A Novel Assay To Accurately Measure TorqueTenovirus Load For Immunosuppression Level Monitoring In Transplant Recipients

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Abstract

Purpose:

- Optimized dosing of immunosuppressive drugs is needed to mitigate the risk of graft rejection at low dosage (under-immunosuppressed) and multiple adverse effects at higher dosages (over-immunosuppressed), including opportunistic infections, cancer, and toxicity to organs.
- Additional diagnostic testing to measuring immunosuppressive drug trough levels is needed to evaluate patient immune status and guide dosing.
- Alphatorquetenovirus (TTV) levels in blood is a new and reliable biomarker for immunosuppression status.

Results:

- Developed new real-time PCR test to quantitate the number of TTV genomic copies from patient blood samples.
- Test precisely measures 27 most common species of TTV over broad range of concentrations, up to single copies, while not picking up high levels (280 million copies per mL) of TTMV and TTMDV.

Introduction

AlphaTorquetenovirus (TTV) is a member of the Anelloviridae family of viruses, along with the Beta- and GammaTorquetenoviruses (TTMV, TTMDV), that have a circular, single stranded, anti-sense genome. TTV is a large component of the human virome, highly prevalent in the general population, across different populations and age groups, and non-pathogenic. The international committee on taxonomy of viruses recognizes 29 different species of TTV (1).

The primary interest in TTV is in its potential utility to reliably monitor the immunocompetence status of patients undergoing immunosuppressive drug treatment and guide dosing to avoid outcomes associated with under or over immunosuppression. Twenty clinical studies on over 1,000 transplant recipients, both adults and children with various types of transplants, have associated low TTV levels with graft dysfunction and high TTV levels with infection. The current method of monitoring the trough level of immunosuppressive drugs helps to guide dosing to reduce risk of overdosing, however, it does not fully reflect the patient's immunocompetence state, which may be further complicated by diabetes and other immunosuppressive conditions. Therefore, with the aim of prolonging graft survival and improving the quality of life for patients, a new diagnostic to measure TTV levels, as an indirect measure of immunocompetence status, is necessary to better guide immunosuppressive drug dosing. However, no IVD product to measure TTV is currently available in the United States.

Assay Optimization

We developed novel quantitative TaqMan assay for TTV quantification. The assay is comprised of a single mix of primers and probes to run TaqMan real-time PCR on a 96-Well plate on the QuantStudio 5 and QuantStudio 5 Dx instruments.

Assay was optimized to reach two important goals: to ensure tight distribution of Cq values among the different species of TTV, and to minimize risk of detecting closely related TTMV and TTMDV with significant amplification delay and possibly providing misleading results.

We tested 27 most common TTV species with 3 lots of our optimized assay and compared data with data generated from a published qPCR primer mix and cycling conditions (2). Fig 1 shows Cq values generated by previously published formulation (2) with significant amplification delay for multiple TTV species including some common TTV variants. Broad range of Cq values among TTV species could lead to differences in calculated TTV titer exceeding two orders of magnitude. In contrast, our new assay demonstrated a tight distribution of Cq values across all 27 species for 3 lots of p-mix tested Fig 1. We tested TTMV and TTMDV templates representing highest similarity to TTV, and demonstrated that our optimized assay does not detect even 5 million copies of template per reaction (equivalent of ~280 millions per 1 mL of blood) Fig.2.

Performance of New TTV Assay

Figure 1. Novel TTV assay improves consistency of TTV variants equivalently. Real-time PCR data of our developed assay (One Lambda) in comparison to a published TTV assay (2). Synthetic DNAs with sequences from 27 TTVs were normalized to 1,500 copies per reaction. Three separate lots of our developed assay mix were tested with all 27 TTV variants in duplicate in two independent experiments by two operators. Previously published TTV quantification conditions (2) were tested with the same TTV dilutions in triplicate in two independent experiments by two operators.

