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MOLECULAR DETECTION OF TORQUE TENO VIRUS (TTV) INFECTION OF PATIENTS WITH HAEMOGLOBINOPATHIES AND HAEMATOLOGICAL MALIGNANCIES

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ABSTRACT

The existence of Torque teno virus (TTV) DNA in different organs indicates the presence of wide affinity to the host cell. The present study aimed to detect TTV molecularly in the samples of haemoglobinopathies and haematological malignancies patients. The results of semi-nested PCR showed a positive amplification in 2 % of serum samples of haemoglobinopathies patients, while no positive amplification was observed in 50 EDTA bloods samples of haematological malignancies patients. One sample of positive cases was sequenced successively. The sequence was compared with deposited copies of TTV in the NCBI using BLAST software and the results showed a sequence 96.64% with KY750543.1. The result of the nucleotide sequence analysis has shown mutations at TTV genome corresponding to ORF1. The phylogenetic analysis results of our case ORF1 sequence with several selected ORF1 copies from NCBI displayed five main clades included grouping of our case ORF1 with the Italian (AJ402211.1) one in the same clade. The result of the amino acids sequence analysis was produced one peptide (58 amino acids) that showed a similarity of 68.97% with unnamed peptide (partial [Torque teno virus] Sequence ID: BAA74998.1 Length: 67) and a 51.72% with ORF1 (partial [Torque teno virus] Sequence ID: AAL08949.1 Length: 84). The present study concluded that the prevalence of TTV infections was remarkably low and it exhibits extensive genetic variation.

KEYWORDS: TORQUE TENO VIRUS, HAEMOGLOBINOPATHIES PATIENTS, HAEMATOLOGICAL MALIGNANCIES, SEQUENCING, AMINO ACIDS.

1. INTRODUCTION

In 1997, Nishizawa *et al.*, [1] discovered Torque teno virus (TTV) in Japanese patient serum with hepatitis. The newly TT-virus was named according to the patient initials. In human, five different geno-groups of TTV have been identified; in addition TTV was isolated from domestic animals recently [2]. The virus name was changed to Torgue teno virus, in latin “torques”, necklace; “tenuis”, thin [3]. TTV is a non-enveloped small virus, about

30 nm in diameter, circular ss negative DNA genome, ~3.6-3.8 kb in length, belongs to the *Circoviridae* Family [4]. After TTV discovery, many publications documented the TTV prevalence among acute or chronic hepatitis patients, blood donors, drug users and health people [5, 6]. Different transmission routes were suggested to describe the distribution of TTV in a wide range of tissues and body fluids. Because of TTV emergence in post-transfusion hepatitis and blood, prevalence of TTV DNA showed higher in patients that received multiple blood transfusions or blood products [7, 8]. However, several studies showed higher TTV prevalence among high-risk patients including 68% in hemophilia patients, 46% in hemodialysis patients, 40% in intravenous drug abusers, and 17.8% in hematological malignancies patients [8, 9]. Thus, this study aimed to investigate the TTV prevalence among patients with haemoglobinopathies and haematological malignancies by PCR technique to amplify N22 region and sequencing.

2. MATERIALS AND METHODS

2.1. Patients and samples

One hundred and fifty blood samples from patients of both sexes were collected, comprising 100 serum samples and 50 EDTA blood samples from patients with haemoglobinopathies and haematological malignancies (without any therapy), respectively.

The samples were collected from March to December, 2018 that obtained from Al-Basrah children's Hospital, Al- Sader educational Hospital and Center of hereditary blood disease of Al-Basrah Hospital for women and obstetrics at Basrah governorate, Iraq. All the samples were stored at -20^{0}C until DNA isolation.

2.2. Molecular detection of TTV

2.2.1. DNA extraction

DNA was extracted from serum and blood samples using a DNA extraction Kit (Promega, USA) according to the manufacturers' instructions.

