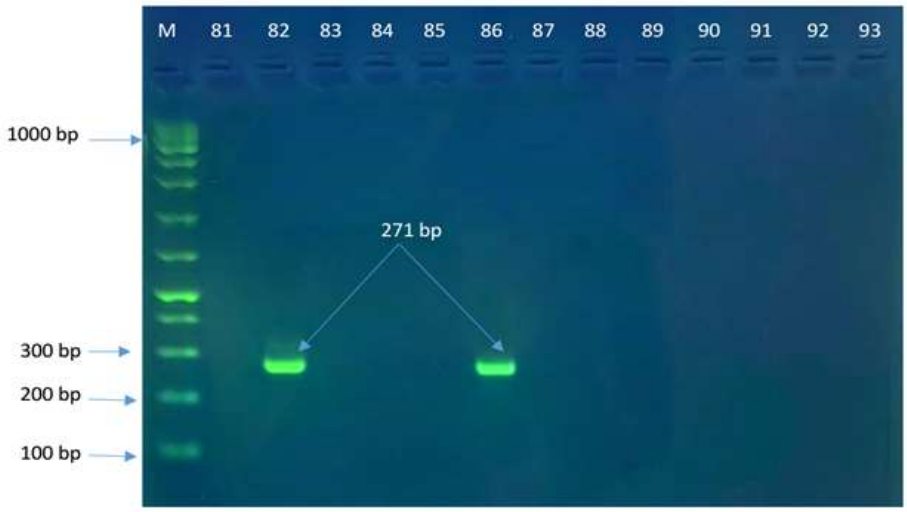


Figure 1. Gel electrophoresis of the second-round PCR amplification for NG061. M: DNA ladder (100-1000 bp). Samples 82 and 86 were positive, showing a product of 271 bp, while other samples were negative. The product (271 bp) was electrophoresed on 0.5% agarose at 7 volts/cm². The gel was run for 1 hour and 30 minutes, stained with red safe stain, and visualized under UV light.

The age-related distribution of positive and negative TTV infection in women with UTI

Four kinds of patients were used in this investigation. There were no positive cases within the age group, whereas the age group (31-46) had the most significant infection rate of 6 (75 percent), followed by one case for each of the age groups (17-30 years) and (63-77 years), respectively (47-62 years). The Table 6 demonstrates a strong relationship with age).

Table 6. Distribution of TTV Infection among women with UT according to age

Age	**Positive**	**Negative**
	No%	**No%**
17-30 years	1 (12.50%)	39 (42.39%)
31-46 years	6 (75.00%)	37 (40.22%)
47-62 years	0 (0%)	14 (15.22%)
63-77 years	1 (12.50%)	2 (2.17%)
Total	8 (100%)	92 (100%)
Chi-Square (χ^2)	6.25 *	42.348 **
Note: * (P≤0.05), ** (P≤0.01).		

Bacterial contamination in TTV positive samples

The Table 7 provides information on how the distribution of positive TTV DNA in urine samples indicated healthy bacterial growth. *Staphylococcus aureus* had three cases (37.5%), followed by two instances (25%) of each *Escherichia coli* and *Proteus mirabilis*. Compared to other species, *Enterococcus fecalis* displayed a lower frequency of 1 (12.5%), and no other species

exhibited any promising signs. No statistically significant differences between them were found, according to the investigation.

Table 7. Distribution of TTV infection according to culture

Bacterial isolates	**Positive**	**Negative**
	No%	**No%**
Staphylococcus aureus	3 (37.5%)	26 (28.26%)
Escherichia coli	2 (25%)	20 (21.73%)
Proteus mirabilis	2 (25%)	1 (1.09%)
Enterococcus faecal	1 (12.5%)	11 (11.96%)
Klebsiella spp.	0	4 (4.35%)
Pseudomonas spp.	0	1 (1.09%)
Streptococcus B-hemolysis	0	7 (7.60%)
Candida spp.	0	1 (1.09%)
No growth	0	21 (22.83%)
Total	8 (100%)	92 (100%)
Chi-Square ($\chi 2$)	1.000 NS	74.891 **
Note: ** (P≤0.01), **NS**=Non-Significant		

Phylogenetic analysis

Eight local isolates of TTV were studied using sequence nucleotide analysis, and strain NA-MU 15 *ORF1* gene, partial cds (ID: KY750543.1), length: 269, showed a significant Figure 2. There are matches. All eight samples' *ORF1* region (271 bp) amplicon was aligned pairwise with reference isolates using the GenBank collection on the NCBI website.

