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Validation of SYBR Green based quantification assay for the detection of human Torque Teno virus titers from plasma

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Abstract

Background: Quantification of titers of ubiquitous viruses such as Torque teno virus (TTV) that do not cause clinical symptoms might be helpful in assessing the immune status of an individual. We hereby describe the validation of a SYBR Green-based TTV quantification method for plasma samples.

Methods: Plasmids with TTV specific inserts were used for preparing standards and absolute quantification of TTV was performed using SYBR Green methodology. The method was assessed for its accuracy and precision (intra and inter-day) on four non-consecutive days. TTV was also quantified from plasma samples of 20 healthy volunteers and from 30 hematopoietic stem cell transplant (HSCT) recipients.

Results: The assay was specific and showed satisfactory efficiency (82.2%, R^2=0.99) with the limit of quantification defined as 100 copies per reaction. The assay had good precision (inter and intra-day coefficient of variation in cycle threshold (C_T) < 4%) and accuracy (100 ± 10%) in the range of 100 to 10^{10} copies/reaction. We found TTV loads ranging from 2.5 – 4.07 log copies/mL of plasma with C_T (mean ± SD) of 33.8 ± 1.77 in healthy individuals and 2.06 – 8.49 log copies/mL of plasma with C_T (mean ± SD) of 24.3 ± 1.04 in HSCT recipients.

Conclusion: SYBR Green-based q-PCR assay combines simplicity with satisfactory sensitivity and may be suitable for monitoring the immune status of transplant recipients, where TTV loads over time may serve as a marker for immune reconstitution in human plasma samples.

Keywords: SYBR Green, Real-time PCR, Human torque teno virus

Background

Torque teno virus (TTV), classified into the family *anelloviridae*, genus *alphatorquevirus*, was first described in a patient with non-A-E hepatitis [1,2]. TTV is a non-enveloped, single-stranded, circular DNA virus present in plasma of >90% of individuals, regardless of geographical origin, age or health status [2,3]. Viral titers in the plasma may reflect the individual's immune status, since immunocompromised patients harbor high loads of TTV [4]. This approach can be used to estimate immune recovery in hematopoietic stem cell transplant (HSCT) recipients by monitoring TTV titers after transplantation [5].

TTV DNA has a total genomic length of approximately 3.8 kilobases [6-9] and contains two large-open reading frames (ORF1 and ORF2) and several smaller ORFs [3]. TTV exhibits a wide range of sequence variability, with at least 38 TTV genotypes and forms five distinct phylogenetic groups [10-13]. The conserved ORF-2 region allows the design of primers expected to amplify most strains of TTV [14].

Several previously described quantification methods for human TTV [15-17] are based on TaqMan technology, which may be less suitable for quantification of highly variable viruses such as TTV. SYBR Green-based PCR with primers annealing to more conserved regions may be preferable. In the present study, we report the

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validation of SYBR Green based quantification assay for routine use by using a set of primer pairs targeted for amplifying a well-conserved sequence of ORF-2 [14].

Results and discussion

The assay was validated with serial dilutions of standards ranging from 100 to 10^{10} copies per reaction. The dual sets of primers used showed satisfactory amplification on four different days with assay efficiencies in the range of 81.8% - 82.9% (slopes of the standard curve −3.81 to −3.85). The assay was linear in the range of standards used (co-efficient of regression, R^2) of 0.99 (Figure 1), which indicates a good correlation between viral copy numbers and cycle threshold (C_T) values. The lower limit of quantification was determined as 100 copies/reaction (equivalent to 3000 copies/mL of plasma) with an accuracy of 100 ± 5% and intra and inter-day coefficient of variations below 4% (Table 1). The observed melting curve (Melting temperature™: 85.01°C ± 0.27 (mean ± SD; Figure 2) that was clearly different from the melting curve of primer-dimers (T_M ranging from 71.1 – 72.9°C) as well as the purity of the TTV-specific 96 bp amplicon in the samples that contained TTV DNA (Figure 3) confirmed the high specificity of the PCR.

