

Expression of TTV-ORF2 Protein for Detection of Anti-TTV IgG Antibodies in Human Sera

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ABSTRACT

The present study describes the cloning and expression of ORF-2 region of TTV genome and the use of expressed peptide in developing immunoassay for detection of anti-TTV antibodies in serum. Presence of TTV-DNA in serum was detected by PCR amplifying N-22 region of ORF-1 of TTV genome. This was followed by genotyping of TTV by RFLP using N-22 amplicon. Using genotype-1 positive serum as the source of TTV, the ORF-2 region was amplified by PCR and subsequently cloned and expressed in pET-19b vector. The expressed protein, identified as 20 kDa protein on SDS-PAGE gel, was purified by affinity chromatography and then used as antigen to develop western blot assay for detection of anti-TTV antibodies in serum. Analysis of sera for anti-TTV antibodies and their comparison with presence of TTV-DNA, produced encouraging results. There was a good relation between presence of anti-TTV and TTV-DNA in these sera samples. Anti-TTV antibodies could be detected in all TTV-DNA positive sera irrespective of the presence of TTV-genotype. This investigation demonstrates that ORF-2 peptide may be used in developing immunoassay for identification of TTV infection.

Keywords: TTV; ORF-2; Anti TTV-IgG; Expression; N-22

1. Introduction

Torque Teno Virus (TTV) is an icosahedral, non-enveloped, circular, single-stranded, negative sense DNA virus with 3852 bases in its genome [1-3]. The genome of TTV contains a highly conserved non-coding region and the coding region. The coding region consists of ORF1 that encodes viral capsid protein and ORF2 encodes non-structural proteins. Beside ORF1 and ORF2, the coding region of TTV also has ORF3 and ORF4 whose products are not known [4-7]. The TTV genome exhibits high diversity and thus, has been classified into several genotypes [8-11]. More than 40 genotypes of TTV have been identified till date [12].

TTV is mainly transmitted via the parenteral route and found in blood and blood products [13,14]. However, TTV can be transmitted by non-parenteral routes also. This is evident by its excretion in feces [7,15] and in exhaled breath [16]. The association of TTV with cryptogenic chronic liver diseases [17,18], post-transfusion hepatitis [5,11,19] and acute hepatitis of unknown etiology [7], suggested a possible etiological role of this agent

in the development of both acute and chronic hepatitis. TTV-DNA has been reported more frequently in patients with liver cirrhosis and hepatocellular carcinoma [20,21], though its role in causing the disease is not known. In several studies, the viral genome has been reported at comparable prevalence rates in the blood of patients and healthy persons. This led to the hypothesis that TTV might be essentially non-pathogenic in nature [22].

Although several reports have been published on various aspects of TTV including physico-chemical characteristic of the virus, however, its prevalence, genomic organization, infective potency and diagnostic procedures for its detection still need investigations. Moreover, there are only few reports available that demonstrate cloning and expression of N-22 and ORF1 but without reaching any final conclusions [8,23-28]. The present investigation was undertaken to express ORF2 region of TTV genome and used the translation product to develop a simple immunoassay for detection of anti-TTV antibodies in serum. This was aimed to have a simple diagnostic assay for TTV infection in all small

diagnostic laboratories.

2. Methodology

2.1. Ethics

The Ethical approval for this study was given by Ethical Committee of All India Institute of Medical Sciences, New Delhi, India.

2.2. Patients and Blood Samples

Sera were obtained, with informed consent, from 110 patients including 50 patients with liver diseases, 50 patients with renal failure and 10 healthy controls.

2.3. Detection of TTV-DNA

Sera were analyzed for the presence and genotyping of TTV-DNA by detecting N-22 region of TTV genome. N-22 region of ~270 bp was amplified by nested PCR using NG059, NG063 and NG061 primers [10]. PCR mixture contained 200 µM of each dNTPs (Qiagen, Germany), 25 pmoles/µl of each primer, 1.5 mM MgCl₂ and 1.5 U of Taq Polymerase (Qiagen, Germany) in 25 µl reaction mixture. First round PCR was performed for 35 cycles and second round PCR for 25 cycles of amplification. Initial denaturation was at 95°C for 5 min, amplification conditions were: 95°C for 30 sec, 55°C for 1 min and 74°C for 1min. Amplicons were detected by agarose gel electrophoresis followed by EtBr staining. Amplified products were confirmed by sequencing and phylogenetic analysis.