The clustering technique is the most often used method of creating the phylogenetic tree. Utilizing the NCBI website and MEGA 6 software, distance-based approaches, like the neighbor-joining method, convert the sequencing data into pairwise distances (dissimilarities) and utilize the matrix to estimate the genetic divergence between eight local isolates and 17 reference isolates. According to the closest, top to bottom, phylogenetic tree results analysis in the current investigation, the local isolate No. 6 was 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 displayed strong similarities to the Brazilian isolate DQ665287.1 and the Italian isolate AF212332.1.

The isolates with the numbers (3 and 7), (2 and 5), and (4 and 8) are highly similar to the Iranian isolates with the ID: GQ179967.1. Lastly, these outcomes validated that they were within P-distance, as demonstrated in Figure 2. Every local isolate was listed in GenBank with ID (MW513364.1, MW513365.1, MW513366.1, MW513367.1, MW513368.1, MW513369.1, MW513370.1 and MW513371.1).

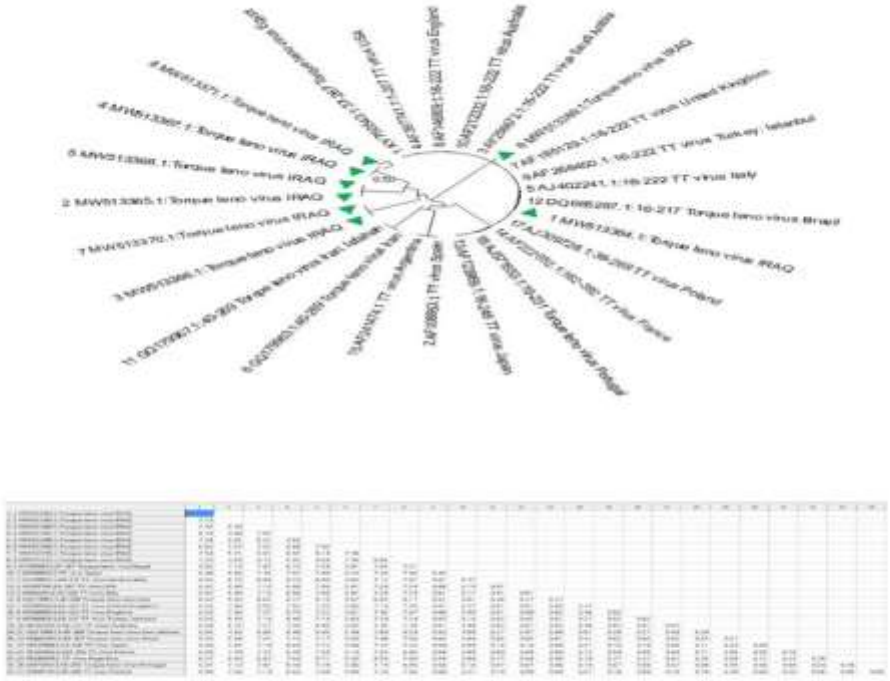


Figure 2. These outcomes validated that they were within P-distance.

DISCUSSION

Compared to the current study, neighboring nations had higher rates of TTV infection, with Iran showing the highest rates at 26.8% of thalassemia patients and 21% of hemodialysis patients. Hemodialysis patients in Saudi Arabia saw a mortality rate of 42.9% compared to the control group (19%). Compared to other research, this one reported a relatively high rate of TTV infection (2%) among patients with hemoglobinopathies and hematological malignancies in the Basrah Governorate. Additionally, an Iranian investigation found the virus in 5.8% of HCV-positive people and 4.0% of healthy people [14].