SYBR Green Real-time PCR quantification assay was reproducible with good inter (co-efficient of variation (CV): 0.22 to 1.23%), and intra-day (CV: 0.01 to 3.10%) precision (Table 1). The calculated standard copy numbers were accurate with intra-day and inter-day accuracies in the range of 86.3 to 102.3% and 88.2 to 102.3%, respectively (Table 1). We also observed good reproducibility of the assay when standards were run in triplicates on two different days with intra- and inter-assay precisions of 0.08 to 3.93% and 0.45 to 2.69%, respectively. The intra and inter-day accuracies were 83.8 to 102.1% and 84.3 to 101.3%, respectively (Table 2). Furthermore, our assay yielded similar TTV titers (± 10% variation) in positive controls (4.75 log copies/mL and 2.94 log copies/mL) that were kindly provided by Maggi's group, Pisa, Italy.

In order to check the robustness of the SYBR Green qPCR assay, we measured TTV titers in plasma samples of 20 healthy individuals and 30 HSCT recipients. Variations in TTV loads in terms of log copy numbers of TTV genomes per mL of plasma were found to be in the range of 2.5 – 4.07 log copies/mL (Figure 4A) with a C_T (mean ± SD) of 33.8 ± 1.77 for healthy individuals and 2.06 – 8.49 log copies/mL (Figure 4A) with a C_T (mean ± SD) of 24.3 ± 1.04 for HSCT recipients. In addition, we observed the differences in melting curves for HSCT recipients (T_M ranging from 82.61°C-84.85°C; Figure 4B) which might be due to possible sequence heterogeneity of human TTV strains, with the presence of TTV specific products (96 bp) on 3% agarose gel electrophoresis (Figure 4C).

Several TTV studies using TaqMan chemistry have reported varying levels of sensitivity, ranging from 120 to 1000 copies/mL for different types of clinical specimens [5,14,15,17-22], which may be the result of differences in the primers used. Although sequence heterogeneity in TTV is high with some variants only sharing 50% of nucleotides [23,24], certain conserved regions can be chosen for primer design in order to amplify more than one subtype of TTV [25]. Focosi *et al.* [5] and

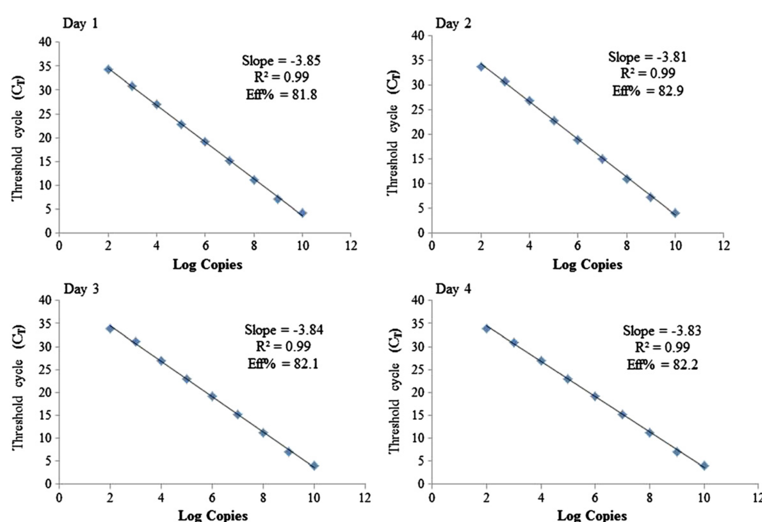

Figure 1 SYBR Green based standard curve from two independent 10 fold serial dilutions of plasmid standards. Standard curve was plotted in the sample plasmid on the x-axis and threshold cycle (C_T) on the y-axis. The x-axis represents human TTV in 10-fold dilutions (Log copies) and the y-axis the fluorescence data used for C_T determinations in ΔRn (baseline-corrected normalized fluorescence). Assay was in linear range of Human TTV with R^2 values (square of the correlation coefficient) of 0.99.