2.4. Amplification of Full Length ORF2 Gene

The amplification of full length ORF2 region of TTV was carried out using serum positive for TTV genotype-1 (G-1). Following set of primers were designed using softwares like DNA star and Primer 3plus : Forward—5'-ATG TTT ATT GGC AGG CAC TA-3' and Reverse—5'-TTA CGT TTC TGC GGC GG-3' (Accession no. AF122914). Amplification conditions were as follows : Initial denaturation was done at 94°C for 7 min, followed by 35 cycles of amplification conducting annealing at 58°C for 45 sec, extension at 74°C for 45 sec and final extension at 74°C for 3 min. The amplicon was resolved on agarose gel and identified as ~459 bp long fragment.

2.5. Cloning of Full Length ORF2 Using TA-Sequencing Vector

The PCR product of ORF2 region (~459 bp) was purified and cloned using TA sequencing vector. The recombinant vector was then transformed into *E. coli* (DH5α) cells that were grown overnight at 37°C on LB agar plate containing ampicillin. The colonies were screened by

sequencing and Restriction Digestion using *NcoI* restriction enzyme. The products were resolved on agarose gel. Presence of ~3000 bp and ~500 bp fragment confirmed correct insertion of ORF2 gene in TA vector.

2.6. Cloning of ORF2 in pET19b Expression Vector

Recombinant plasmid was isolated from *E. coli* cells and ORF2 was amplified with primers designed with *NdeI* and *BamHI* restriction enzyme sites in between. Primers were as follows: Forward 5'-CTG ATT **CAT ATG** ATG TTT ATT GGC AGG CAC-3', Reverse: 5'-ATA TAT **GGA TCC** TTA CGT TTC TGC GGC GGC-3'. In each primer, bold sequences are the site for restriction enzymes to facilitate cloning in expression vector. After digestion with *NdeI* and *BamHI* restriction enzymes, the vector (pET19b) and the amplicon were ligated using T4 DNA ligase (Fermentas, Canada). The recombinant vector was transformed in *E. coli* BL21 cells and incubated at 37°C for overnight on LB agar (containing ampicillin). The colonies grown were confirmed by sequencing and restriction digestion of vector with *NdeI* and *BamHI* enzymes.

2.7. Expression of ORF2 Gene in *E. coli*

ORF2 region was expressed as a fusion protein containing 6 histidine residues at N-terminal of expressed protein. Expression was carried using IPTG in 1mM concentration for 4, 8, 12, 16 and 20 hrs at 37°C. Finally, the culture was harvested at room temperature. A parallel culture was set up for control without adding IPTG. Expression of desired protein was confirmed by running the cell lysate of induced and un-induced culture on 12% SDS-PAGE. Purification of recombinant ORF2 protein was done by affinity chromatography using QIAexpress Ni-NTA fast start protein purification kit, Qiagen (Germany).

2.8. Western Blot Analysis for Identification of Expression Product

After SDS-PAGE, protein was transferred on PVDF membrane. The membrane was blocked with 3% BSA in PBS (pH 7.4) and incubated with primary antibodies (Mouse anti-His antibodies, Qiagen, Germany) in 1:1000 dilutions for 3 hours at room temperature. Following this, secondary antibody (Goat-anti-mouse antibodies—HRP conjugate, Santa Cruz, USA) was added in 1:3000 dilutions and incubated for 2 hours at room temperature. Finally, blot was developed by adding Diaminobenzidine (DAB, SRL, India).

2.9. Blot Assay for Anti-TTV Antibodies Using ORF2 Protein as Antigen

The protein was transferred from gel to PVDF membrane.

The membrane was blocked with 3% BSA. Human sera in different dilutions (1:100, 1:1000, 1:3000 and 1:5000) were used for detection of IgG antibodies against TTV infection. Anti-TTV antibodies were detected by secondary antibody (anti-human IgG HRP conjugated antibody, Qiagen, Germany) added in 1:6000 dilutions in PBST. Secondary antibodies were detected with substrate (DAB, SRL, India).