The variations in TTV infection rates across studies could be caused by various things, including the methods used for detection. Therefore, a nested PCR approach was adopted in the current investigation. Others utilize quantitative polymerase chain reaction at the same time (qPCR). Due to the qPCR's inability to cross-react with other viruses, it was able to detect the most common human TTV genotypes.

The sample size and the research population's nature are the second elements that might impact TTV infection rates. The sample size for the current investigation was one hundred urine samples. Al-Qahtani et al. examined 607 blood samples and found that (96.3%) of TTV/TTMV co-infections of an HBV group in various nationalities were found in Qatar. At the same times, (55.4%) of co-infected TTV/TTMV in a healthy population were also found there. Due to the significant danger of contamination from proliferating germs, urine samples should be tested as soon as possible (within 1.5 to 2 hours) of sample collection. Additionally, it is challenging to preserve since chemical preservatives alter the pH and specific gravity of the product. After DNA encapsulation, viral capsids' empty proteinaceous shells can undergo morphological modifications due to environmental pH variations [15].

The third reason is the utilization of numerous genes in various parts of the genome to identify TTV infection. TTV DNA was found in samples from eastern Taiwan natives in 95% of cases using the N22 area discovered (11%) and reanalyzed TTV prevalence with the UTR region. Results from the detecting 5'-UTR primer revealed that TTV DNA was more prevalent in patients and healthy controls than with the N22 primer. Intriguingly, non-coding portions (UTRs) in TTV and GC-rich regions, a

poly-A sequence downstream, a TATA box, and upstream are the conserved parts of the genome. Other variables that could impact include immunological function, diet, medicines, genetic makeup, regional distribution, seasonal considerations, way of life (smoking, drinking, physical activity), and transmission of zoonotic diseases [16].

The current investigation revealed that 6 TTV infections, or 75% of all cases, occurred in people aged 31 to 46. Only 1 case, or 12.5%, occurred in people aged 17 to 30 and 63 to 77. These findings were consistent with a study conducted in Canada using stool samples from individuals exhibiting signs of enteritis (954) and non-diarrheic people (76), which discovered that TTV prevalence was significantly higher in the young and elderly. This study also suggested that immunological status is crucial for infection by using qPCR assay.

This virus TTV load was significantly higher in the elderly (50-60 years old) compared to the young group during the investigation of TTV DNA load in the plasma of (313) healthy individuals using real-time PCR in Austria. The study investigated the association between TTV load and age and found a slightly positive correlation with age within a cohort of (379) elderly subjects in Italian elderly subjects. These findings are connected to positive TTV infection occurring in all age groups, and practically everyone was infected for an extended period without developing any symptoms, establishing favorable and productive contact with the host [17].

According to Murray et al., the current study revealed a moderate rate of bacterial growth in TTV-positive patients who were identified using numerous biochemical tests, such as three samples (37.5 percent) of *Staphylococcus aureus* followed by two samples (25 percent) of each *Escherichia coli* and *Proteus mirabilis*. *Enterococcus fecalis* 1 (12.5%) sample is the last. This conclusion is consistent with the study's finding that, in contrast to none in the controls in Washington, 57.1 percent of culture-positive samples and 100 percent of culture-negative samples showed the presence of TTV DNA. The current study disagreed with the study's findings that TTV occurs frequently in pregnant women who have average pregnancies and that it is linked to Brazil's lack of *L. crispatus* dominance, rise in vaginal MMP-8, and fall in D-lactic acid levels. Few resident *L. gasseri* and *L. crispatus* strains could produce bactericidal solid action, demonstrating each strain's unique capacity to manufacture and release antibiotic-like substances. It eliminated the microorganisms already linked with the cervix epithelial cells' surface and effectively protected the cells from the adverse effects of *G. vaginalis* toxin production and uropathogenic *E. coli*. Because changes in the vaginal microbiota's features lead to the loss of typically protective *Lactobacillus* spp., which raises the risk of UTI, the vaginal microbiota is a dynamic and frequently crucial part of this pathogenic interplay. These changes could result from estrogen insufficiency, antibiotic treatment, contraception, or other factors [18].