Table 1 SYBR Green real-time PCR assay for TTV using two independent standards series

Plasmid – standards	Day	C_T Mean ± SD (n=2)*	Intra-day precision	Inter-day precision	Intra-day accuracy	Inter-day accuracy
10×10^9	1	4.11 ± 0.09	2.22	0.74	99.9	100.1
	2	4.17 ± 0.08	2.03		99.8	
	3	4.11 ± 0.09	2.36		99.7	
	4	4.09 ± 0.02	0.50		100.2	
10×10^8	1	7.14 ± 0.07	1.10	0.92	102	102.3
	2	7.24 ± 0.22	3.10		101.8	
	3	7.08 ± 0.06	0.88		102.1	
	4	7.11 ± 0.10	1.53		102.3	
10×10^7	1	11.15 ± 0.05	0.45	0.85	101.2	101.4
	2	11.03 ± 0.16	1.49		101.6	
	3	11.26 ± 0.12	1.07		100.8	
	4	11.19 ± 0.06	0.58		101.1	
10×10^6	1	15.21 ± 0.03	0.20	0.31	99.8	100.2
	2	15.10 ± 0.02	0.15		100.1	
	3	15.19 ± 0.01	0.11		100	
	4	15.19 ± 0.003	0.02		99.9	
10×10^5	1	19.15 ± 0.009	0.04	0.28	98.5	98.9
	2	19.03 ± 0.06	0.35		98.8	
	3	19.14 ± 0.02	0.10		98.9	
	4	19.11 ± 0.02	0.12		98.6	
10×10^4	1	22.89 ± 0.009	0.03	0.30	97.9	97.8
	2	22.86 ± 0.09	0.43		97.5	
	3	22.98 ± 0.13	0.57		97.9	
	4	23.00 ± 0.05	0.25		96.8	
10×10^3	1	26.95 ± 0.06	0.24	0.22	94.2	94.3
	2	26.87 ± 0.04	0.17		93.9	
	3	26.95 ± 0.005	0.01		95.3	
	4	27.02 ± 0.13	0.50		92.9	
10×10^2	1	30.83 ± 0.16	0.52	0.35	89.6	88.2
	2	30.89 ± 0.08	0.26		87.2	
	3	31.08 ± 0.03	0.12		88.5	
	4	30.97 ± 0.13	0.42		86.6	
10×10^1	1	34.29 ± 0.30	0.89	0.66	86.3	92.7
	2	33.78 ± 0.57	1.70		93.8	
	3	33.93 ± 0.77	2.29		97.8	
	4	33.87 ± 0.56	1.65		92.3	

*2 independent 10-fold dilution series (n=2) of plasmid standards.

Maggi *et al*. [19] used probes directed against the conserved portion of untranslated region (UTR), while others used probes specific for highly conserved region of ORF2 and ORF1 of TTV [14,15,17].

For our qPCR protocol we used the primers described by Moen *et al.* [14] that differentiate between TTV and TTV-like mini virus (TLMV). Although TaqMan based assays may be somewhat more sensitive, we opted for SYBR Green assay using a primer pair rather than TaqMan technology, which uses two primers and one probe, and is probably more prone to the problem of variable amplification efficacy of strains differing for single nucleotides. Indeed, in our sequencing results for 12 separate clones (Figure 5) from one single patient we