2.2. 2. Semi nested PCR protocol

The semi nested PCR protocol was used to amplify two DNA fragment of TTV in the N22 region on ORF1. The first round, involved usage of outer primers: NG059 and NG063 (sequences 5´-ACA GAC AGA GGAGAA GGC AAC ATG-3´and 5´-CTG GCA TTT TAC CATTTC CAA AGT T-3´) to amplify 286 bp of target region. The reaction mixture (50µl), was composed of 10 µl of DNA templet, 10 pmol of NG059 and NG063 primers, 25 µl of master mix (Biolab, UK) and the volume completed to 50µl with DD-Water, while in the second round, semi inner primer NG061 (sequence 5´-GGC AAC ATGTTA TGG ATA GAC TGG-3´) and the outer primer NG063 were used to amplify 271 bp of first product. The reaction mixture (25µl), was composed of 5 µl of the first PCR product as a templet, 10 pmol of NG061 and NG063 primers, 12.5 µl of master mix and the volume completed to 25µl with DD-Water, The reaction conditions for first round were 94^{0}C for 30s, 35 cycles of 60^{0}C for 45s, 72^{0}C 45s, with a final extension at 72^{0}C for 7 min., while the second round conditions were 94^{0}C 30s, 30 cycles of 59^{0}C for 45s, 72^{0}C for 45s, followed by a final extension at 72^{0}C for 7 min. The Amplified products were visualized on 1.5 % agarose gel, and the purified products were sent to Zhejiang Youkang Biotechnology Co., Ltd (China) for sequencing.

2.2. 3. Phylogenetic analysis

DNA sequences of positive samples of TTV ORF1 were compared with similar selective copies from the database of American NCBI; National Center for

Biotechnology Information (www.ncbi.nlm.nic.gov). The phylogenetic analysis and the tree construction according to neighbor-joining algorithm using Mega 5 software. The nucleotide sequence of our case study was translated to amino acid sequence using CAP3 sequence assembly software and the resulted peptide sequence was compared with NCBI database for best alignment using BLAST software.

3. RESULTS AND DISCUSSION

3.1. RESULTS

The 100 of haemoglobinopathies patients including 73 Sickle cell anemia, 21 thalassemia and 6 with mixing of both were male (44%) and (56 %) of them were female with age range 2-45 years old. A 26 of 50 (52.3%) patients with haematological malignancies were males and (24 of 50; 48%) were females, with age range 2-93 years old. The prevalence of different types of haematological malignancies in patients were as follow: 20 of 50 (40%) patients with acute lymphocytic leukemia (ALL), 19 of 50 (38%) patients with Non-Hodgkin's disease (NHD) and 11 of 50 (22%) patients with Hodgkin's disease (HD).

3.1.1. Molecular detection

In order to detect the prevalence of TTV DNA in the studied patients, nested PCR was employed to amplify N22 region. The TTV was detected in 2 of 100 (2%) of haemoglobinopathies patients. Amplicons of 271 bp were visualized under UV light are shown in Figure (1). The two people who infected with TT virus are a 40-year-old woman with sickle cell anemia and an eight-year-old child (male) suffering from obviously thalassemia. Interestingly, in the patients with haematological malignancies, there was no evidence of any TTV DNA.

3.1.2. DNA Sequencing

The amplified products of the 2 positive cases were sent to sequencing. One sample was sequenced successively. The assembly of the forward and reveres primers were produced sequence of 271 bp. The DNA sequence from this case compared with other sequences available on NCBI databases using BLAST software.

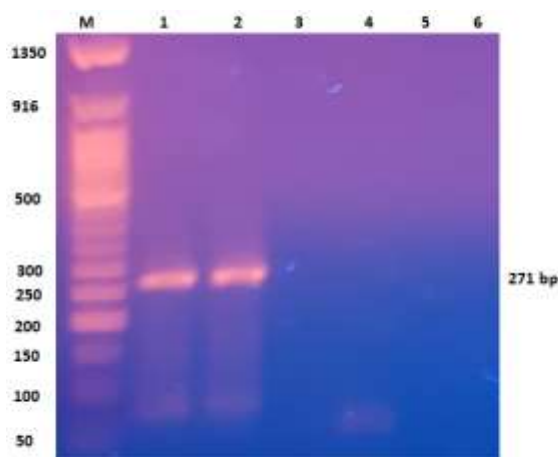

Figure 1: Agarose electrophoresis of TTV suspected samples using N22 specific primers. Conventional PCR amplicons were loaded on 1.5% agarose gel in order to visualize N22-encoding fragment. Lane M, 50 bp DNA size marker, Lanes 1-6 TTV examined samples.