The current study is regarded as the first in the Diyala Governorate to deal with phylogenetic analysis of TTV, according to the information that is currently accessible. Sending the PCR product and the appropriate primers to a Macrogen business in Korea allowed for the sequencing of the product, which showed eight local isolates. After analyzing the sequence's nucleotides and amino acids, MEGA 6 software from NCBI was used to produce a substantial alignment with the TTV strain NA-MU 15 *ORF1* gene, partial with accession number (ID: KY750543.1) length: 269. Except for the local isolate (No. 3), which was 100% similar to the reference isolate TTV strain NA-MU 15 *ORF1* gene, the findings of the sequencing analysis revealed several changes (transition and transversion) at the TTV genome-related by ORF1-N22 area. Nucleotide sequence analysis revealed substantial similarity between all eight and reference isolates, ranging from 98 to 100 percent. Additionally, a high identification rate of all local isolates using this reference isolates' amino acid sequences ranged from 96 to 100 percent [19,20].

According to Sanjuan and Domingo-Calap, the local isolates may have undergone several recombinations and mutations in the Iraqi population, resulting in numerous TTV variants driven by a high mutation rate more similar to RNA viruses. There is much variation among the eight local isolates analyzed using a phylogenetic tree. Using MEGA 6 and NCBI software, the phylogenetic tree by the neighbor-joining method, as described by Saitou and Nei, revealed that local isolates clustered with the Egyptian isolate discovered by Hassuna et al., are reported TTV to be highly prevalent among children with thalassemia and non-thalassemic with genotypes 1 and 2. Isolate No. 6 was linked to the following isolates: Saudi Arabian isolate ID: AY256672.1, Australian isolate ID: AF212332.1, American isolate ID: AF397741.1, English isolate ID: AF146809.1 and Egyptian isolate ID: KY750543.1. Local isolates (No. 1) clustered with isolates from Italy (AJ402241.1) and Brazil (DQ665287.1), whereas isolates (No. 3) and (No. 2) exhibited close relationships to isolate (No. 7) and isolate (No. 4) clustered with isolate (No.8). All six of these isolates are related to Iranian isolates identified by accession number ID: GQ179967.1. The findings of this study concurred with those of a study by Al-Mozaini et al., which discovered that the most prevalent genotype of TTV in Saudi Arabia is 2. In addition, the study discovered that the results show that TTV is common in genotypes 1, 2, and 3 in Italy in people who were exposed to parenteral means and that the infection frequently persists.

The Figure 3 demonstrates that all six isolates are closely linked to Iranian reference isolates. The present study concurred with Iraqi studies interested in the phylogenic analysis of TTV (Figure 4), which discovered that sequence analysis revealed a mutation in the genome for TTV matching to ORF1 (N22). Additionally, utilizing ORF N22 primers, a Brazilian study discovered that TTV prevalence in healthy adults was 69.0 percent. The two nations' growing amity, the sharing of water resources, commerce interactions, religious tourism, and international travel for research and medical care can all be used to explain these findings.

Even within the same region, various reasons lead to considerable variance in the TTV genome. When utilized in phylogenetic analysis, ORF1 (N22 region) amplification results in high-diversity strains.