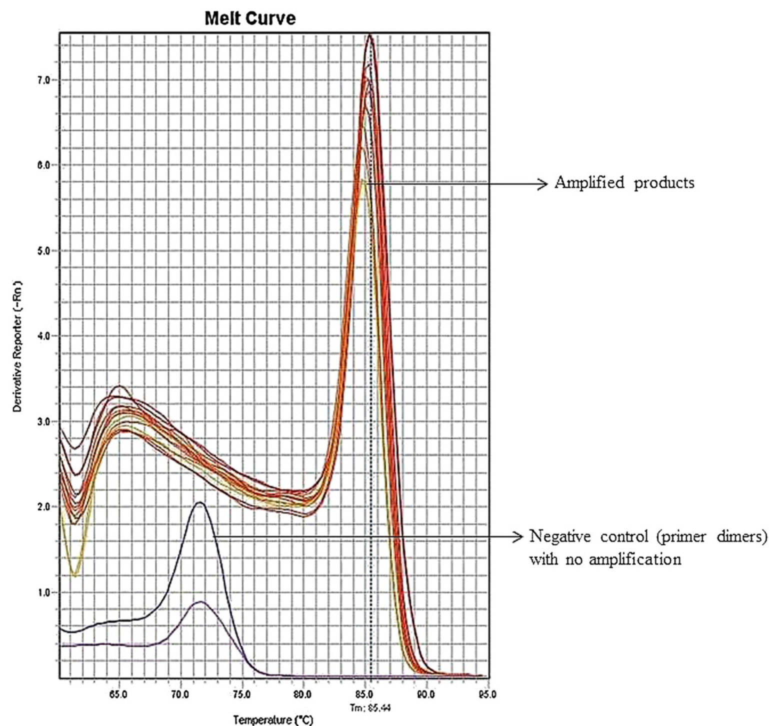

Figure 2 Melting curve analysis of TTV real-time PCR products. Y-axis represents the derivative reporter (ΔRn) while x-axis represents the temperature (°C). The figure shows a melting temperature (T_m) of human TTV PCR products as 85.4°C with no amplifications detected in negative controls except primer-dimers.

observed sequence heterogeneity of TTV in the region that has been used for TaqMan probe [14]. In general, SYBR Green methodology may be more suitable for viral studies where sequence heterogeneity is high in comparison to TaqMan probe-based assays which require high sequence identity for successful probe binding to avoid frequent variable results [14]. In addition, the assay's threshold of 3000 copies/mL of plasma may be low enough for monitoring TTV in immune deficient patients.

Conclusion

This report describes the validation of a SYBR Green assay for quantification of TTV viral load in human plasma samples. The developed assay was accurate with satisfactory efficiency, reproducibility in the range of

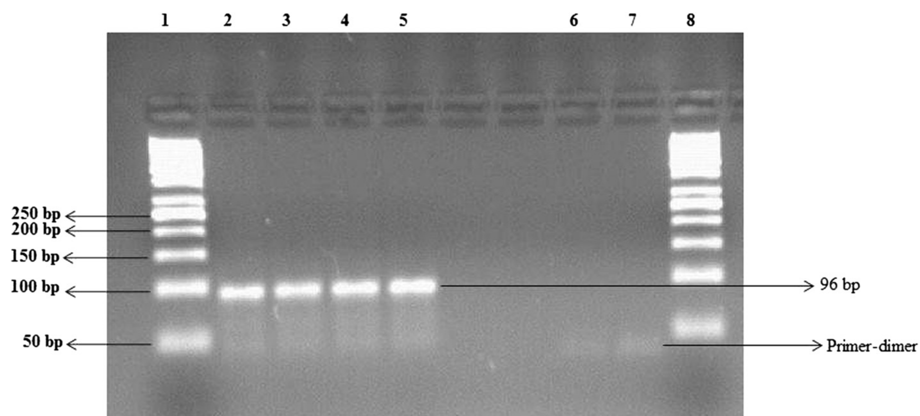

Figure 3 Agarose gel (3%) electrophoresis of SYBR Green real-time PCR products. The gel picture shows the presence of primer-dimers in negative control (Lane 6 and Lane 7) and specific TTV amplified products (96 bp) in Lanes 2, 3, 4 and 5. Lane 1 and Lane 8 contained 50 bp DNA ladder.

Table 2 SYBR Green PCR assay for TTV using single series of plasmid standards

Plasmid standards	Day	C_T Mean ± SD (n=3)*	Intra-day precision	Inter-day precision	Intra-day accuracy	Inter-day accuracy
10×10^9	1	4.21 ± 0.02	0.50	0.45	99.6	99.6
	2	4.18 ± 0.01	0.39		99.6	
10×10^8	1	7.59 ± 0.05	0.75	0.65	101.3	101.3
	2	7.52 ± 0.05	0.78		101.2	
10×10^7	1	11.74 ± 0.05	0.45	1.10	100.8	100.8
	2	11.56 ± 0.01	0.14		100.8	
10×10^6	1	15.83 ± 0.04	0.28	1.22	100.4	100.4
	2	15.56 ± 0.01	0.08		100.4	
10×10^5	1	19.75 ± 0.05	0.26	1.39	100.6	100.7
	2	19.37 ± 0.01	0.10		100.8	
10×10^4	1	24.15 ± 0.03	0.12	1.52	98.1	98.4
	2	23.64 ± 0.02	0.10		98.6	
10×10^3	1	28.40 ± 0.03	0.12	1.64	95.3	95.8
	2	27.75 ± 0.05	0.19		96.4	
10×10^2	1	33.21 ± 0.20	0.62	1.51	83.8	84.3
	2	32.51 ± 0.06	0.21		84.8	
10×10^1	1	35.59 ± 0.39	1.11	0.67	102.1	99.0
	2	35.25 ± 0.06	0.17		95.7	