In this study, the TTV sequence showed a sequence similarity of 96.64% with KY750543.1. Then, amplicon sequence of 271 bp compared with selected sequences of ORF1 region (Figure, 2) by sequence alignment one to others. The results of sequencing showed mutations including, replacements of G by C, A by G, A by G, C by T and T by G at nt. positions 125,65,79,88 and 98 respectively.

The phylogenetic tree analysis (Figure, 3) displayed five main clades, the present results revealed that the studied sample (H-C11_12_Basrah-Iraq) showed similarity or clustered with one sequence (AJ402211.1 TT virus partial n22 gene for ORF1 protein Italy) forming one of the clades with genetic distance or sequence divergence 0.02124, reflecting a close relationship of

these isolates. Also the studied sample clustered with sequence (AF395926.1: GI-5 ORF1 gene Greece) in other clade with sequence divergence 0.00137.

When nucleotide sequence of the studied sample converted to amino acids was produced six frames, one of them had length 58 amino acids with sequence correctly. When compared this amino acids by using BLAST software, the result showed similarity of 68.97% to unnamed protein product, partial [Torque teno virus] Sequence ID: BAA74998.1 Length: 67 (Figure, 4), and a 51.72% to ORF1, partial [Torque teno virus] Sequence ID: AAL08949.1 Length: 84.

The results of protein sequencing showed a several mutations along the protein when compared to sequence ID: BAA74998.1, including ten replacements at positions 2, 7, 8,29,30,42,44,47,49 and 51, also eight insertions at positions 5, 18, 20, 23,34,36,38 and 55. Furthermore, the protein sequence showed different mutations when compared to sequence ID: AAL08949.1, including twenty two replacements at positions 2,3,5,6,8,11,12,15,16,20,26,27,34,38,42,43,45,46,51,53,54 and 57, also six insertions at positions 7, 18,23,37,44 and 55.

3.2. DISCUSSION

The existence of TTV DNA in different organs indicates the presence of wide affinity to the host cell. The TTV history indicates as a potential hepatitis virus, so the main target organ (liver) has also been studied extensively. By *in situ* methods, TTV is found in the nucleus and/or the cytoplasm of hepatocytes in patients with liver damage [10, 11], but with no cytopathogenic effect (CPE) in liver cells [12, 13]. Several diseases are possible association with TTV infection such as aplastic anemia, thrombocytopenia, or idiopathic pulmonary fibrosis [14, 15]. In Iraq, The TTV prevalence has not been well documented. The results of semi-nested PCR reaction showed positive result in 2% of haemoglobinopathies patients out of the 100 serum samples. However, no amplification was observed in 50 EDTA bloods of the patients with haematological malignancies submitted to the same reaction. The low prevalence of TTV infections may be due to PCR primers and conditions, low virus loads and sequence variation, also the presence of TTV-like particles. In addition, in the Patients with leukemia and lymphoma may be due to reactivation of viruses especially TT virus following chemotherapy, while our samples were diagnosed at the beginning of the disease without any chemotherapy for the purpose of knowing the role these viruses with haematological malignancies.

In humans, distribution of TTV infection is still a subject of discussion, may be because of genotypes variability of TTV, also to inability to design of PCR primers to detection majority of genotypes [16]. Furthermore, with reason little known about distribution of TTV in healthy adults [17, 18]. Prevalence of TTV infection is changing due to development of new variants or new detection methods [19, 20]. DNA amplification by PCR is often used to detect TTV genome using two regions either (N22) coding region of the ORF1 or (UTR) non-coding region [21]. Amplification of the N22 only detects number of TTV genotypes [22, 23].

Contradiction in results was produced when using differed PCR conditions and primers, leads to detecting fewer genotypes than others [24-26]. Also, the turn and low of TTV loading may be because using suboptimal conditions of PCR [27].

Chemotherapy and direct contact increase the viral infections risk especially TT virus in leukemia patients [28-31]. Detection of TTV DNA in peripheral blood can be used as a biomarker for

hematological cancers screening. However, the high of TTV DNA titer in leukemia patients may be caused the cellular immune responses impairment without having any relation to cancer [32]. Several studies showed there is no relationship of TTV infection with hematological cancers especially in leukemia and lymphoma patients [33, 34]. In addition, DNA of TTV was not detected in leukocytes of leukemia patients [35]. Therefore the spread of TTV infection in these patients is silent [36]. Several researchers detected TTV-like particles from cell lines of lymphoma and leukemia [37]. In addition, other researchers detected virus-like particles in the Hodking's lymphoma [38]. Therefore, the huge of data of TTV sequencing allow us to design primers detect the most TTV genotypes.