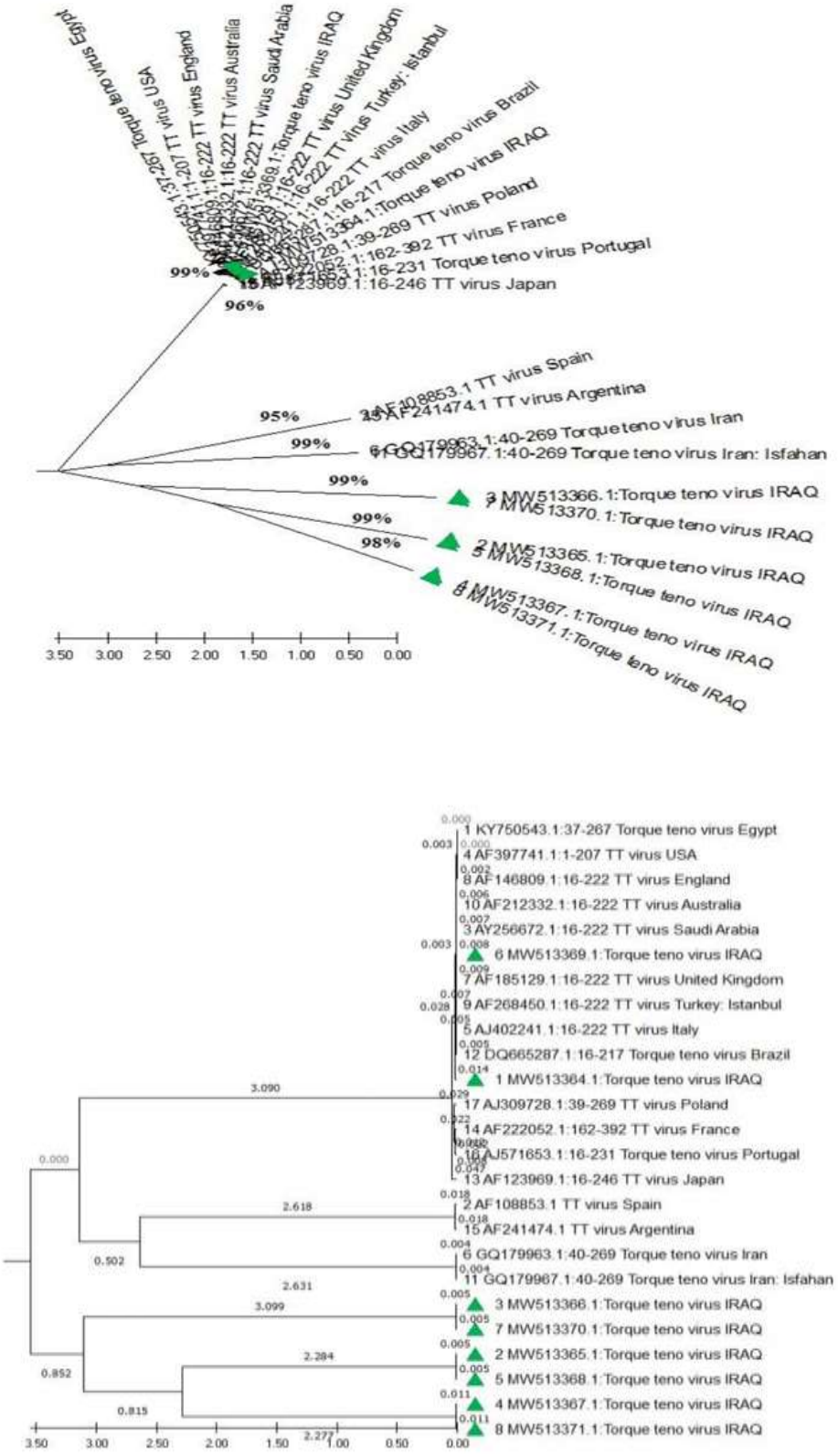