*Tenfold dilutions run in triplicates.

100-10^{10} copies/ reaction. This simple assay can be used in studies assessing TTV plasma loads as a marker of immune reconstitution. A prospective study is currently ongoing in our institution to validate the correlation of TTV titer and the immune status post HSCT.

Methods

Reagents and consumables

QIAamp® MinElute virus spin kit for DNA extraction, QIAprep® Spin Miniprep kit for plasmid extraction and QIAquick® PCR purification kit were obtained from Qiagen, Germany. TA cloning kit dual promoter (pCRII) with One Shot TOP10F`competent cells and ampicillin were obtained from Invitrogen, San Diego, California. DNA Taq polymerase, *BamHI* and *EcoRI* restriction enzymes were obtained from New England BioLabs, USA. SYBR®-Green PCR master mix, 96 well MicroAmp® fast optical reaction plates (0.1 mL capacity) and MicroAmp® optical adhesive films for real-time PCR assay were obtained from Applied Biosystems, Fostercity, CA. All the experiments were performed on StepOnePlus®-Real Time PCR Systems by Applied Biosystems, Fostercity, CA. For amplification of human torque teno virus (TTV), a set of primer pairs described previously were used (Table 3). Primers were made according to the reference strain of TTV genome TA 278 (Gen Bank acc. No. AB008394) and were synthesized by Microsynth (Switzerland) at a scale of 0.2 μmol. DNA ladders, $MgCl_2$, dNTP's and buffers were obtained from Fermentas Life sciences, Germany.

Samples and DNA extraction

Blood samples (5 mL) collected in EDTA tubes from 20 healthy adult volunteers and 30 randomly selected adult HSCT recipients were centrifuged at 900 g for 10 minutes to separate plasma which was immediately frozen at -20°C until used for DNA extraction. Two independent DNA extractions were performed for each of the healthy individuals along with one independent DNA extraction for HSCT recipients, each from 200 μl of plasma using QIAamp MinElute Virus Spin kit according to the manufacturer's recommendations. DNA was eluted in 30 μL of Milli-Q water. All extracted DNA samples were stored at -20°C until the analysis. The study protocol was approved by the institution's ethics committee and healthy donors and HSCT recipient's samples were used after obtaining informed consent.

Construction of plasmids for standards preparation

A region of 119 bp PCR fragment of TTV genome was amplified using primers TTVf and TTVr (Table 3). Resulting amplicon was purified using QIAquick PCR Purification kit, quantified by spectrophotometer and then cloned into the TA cloning vector. The resulting plasmid was transformed into One Shot TOP10F` competent cells according to instructions provided by the