The TTV sequence in the studied case showed a sequence similarity of 96.64% with KY750543.1, the result of the sequence analysis has shown mutations at TTV genome corresponding to ORF1. The phylogenetic tree analysis displayed five main clades. The amino acid sequence in this study was the first reported in Iraqi patients. The amino acid identity between H-C11_12_ Basrah-Iraq isolate and known sequences isolates noticed in the present study boosts the evidence that TTV has high heterogeneity.

Using capsid gene ORF1 as a marker, may be give a good idea for origin of evolutionary of the sequences. Furthermore, amplification of the complete genome may be useful for phylogenetic analysis [39, 40]. The phylogenetic analyses based on a small region on genome of TTV shows huge sequence heterogeneity [41]. The TTV heterogeneity may produce lower replication mutations, prolong of human infections, low in genetic matching, and absence of competition among isolates [42]. The TTV has a wide range of extreme in sequence divergence, so temporarily TTV genomes are classified into 23 genotypes with sequence divergence of 30% from one another [43], or into 4 major phylogenetic groups [44].

In conclusion, the TTV sequence in the studied case showed a sequence similarity of 96.64% with KY750543.1. The result of the sequence analysis has shown mutations at TTV genome corresponding to ORF1. The phylogenetic tree analysis displayed five main clades. The amino acid sequence in this study was the first reported in Iraqi patients. The amino acid identity between H-C11_12_ Basrah-Iraq isolate and known sequences isolates noticed in the present study boosts the evidence that TTV is highly heterogenic.

```
GQ179963.1              -GGCAACATGCTGTGGATAGACTGGCTAACTAAAGATGACTCAGCCTACTCAA-AACAAG  58
AF222052.1:             GGGCAATATGCTATGGATAGACTGGCTAACTAAAGATGACTCAGTCTACTCCAAAACAAG  60
AY032893.1              -GGCAACATGTTATGGATAGACTGGCTAACTAAAGATGACTCAAGCTACTCAAAAACAAG  59
KY750543.1NA_MU         ----AACATGTTATGGATAGACTGGCTAACTAAAGATGACTCAGCTTACTCAAAAACAAG  56
KY750545.1:             -------------------------------------GACTCAGCTTACTCAAAAACAAG  23
AF395926.1:             -GGCCACATGTTATGGATAGACTGGCTAACTAAAGATGATTCAGCCTACTCAAAAACAAG  59
H-C11_12_Basrah-Iraq    -GGGCAACATGTTTGGATAGACTGGCTAACTAAAGATGACTCAGCCTACTCAAAAACAAG  59
AJ402211.1              -------------------------CTAACTAAAGATGACTCAGCCTACTCAAAAACAAG  35
                                                             ** ***   ***** * ******

GQ179963.1              CAGTAAATGCCTTATAGAAAATCTGCCCCTGTGGGCCTCAGTATACGGATACAAAGAGTA  118
AF222052.1:             TAGCAAGTGCCTCATAGAAAACTTACCCCTGTGGGCCTCAGTATACGGATACAAAGAGTA  120
AY032893.1              TAGCAAGTGTCTCATAGAAAACCTACCCCTGTGGGCCTCAGTATACGGATACAAAGAATA  119
KY750543.1NA_MU         CAGTAAATGCCTCATAGAAAACCTGCCCCTGTGGGCCTCAGTATACGGATACAAAGAGTA  116
KY750545.1:             CAGTAAATGCCTCATAGAAAACCTGCCCCTGTGGGCCTCAGTATACGGATACAAAGAGTA  83