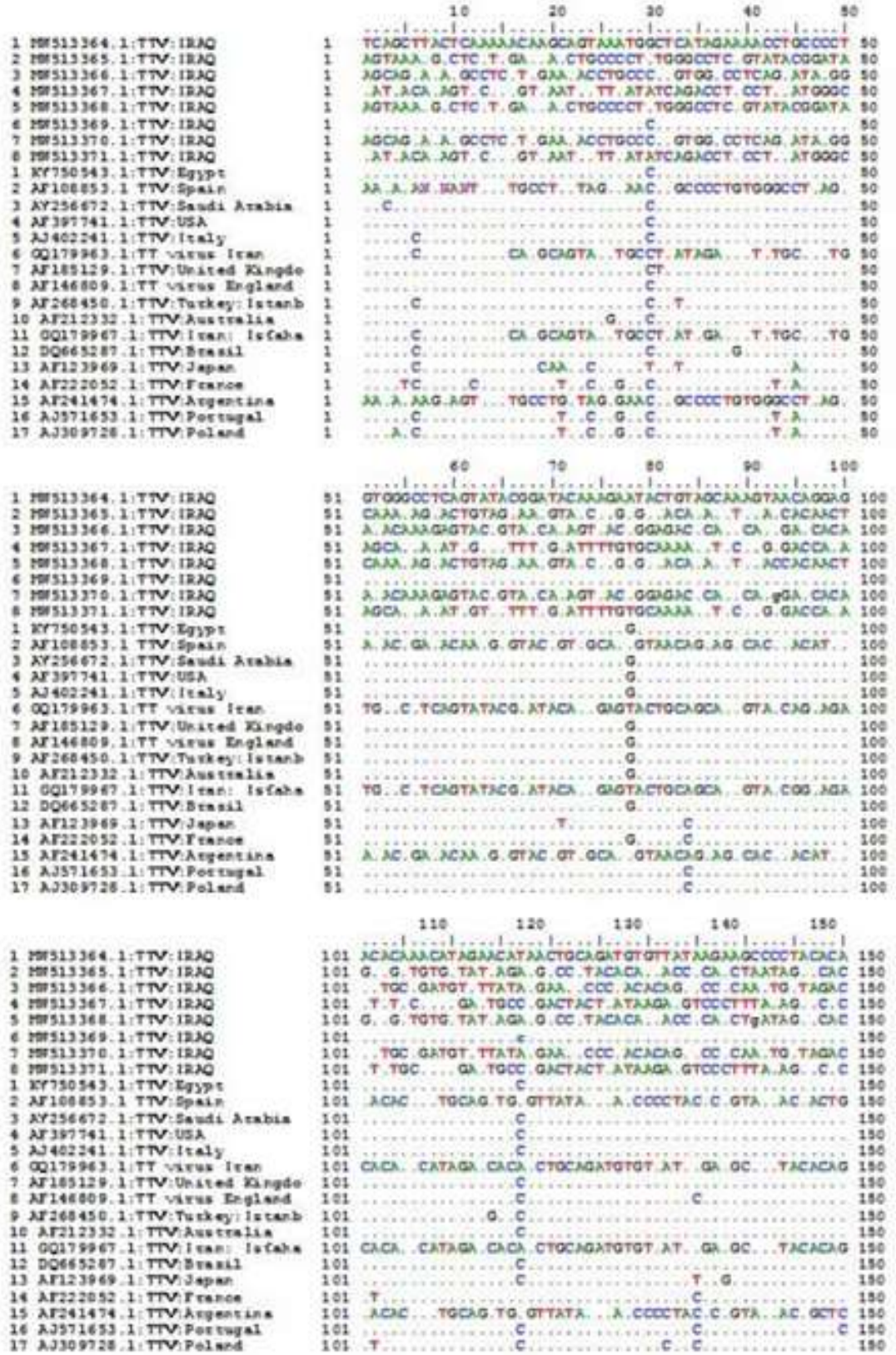