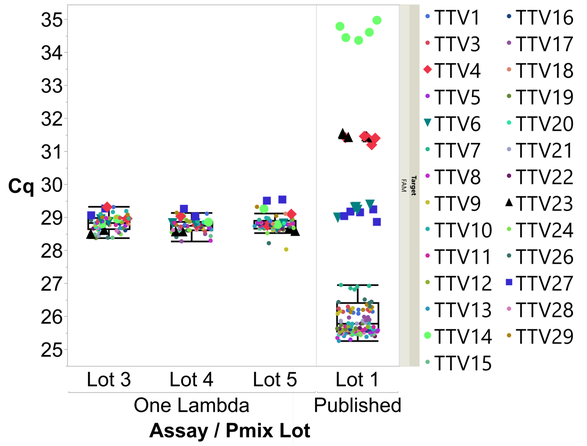
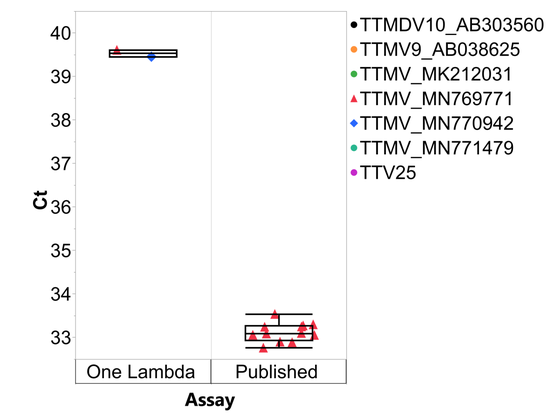
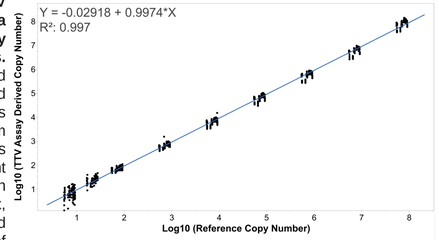


Figure 2. Novel TTV assay improves specificity in detecting TTV over other Anelloviridae. To really challenge assay specificity, we tested TTMV and TTMDV variants with closest sequence similarity to TTV at high concentration. Three lots of new TTV assay were tested with 5 million copies per reaction (equivalent of ~280 mil per mL of blood) of 5 TTMV and one TTMDV controls. To confirm that our newly optimized assay is not inferior to previously published design (2), we tested it with the same negative controls. Both, previously published assay and new One Lambda assay demonstrated no reactivity to TTV25. Here are combined data from 3 lots of our assay (One Lambda) and previously published formulation (2). All assays were tested with 6 replica-reactions per template, twice by two operators on two different types of qPCR instrument. One Lambda assay generated negative results (below 1 copy per reaction) for all tested samples. In contrast, published assay detected one of TTMV variants generating Cq values expected for significantly lower titers of TTV, thus presenting risk of misleading results for patients infected by certain members of Anelloviridae family.



TTV Assay Linearity Study

Figure 3. Novel TTV assay quantitative over a broad range of clinically relevant concentrations. Copy number generated by our qPCR assay tested with 16 TTV variants tested in the range from ~5 to 500 million copies per reaction. Each variant was tested in triplicate with 2 lots of our assay mix, qPCR was performed using both types of instruments

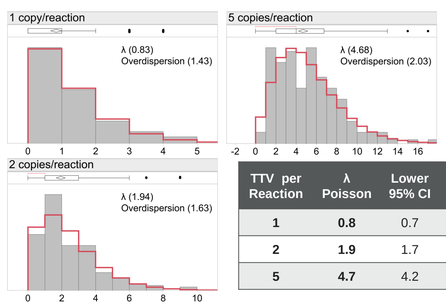


Data for 500 million copies per reaction are not included into analysis. Due to very early Ct values data for ~ 500 million copes falling outside of the linear range. As expected, data for ~5 copies per reaction are affected by sampling variations. Limits of quantification were evaluated in following study.