3. Results

Using N-22 region of TTV genome for detection and genotyping of TTV, a total number of 110 patient's sera were analyzed. TTV viraemia (TTV-DNA) was detected in 25 of 50 (50%) patients with liver disease, 28 of 50 (56%) patients with renal disease and 2 of 10 (20%) sera from healthy control. All positive samples were subjected to sequencing. Retrieved sequences were BLAST analyzed with known TTV sequences available in the database. Genotype 1 was found to be the predominant genotype among studied group followed by detection of genotype 2 in few cases. Since genotype 1 was the predominant genotype, JA-20 isolate of TTV genotype-1 was used as a reference isolate for amplification of ORF2 region. ORF2 was amplified and cloned in TA sequenc-

ing vector and was confirmed by sequencing. Retrieved sequences were BLAST analyzed and showed 100% similarity with ORF2 sequence of TTV genotype-1 (JA20 isolate) (**Figure 1**).

ORF-2 region of TTV genotype-1 was amplified and cloned in expression plasmid containing histidine tag. It expressed the protein at 8, 12, 16 and 20 hour post incubation at 37°C (**Figure 2**). Optimal expression of protein was obtained after 16 hr incubation at 37°C. ORF-2 of JA-20 isolate often produces a truncated protein of molecular mass ~20 KDa. The protein was purified by affinity chromatography and was 1 mg/ml in concentration. Expression was confirmed by immuno-blotting with anti-His antibodies (**Figure 3**). The ORF2 protein was evaluated for its immunogenic nature by detection of anti-TTV antibodies in human sera (**Figure 4**) with different dilutions. In order to detect presence of antibodies in different patient group, 1:1000 dilution of human sera were used.

The result of blot assay demonstrated that TTV-DNA negative samples, from both disease groups as well as healthy controls, could not develop the Western blot, showing the absence of anti-TTV antibodies in these sera. Since, genotype 1 and 2 are prevalent in Indian population [29], only sera positive for TTV-DNA of genotype 1

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>lcl|8559, Length=622, Score = 837 bits (453), Expect = 0.0,
Identities = 453/453 (100%), Gaps = 0/453 (0%), Strand=Plus/Plus

Query 1   ATGTTTATTGGCAGGCACTACAGAAAGAAAGGGCGCTGCTACTGCTATCTGTGCATTCT 60
          |||
Sbjct 139 ATGTTTATTGGCAGGCACTACAGAAAGAAAGGGCGCTGCTACTGCTATCTGTGCATTCT 198

Query 61   ACAAAGACAAAAGGGAACTTCTGATAGCTATGTGGACTCCCCACGCAATGATCAACAA 120
          |||
Sbjct 199 ACAAAGACAAAAGGGAACTTCTGATAGCTATGTGGACTCCCCACGCAATGATCAACAA 258

Query 121  TACCTTAACTGGCAATGGTACAGTTCTGTACTTAGCTCCCACTCTGCTATGTGCGGCTGT 180
          |||
Sbjct 259 TACCTTAACTGGCAATGGTACAGTTCTGTACTTAGCTCCCACTCTGCTATGTGCGGCTGT 318

Query 181  CCCGACGCTATCGCTCATCTTAATCATCTTGCTAATCTGCTTCGTGCCCCGCAAAATCCG 240
          |||
Sbjct 319 CCCGACGCTATCGCTCATCTTAATCATCTTGCTAATCTGCTTCGTGCCCCGCAAAATCCG 378

Query 241  CCCCCGCTGATAATCCAAGACCCCTACCCGTGCGAGCACTGCCTGCTCTCCCGGCTGCC 300
          |||
Sbjct 379 CCCCCGCTGATAATCCAAGACCCCTACCCGTGCGAGCACTGCCTGCTCTCCCGGCTGCC 438

Query 301  CACGAGGCGGCCGGTGATCGAGCACCATGGCCTATGGGTGGTGGAGGAGACGCCGAGGC 360
          |||
Sbjct 439 CACGAGGCGGCCGGTGATCGAGCACCATGGCCTATGGGTGGTGGAGGAGACGCCGAGGC 498