Figure 3. demonstrates that all six isolates are closely linked to Iranian reference isolates



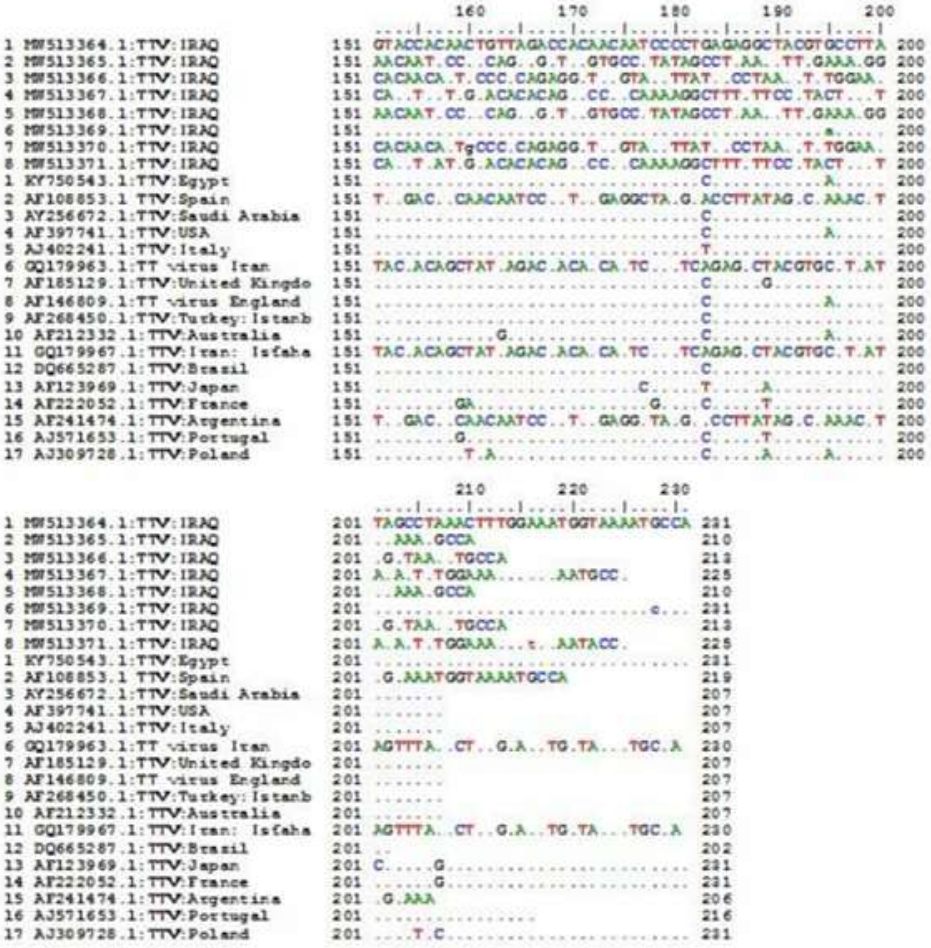


Figure 4. phylogenic analysis of TTV.

Various transmission mechanisms may cause widespread TTV infection across human populations. This might be connected to increased communication and travel to healthcare, tourism, and educational destinations. Additionally, TTV can spread through various channels, as evidenced by the fact that it can spread through exhalation and that TTV particles shed into feces may be highly resistant to the aquatic environment (Figure 5).

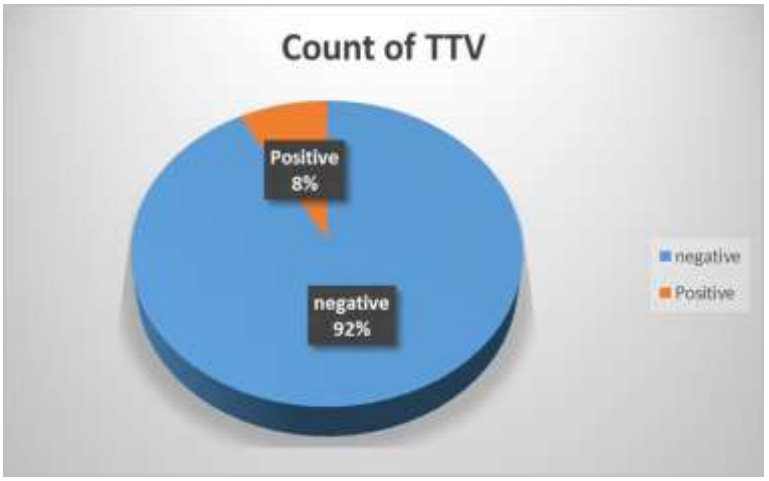


Figure 5. Count of TTV.

CONCLUSION

Torque Teno Virus genotypes 1 and 2 are shared by all isolated groups of study participants who are female and have UTI. Closed to isolates from other countries, including Egypt, the USA, England, Australia, and Saudi Arabia, was the local isolate (No. 6). To isolate Italian and Brazilian isolates, local isolates (No. 1) were shut down. The remaining six samples were similar to the Iranian isolates as well.

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DECLARATIONS

Interest-based conflict. There are no conflicts of interest, according to the authors.

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