AF395926.1:             CAGTAAATGCCTCATAGAAAACCTGCCCCTGTGGGCCTCAGTATACGGATACAAAGAGTA  119
H-C11_12_Basrah-Iraq    CAGTAAATGCCTTATAGAGAACCTGCCTCTGTGGGCCGCAGTATACGGATACAAAGAGTA  119
AJ402211.1              CAGTAAATGCCTTATAGAGAACCTGCCCCTGTGGGCCTCAGTATACGGATACAAAGAGTA  95
                         ** ** ** ** ***** **  * ** ********* ******************* **

GQ179963.1              CTGCAGCAAAGTAACAGGAGACACAAACATAGAACACAACTGCAGATGTGTTATTAGAAG  178
AF222052.1:             CTGCAGCAAAGTAACAGGAGATACAAACATAGAACATAACTGCAGATGTGTTATCAGAAG  180
AY032893.1              CTGCAGCAAAGTAACAGGAGACACAAACATAGAACACAACTGTAGATGTGTTATTAGAAG  179
KY750543.1NA_MU         CTGTAGCAAAGTAACAGGAGACACAAACATAGAACACAACTGCAGATGTGTTATAAGAAG  176
KY750545.1:             CTGTAGCAAAGTAACAGGAGACACAAACATAGAACACAACTGCAGATGTGTTATAAGAAG  143
AF395926.1:             CTGTAGCAAGGTAACAGGAGACACAAACATAGAACACAACTGCAGATGTGTTATAAGAAG  179
H-C11_12_Basrah-Iraq    CTGTACCAAAGTAACAGGAGACACAAACATAGAACACAACTGCAGGTGTGTTATAAGAAG  179
AJ402211.1              CTGTAGCAAAGTAACAGGAGACACAAACATAGAACACAACTGCAGATGTGTTATAAGAAG  155
                        *** * *** *********** ************** ***** ** ******** *****

GQ179963.1              CCCCTACACAGTACCACAGCTATTAGACCACAACAATCCCCTCAGAGGCTACGTGCCTTA  238
AF222052.1:             CCCCTACACAGTACCACAGATGTTAGACCACAACAATGCCCTCAGAGGTTACGTGCCTTA  240
AY032893.1              CCCCTACACAGTACCACAGCTGTTAGACCACAACAATCCCCTCAGAGGCTACGTACCTTA  239
KY750543.1NA_MU         CCCCTACACAGTACCACAACTGTTAGACCACAACAATCCCCTCAGAGGCTACGTACCTTA  236
KY750545.1:             CCCCTACACAGTACCACAACTGTTAGACCACAACAATCCCCTCAGAGGCTACGTACCTTA  203
AF395926.1:             CCCCTACACAGTACCACAACTGTTAGACCACAACAATCCCCTCAGAGGCTACGTGCCTTA  239
H-C11_12_Basrah-Iraq    CCCCTACACAGTACCACAACTGTTAGACCACAACAATCCCCTCAGAGGCTACGTGCCTTA  239
AJ402211.1              CCCCTACACAGTACCACAACTGTTAGACCACAACAATCCCCTCAGAGGCTACGTGCCTTA  215
                        ******************   * *************** ********** ***** *****

GQ179963.1              TAGTTTAAACTTTGGAAATGGTAAAATGCCAG      270
AF222052.1:             TAGCCTGAACTTTGGAAATGGTAAAATGCCAG      272
AY032893.1              TAGCCTCAACTTTGGAAATGGTAAAATGCCAG      271
KY750543.1NA_MU         TAGCCTAAACTTTGGAAATGGTAAAATGCCAG      268
KY750545.1:             TAGCTTTAACTTTGGAAATGGTAAAATGCCAG      235
AF395926.1:             TAGCCTAAACTTTGGAAATGGTAAAATG----      267
H-C11_12_Basrah-Iraq    TAGCCTAAACTTTGGAAATGGTAAAATGCCAG      271
AJ402211.1              TAGCCTA-------------------------      222
```

Figure 2: Comparison of nucleotide sequence of TTV N22 with selected ORF1 sequences

H-C11_12_Basrah-Iraq
0.02124
AJ402211.1 TT virus partial n22 gene for ORF1 protein Italy
0.00958
-0.00137
0.00247
AF395926.1: GI-5 ORF1 gene Greece
0.01066
0.01053
KY750543.1NA_MU 15 ORF1 gene Egypt
0.00086
0.00773
0.01322
KY750545.1: NA_MU 49 ORF1 gene Egypt
0.00888
GQ179963.1 Isfahan MEF4 nonfunctional ORF1 gene Iran
0.02562
AY032893.1 17old DLBCL ORF1 gene Italy
0.03212
0.01461
AF222052.1: TTVm-ty2 putative ORF1 gene France
0.04242