Query 361  GCTGGCGCAGGTGGAGACGCCACCATGGAGGCGCCGCTGGAGGACCCGAGACGCAGAC 420
          |||
Sbjct 499 GCTGGCGCAGGTGGAGACGCCACCATGGAGGCGCCGCTGGAGGACCCGAGACGCAGAC 558
```

Figure 1. Sequence alignment of cloned ORF2 region with known TTV sequences using BLAST analysis. All the sequences retrieved after sequencing of cloned ORF2 were aligned using BLAST and shows 100% similarity with known TTV sequences of genotype1.

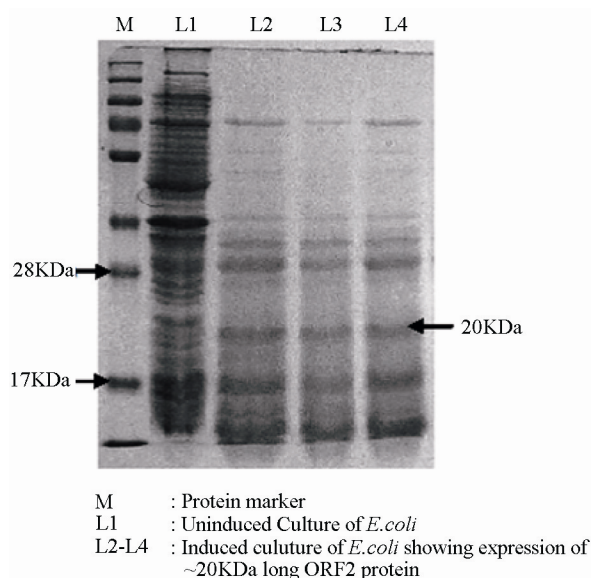


Figure 2. SDA-PAGE analysis of expressed ORF2 protein. ORF2 protein was expressed at 37°C for 16 hrs with 1mM IPTG. Gel picture shows expression of ORF2 protein in induced bacterial culture in comparison to un-induced culture.

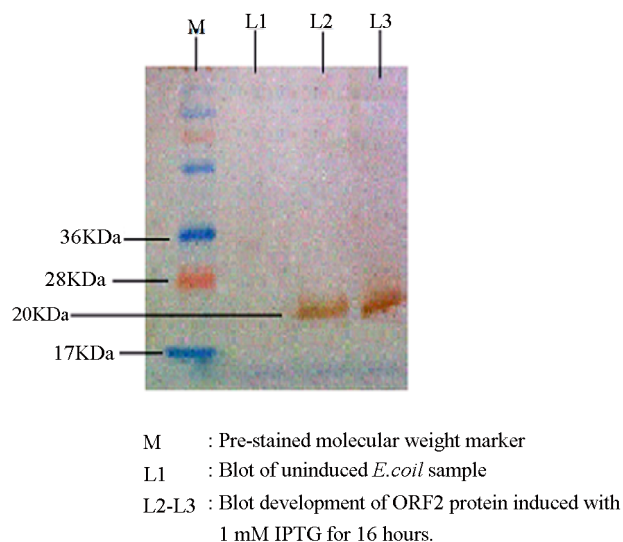
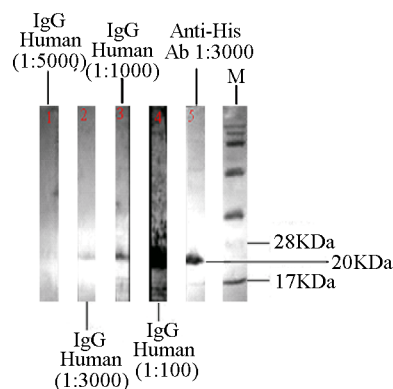


Figure 3. Western blot analysis using anti-His antibodies. Expressed protein was confirmed by Western blot using anti-His antibodies and was compared with uninduced bacterial culture.

and 2 were used for antibody detection. Although, ORF2 used for Western Blot assay was derived from genotype1 (G1), however, all these samples developed the blot indicating presence of anti-TTV antibodies in them. Therefore, it is suggested that ORF2 protein is not genotype specific and can detect antibodies in all TTV-DNA positive samples.

A total number of 110 sera samples including 50 sera



M : Pre-stained molecular weight marker

- 1 : Detection of ORF2 protein (20KDa) with human serum (Primary ab) in 1:5000 dilution
- 2 : Detection of ORF2 protein (20KDa) with human serum (Primary ab) in 1:3000 dilution
- 3 : Detection of ORF2 protein (20KDa) with human serum (Primary ab) in 1:1000 dilution
- 4 : Detection of ORF2 protein (20KDa) with human serum (Primary ab) in 1:1000 dilution
- 5 : Detection of ORF2 protein (20KDa) with anti-his antibody (Primary ab)

Figure 4. Western blot analysis using Human Sera. ORF2 protein was used as antigen to detect anti-TTV antibodies in human sera. Expressed protein was transferred on PVDF membrane and was reacted with human sera in different dilutions. 1:1000 dilution was used for further analysis of different sera samples.

from patient with liver disease, 50 sera from renal diseases and 10 sera from healthy controls were analyzed for anti-TTV antibodies. These samples were first analyzed for the presence of TTV-DNA by PCR. Analysis of these TTV-DNA positive samples shows the presence of anti-TTV antibodies in 25 of 25 (100%) cases with liver disease, 28 of 28 (100%) cases with renal disease and 2 of 2 (100%) sera from healthy control (**Table 1**). Thus, all the samples positive for TTV-DNA also showed the presence of anti-TTV antibodies. These results indicate a successful development of Western blot assay using ORF2 expressed protein for detecting anti-TTV antibodies in human sera. There is a good scope of this protein for use in developing a simpler immunoassay for the diagnosis of TTV infection.

4. Discussion

TTV is a DNA virus with global prevalence and very little evidence of being a potent pathogen associated with some known diseases. Its relatively recent discovery and association with certain carcinoma causes an interest to study this virus in more detail [30-33]. TTV infection is diagnosed by detecting TTV-DNA in blood. Its genome is not very complex and there are several regions that may be cloned and expressed for producing a protein for developing immunoassay for its serodiagnosis. ORF2 region is used as an option to clone and express it to meet this aim, particularly because it produces nonstructural protein and may help in developing immunoassays for detection of antibodies in sera against this non-structural