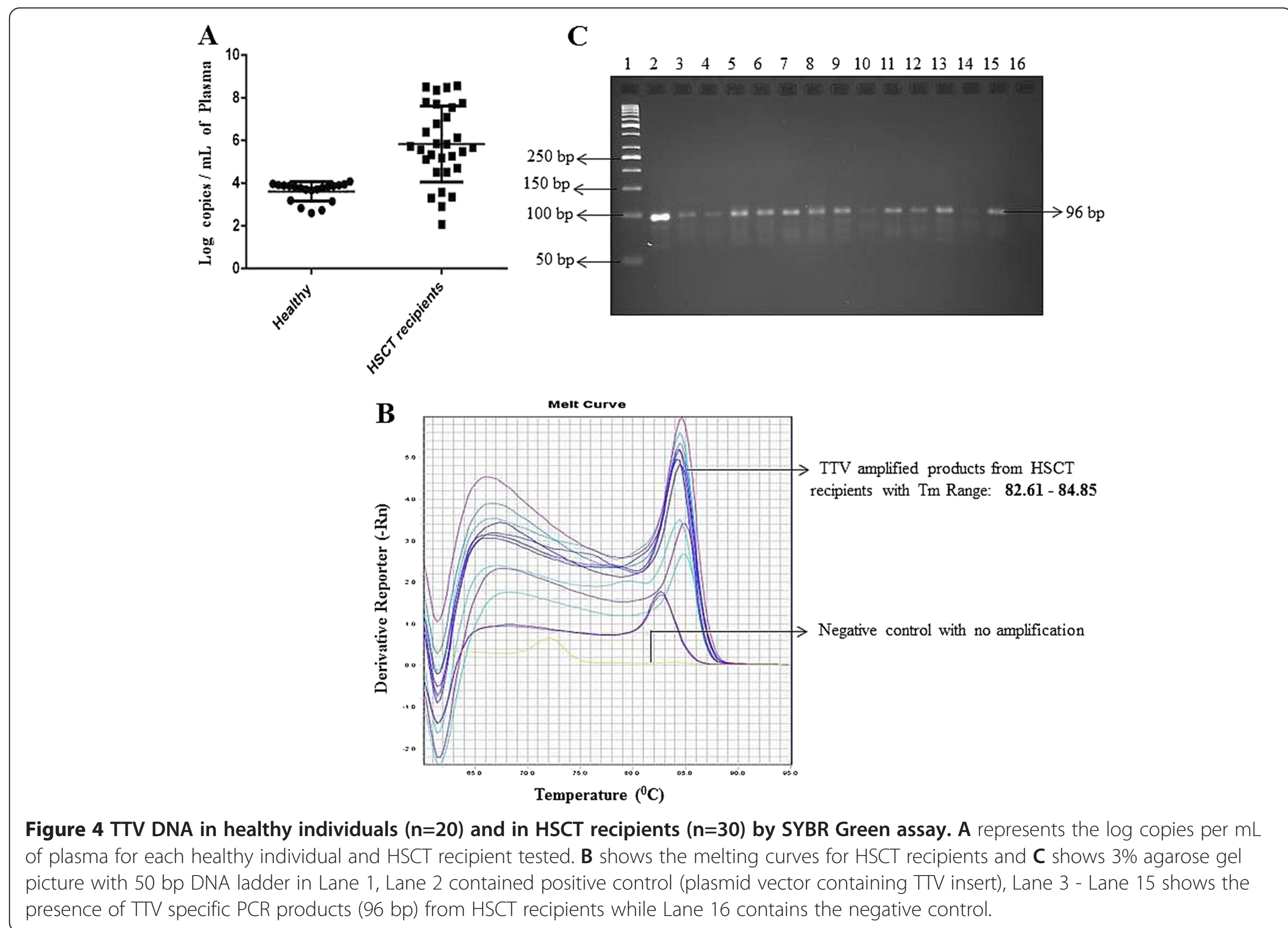

Figure 4 TTV DNA in healthy individuals (n=20) and in HSCT recipients (n=30) by SYBR Green assay. A represents the log copies per mL of plasma for each healthy individual and HSCT recipient tested. **B** shows the melting curves for HSCT recipients and **C** shows 3% agarose gel picture with 50 bp DNA ladder in Lane 1, Lane 2 contained positive control (plasmid vector containing TTV insert), Lane 3 - Lane 15 shows the presence of TTV specific PCR products (96 bp) from HSCT recipients while Lane 16 contains the negative control.

manufacturer. Twelve, isolated colonies of transformed competent cells from solid luria-bertani medium containing ampicillin (100 μg/mL) were subjected for TTV insert confirmation. Each individual colony was suspended separately into 3 mL of liquid luria-bertani medium containing 100 μg/mL of ampicillin for overnight in a shaking incubator at 37°C with a speed of 225 rpm. Following overnight incubation, plasmids purification was done using QIA prep Spin Miniprep kit according to manufacturer's instructions. Restriction enzyme digestion with *EcoRI* for the purified plasmids was done to confirm the presence of cloned TTV insert (119 bp) on 1.5% agarose gel electrophoresis (data not shown). TTV insert (119 bp) cloned into TA vector were sequenced for all the 12 separate clones using M-13 forward and reverse primers and confirmed by aligning with the TTV sequence (Gen Bank acc. no. AB008394). This plasmid with TTV inserts was linearized with *BamHI* enzyme and then used for preparation of standards in serial 10 fold dilutions from 10×10^9 copies to 20 copies/μL.