Figure 3: Phylogenetic tree of nucleotide sequence of TTV N22 with selected ORF1 sequences

unnamed protein product, partial [Torque teno virus]
Sequence ID: **BAA74998.1** Length: 67 Number of Matches: 1
Range 1: 10 to 67

Score	Expect	Method	Identities	Positives	Gaps	Frame
81.3 bits(199)	6e-19()	Compositional matrix adjust.	40/58(69%)	48/58(82%)	0/58(0%)	

Features:

```
Query  1   MPYREPASVGRSIRIQRVLYQSNRRHKHRTQLQVCYKKPLHSTTTVRPQQSPQRLRAL  58
           M YR+P  VGRSIRIQR+L+QS+RRHKH  QLQ+C+K+PLH T TV P Q PQR+RAL
Sbjct  10  MSYRKPTPVGRSIRIQRILHQSHRRHKHTPQLQMCHKEPLHRTPTVSPYQPPQRIRAL  67
```

ORF1, partial [Torque teno virus]
Sequence ID: **AAL08949.1** Length: 84 Number of Matches: 1
Range 1: 24 to 81

Score	Expect	Method	Identities	Positives	Gaps	Frame
54.7 bits(130)	3e-08()	Compositional matrix adjust.	30/58(52%)	36/58(62%)	0/58(0%)	

Features:

```
Query  1   MPYREPASVGRSIRIQRVLYQSNRRHKHRTQLQVCYKKPLHSTTTVRPQQSPQRLRAL  58
           M  R  + VG  IR  R+L QS+RR  HRTQLQ CY+ PLH  +  RPQQ P  +R L
Sbjct  24  MSNRRLSXVGLGIRXXRILQQSHRRLSHRTQLQSCYEVPLHXPSXYRPQQPPVGVRPL  81
```

Figure 4: Comparison between TTV N22 amino acid sequences with other amino acids

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التشخيص الجزيئي لفيروس TORQUE TENO VIRUS (TTV) لدى مرضى اعتلال خضاب الدم و الاورام الخبيثة الدموية

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الخلاصة

يدل وجود دنا فيروس TTV في الاعضاء المختلفة الى الالفة العالية الى خلايا العائل. تهدف الدراسة الحالية الى التشخيص الجزيئي لفيروس TTV في دم المرضى الذين يعانون من اعتلال خضاب الدم والاورام الخبيثة الدموية بواسطة استخدام تقنية تفاعل سلسلة البوليمريز PCR وتحليل تعاقب تسلسل القواعد النتروجينية Sequencing. اظهرت نتائج تفاعل PCR Semi-nested الى حصول تضخيم في 2 (2%) عينة من عينات 100 مصل مرضى اعتلال خضاب الدم. على اي حال، لم يلاحظ اي تضخيم في 50عينة دم لمرضى الاورام الخبيثة الدموية التي خضعت الى نفس التفاعل. جرى بنجاح تحليل تعاقب تسلسل القواعد النتروجينية لعينة واحدة من العينات الموجبة. اظهر تعاقب تسلسل القواعد النتروجينية للعينة المدروسة لفيروس TTV تشابه لتسلسل قواعد العينات في بنك الجينات بنسبة 96.64% مع KY750543.1، حيث اظهرت نتائج تحليل تسلسل القواعد الى وجود طفرات في جينوم الفيروس في منطقة ORF1. كما اظهر تحليل شجرة النشوء والتطور Phylogenetic tree الى وجود خمس مجاميع Clades رئيسية. واظهرت نتائج تحليل الاحماض الامينة الى انتاج تسلسل تعاقب لبروتين واحد بصورة صحيحة طوله 58 من الاحماض الامينية، الذي اظهر تشابه بنسبة 68.97% الى unnamed protein product, partial [Torque teno virus] Sequence ID: BAA74998.1 Length: 67، وتشابه بنسبة 51.72% الى ORF1, partial [Torque teno virus] Sequence ID: AAL08949.1 Length: 84. تستنتج الدراسة الحالية الى انتشار واطيء لفيروس TTV مع وجود تغاير جيني مكثف في تعاقب تسلسل جينوم الفيروس.