Table 1. Detection of anti-TTV antibodies in Liver and renal disease cases.

Disease group	No. of Cases (n)*	TTV-DNA Positive	Anti-TTV Positive	% Positive
Liver Disease	50	25	25	50
Renal Disease	50	28	28	56
Healthy Control	10	2	2	20

*n: number of cases studied. Anti-TTV antibodies were detected in Liver and Renal diseases cases as well as Healthy controls. All the sera sample were first analyzed for the presence of TTV-DNA by amplifying N22 region and then genotyped using different restriction enzyme [10]. All samples that were found positive of TTV-DNA also shows the presence of anti-TTV antibodies in them by using expressed protein as antigen.

protein for diagnosis of TTV infection.

Full length ORF2 gene of TTV genotype-1 was amplified using primers designed from JA20 isolate [3]. The selection of genotype-1 was done for the reason that this genotype showed significantly high prevalence in Indian populations [29]. ORF2 of JA-20 isolate of TTV genotype-1 produces a truncated protein as compared to the one produced by full length genomic expression of TA278 isolate.

As discussed earlier, ORF2 region encodes a protein of near about 20 KDa [8] which is a non-structural protein and has been reported to induce host immune response against TTV [8,34-36]. Although, earlier attempts in several studies focused on the expression of N22 region [26] and ORF1 [28,37], however, their protein product could not prove successful antigenic agent to induce host immune response or for their use in developing immunoassays for sero-detection of TTV infection. As a result, there was a focus exclusively on ORF2 this time, assuming that it may play a significant role in host immunity against TTV infection.

The Western blot technique was developed and used for analysis of anti-TTV antibodies in sera. This study included a total number of 110 sera from healthy control as well as patients belonging to different types of liver diseases and renal ailments. The results of Western blot demonstrated a distinct difference in antibody positive and antibody negative sera. Results show the prevalence of anti-TTV antibodies in sera from TTV DNA positive healthy controls and patient groups. The results were encouraging and comparable to those reported earlier in several other studies [8,24,28]. In all these sera, TTV-DNA and anti-TTV were detected simultaneously. From these results, it is evident that serological assays can be used for detection of TTV infection in small and routine diagnostic laboratories. The reports from few other studies have shown the presence of IgM antibodies [8]. However, these were detected to be the short lived antibodies [37,38] and could not be frequently found in sera by different techniques used. Possibly, the screening of sera samples in this study had a similar problem where

presence of IgM cannot be ruled out.

TTV infection is an infection posing several challenges to the understanding of biologist and virologist. A large amount of work has already been done on the virus for its structural, molecular and epidemiological aspects. However, its role in causation of disease, promoting pre-existing infection or damaging cellular integrity and its functional fabrics, is still mysterious. Moreover, its role in causing or promoting malignant cell transformation faces another question mark to its disease causing potency. This study has touched some of these aspects in the direction of developing a model for its easy and early diagnosis in large population to peep into more intricacies related to pathogenicity of TTV.

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