Absolute quantification of TTV DNA

PCR reaction for absolute quantification of TTV DNA using SYBR Green in a 25 μL reaction is as follows: each reaction contained 12.5 μL SYBR Green PCR master mix, 5 μL of template (serial 10 fold dilutions of the linearized plasmid standards or/ extracted DNA from the plasma samples of healthy blood donors), 1.25 μL (500 nm) of each primer (TTVF-1, TTVF-2, TTVR-1, TTVR-2) and 2.5 μL of Milli-Q water. The cycling conditions included initial activation of AmpliTaq Gold DNA polymerase (present in SYBR Green master mix) for 10 minutes at 95°C. The subsequent PCR conditions consisted of 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute per cycle. After realtime data acquisition, the cycle threshold value was calculated by determining the point at which the fluorescence exceeds an arbitrary threshold limit. Standards with known TTV DNA copies were prepared in two independent serial dilutions and were run in the range of 100 copies to 10×10^9 copies on four non-consecutive days to evaluate biological, inter, intraday variability and accuracy of the assay. In addition, a series of standards from one serial dilution were also run in triplicates on two different days to evaluate the intra-day and inter-day variations. The variability of the assay was evaluated by comparing the C_T values run on the same day (intra-day) and on different days (inter-day) and was represented as co-efficient

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TTV12      TCCGAATGGCTGAGTTTTCCACGCCCGTCCGCAGCGAGAACGCCACGGAGGGAGATCCTC 60
TTV11      TCCGAATGGCTGAGTTTTCCACGCCCGTCCGCAGCGAGAACGCCACGGAGGGAGATCCTC 60
TTV10      TCCGAATGGCTGAGTTTTCCACGCCCGTCCGCAGCGAGAACGCCACGGAGGGAGATCCTC 60
TTV9       TCCGAATGGCTGAGTTTTCCACGCCCGTCCGCAGCGAGAACGCCACGGAGGGAGATCCTC 60
TTV8       TCCGAATGGCTGAGTTTTCCACGCCCGTCCGCAGCGAGAACGCCACGGAGGGAGATCCTC 60
TTV7       TCCGAATGGCTGAGTTTTCCACGCCCGTCCGCAGCGAGAACGCCACGGAGGGAGATCCTC 60
TTV2       TCCGAATGGCTGAGTTTTCCACGCCCGTCCGCAGCGAGAACGCCACGGAGGGAGATCCTC 60
TTV1       TCCGAATGGCTGAGTTTTCCACGCCCGTCCGCAGCGAGAACGCCACGGAGGGAGATCCTC 60
TTV3       TCCGAATGGCTGAGTTTTCCACGCCCGTCCGCAGCGAGAACGCCACGGAGGGAGATCCTC 60
TTV4       TCCGAATGGCTGAGTTTTCCACGCCCGTCCGCAGCGAGAACGCCACGGAGGGAGATCCTC 60
TTV5       TCCGAATGGCTGAGTTTTCCACGCCCGTCCGCAGCGAGAACGCCACGGAGGGAGATCCTC 60
TTV6       TCCGAATGGCTGAGTTTCCCACGCCCGTCCGCAGCGAGAACGCCACGGAGGGAGATCCTC 60
AB008394   TCCGAATGGCTGAGTTTTCCACGCCCGTCCGCAGCGGTGAAGCCACGGAGGGAGATCTCC 60
AB008394                     T
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TTV12      GCGTCCCGAGGGCGGGTGCCGGAGGTGAGTTTACACACCGCAGTCAAGGGGCAATTCG 118
TTV11      GCGTCCCGAGGGCGGGTGCCGGAGGTGAGTTTACACACCGCAGTCAAGGGGCAATTCG 118
TTV10      GCGTCCCGAGGGCGGGTGCCGGAGGTGAGTTTACACACCGCAGTCAAGGGGCAATTCG 118
TTV9       GCGTCCCGAGGGCGGGTGCCAGAGGTGAGTTTACACTCCGCAGTCAAGGGGCAATTCG 118
TTV8       GCGTCCCGAGGGCGGGTGCCGGAGGTGAGTTTACACACCGCAGTCAAGGGGCAATTCG 118
TTV7       GCGTCCCGAGGGCGGGTGCCGGAGGTGAGTTTACACACCGCAGTCAAGGGGCAATTCG 118
TTV2       GCGTCCCGAGGGCGGGTGCCGGAGGTGAGTTTACACACCGCAGTCAAGGGGCAATTCG 118
TTV1       GCGTCCCGAGGGCGGGTGCCGGAGGTGAGTTTACACACCGCAGTCAAGGGGCAATTCG 118
TTV3       GCGTCCCGAGGGCGGGTGCCGGAGGTGAGTTTACACACCGCAGTCAAGGGGCAATTCG 118
TTV4       GCGTCCCGAGGGCGGGTGCCGGAGGTGAGTTTACACACCGCAGTCAAGGGGCAATTCG 118
TTV5       GCGTCCCGAGGGCGGGTGCCGGAGGTGAGTTTACACACCGCAGTCAAGGGGCAATTCG 118
TTV6       GCGTCCCGAGGGCGGGTGCCGGAGGTGAGTTTACACACCGCAGTCAAGGGGCAATTCG 118
AB008394   GCGTCCCGAGGGCGGGTGCCGAAGGTGAGTTTACACACCGAAGTCAAGGGGCAATTCG 118
AB008394                                           G
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Figure 5 Sequence alignments of 12 separate TTV clones from a single patient with TTV sequence (AB008394). The figure shows forward and reverse primers location highlighted in green along with base changes highlighted in blue that were used in dual set of primers for SYBR Green assay and other base changes detected in primer region are highlighted in red. Probe region used previously for TaqMan [14] highlighted in brown.