Limits of Detection and Quantification

To determine the real analytical limit of quantification for our new assay we had to address sampling variabilities expected for DNA concentrations below 10 copies per reaction. We used a previously described (3) method using a Gamma Poisson mixed model to assess the actual quantification parameters of the assay despite sampling variabilities at low copy number of target DNA. Our tests demonstrated that new TTV quantification method can detect and quantify single copies of TTV per reaction (Fig. 4).

Figure 4. Novel TTV quantification assay can measure single copies of DNA. Two lots of p-mix were used to measure three common TTV variants at 1, 2, and 5 copies per reaction using 2 types of qPCR instrument. As recommended by relevant publications, for each TTV, each concentration was tested in 30 replicas per condition. The distribution of TTV copy numbers derived from combined qPCR data (n = 180) was fitted with Gamma Poisson mixed model (red line) to give the mean estimated copy number (λ). Gamma adjustment to Poisson model allowed for minor experimental variabilities and TTV dilutions variation. Though we incorporated 3 additional sources of variability, overdispersion – fold increase relative to the Poisson variance was modest. Thus, for 1 copy per reaction, standard deviation was increased by less than 20% compared to normal Poisson distribution. Table show the Gamma Poisson fit parameters and the goodness of fit of the Gamma Poisson (Pearson Chi Squared).



TTV per Reaction	λ Poisson	Lower 95% CI	Upper 95% CI	Prob>X2
1	**0.8**	0.7	1.0	0.63
2	**1.9**	1.7	2.2	0.36
5	**4.7**	4.2	5.2	0.47

Table 1. Upper limit of quantification. To identify highest measurable level of TTV we tested 6 common variants at two concentrations close to upper linear range. For each concentration, each TTV was tested in 6 replicas with two lots of p-mix bringing total replicates for each concentration across 2 lots and 2 instrument types, to 72.

TTV Copy Number added per reaction	Mean TTV	CV (%)
50 million	50.1 million	26
100 million	120.2 million	29

TTV Measurement in Whole Blood

Table 2. Demographic and measured TTV loads in primary blood samples. Whole blood from 50 random donors were used to extract total DNA. TTV was quantified using our novel assay confirming TTV prevalence/amounts similar to expected for random donors.

Characteristics	Total Cohort (n = 50)	
Age; years, median (IQR)	39.5	(31.3 - 56)
Age; years, min - max	22 – 72	
Gender; male / female, n (%)	22 / 28	(44 / 56)
Race; n (%)		
Asian	2	(4)
Black	25	(50)
White	22	(44)
Other	1	(2)
Weight; pounds, median (IQR)	195.5	(155.5 – 241.3)
Weight; pounds, min - max (range)	120 - 318	(198)
TTV; prevalence, n [TTV Positive] / n (%)	42	(84)
TTV Conc., copies per mL, median (IQR)	3.5×10^3	($171 - 2.25 \times 10^4$)
TTV Conc.; copies per mL, min – max	$0 – 2.9 \times 10^6$	

Conclusions

We developed a new real-time PCR-based assay to reliably measure the amount of TTV in blood.

Assay demonstrated high level of sensitivity, specificity and wide linearity range.

Assay is highly accurate for 27 most common TTV species. The tight distribution of Cq values across the 27 species shall improve measurement accuracy in clinical samples.

Assay performance is consistent among multiple lots of assay mix and two between the QS5 and QS5Dx instruments tested

References

1. Biagini P et al. Family Anelloviridae. In: King AMQ, Adams EB, Carstens EB, EJ L (eds) Virus taxonomy: ninth report of the international committee on taxonomy of viruses. Academic press, London 2012, pp 331-341.
2. Blatter JA et al. Anellovirus loads are associated with outcomes in pediatric lung transplantation. Pediatr. Transplant. 2018 Feb;22(1):10.1111/petr.13069. doi: 10.1111/petr.13069. Epub 2017 Oct 29.
3. Asogawa M. Framework for qPCR modeling and analysis of low copy number sample. Forensic Science International: Genetics Supplement Series 2022 Oct;8:344-346. https://doi.org/10.1016/j.fsigss.2022.10.084.

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