of variations (CV). Accuracy was calculated by taking the ratio of back calculated copies from the standard curve to the theoretical copy number of the reactions. Real-time PCR assay for test samples (HSCT recipients) and for biological replicates of each healthy individual were performed with the inclusion of TTV plasmid standards and negative controls in each run. In addition to this, precision of the assay was also checked by running known TTV positive DNA (positive controls with exact log copies/mL). The viral genomic copies per mL of plasma was calculated as described by Huang *et al.* [26] i.e., by multiplying the copies per reaction by a factor of 30 [30 μL extracted DNA/5 μL of template x (1 mL/200 μL plasma)].

Table 3 Oligonucleotide primers used for human torque teno virus (TTV)

Primers ID	Sequence (5` to 3`)	Nucleotide position*	Reference
TTVf	TCCGAATGGCTGAGTTT	102-118	Moen EM *et al.* [19]
TTVr	CGAATTGCCCCTTGACT	219-203	
TTV-F1	GTTTTCTACGCCCGTCC	115-131	
TTV-F2	GTTTTCCACGCCCGTCC	115-131	
TTV-R1	CCTTGACTCCGGTGTGTAA	210-192	
TTV-R2	CCTTGACTTCGGTGTGTAA	210-192	

*According to the reference strain of TTV genome TA 278 (Gen Bank acc. No. AB008394).

Melting curve analysis for specificity

Following amplification, melting curve or dissociation curve analysis was performed to measure the specificity of the PCR product. The temperature program used for the melting curve analysis was 95°C for 15 seconds followed by 60°C for 1 minute and then 95°C for 15 seconds with ramp rate of +0.3°C/second.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AKT and AP performed the experiments. AKT, AP, PHD, CRSU and MA analyzed and contributed to the interpretation of data. MA and ER designed the research. AKT and MA drafted the article. All the authors revised the manuscript critically. All authors read and approved the final manuscript.

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