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Comparative evaluation of molecular methods for the quantitative measure of torquetenovirus (TTV) viremia, the new surrogate marker of immune competence

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ABSTRACT

Background: Torquetenovirus (TTV) viremia is emerging as a promising tool to assess functional immune competence, to predict post-transplant immune-related complications, and eventually to customize immunosuppression.

Methods: In this study, 327 blood samples were tested using two real-time PCR (rtPCR) assays both targeted to the untranslated region of TTV genome. The first assay was an in-house rtPCR developed by our group, the second one was the recently marketed TTV R-GENE® assay. **Results:** In the validation study, the TTV R-GENE® showed good performances in precision and reproducibility, and a sensitivity as low as 12 TTV DNA copies/ml, like previously reported for the in-house rtPCR. Bland-Altman analysis showed that the mean difference between the two methods was -0.3 Log copies/ml. In the comparison study, 69% and 72% of samples were detected positive by rtPCR and TTV R-GENE®, respectively (94% concordance, $\kappa = 0.88$). Performances did not differ between the two rtPCRs by type of TTV group examined. When a newly-developed in-house digital droplet PCR was applied for TTV quantification and used as alternative method of comparison on 94 samples, the results strongly correlated with those obtained by the two rtPCR methods (99% concordance).

Conclusion: In summary, all the molecular methods assayed are highly sensitive and accurate in quantitation of TTV DNA in blood samples.

KEYWORDS

TTV; real-time PCR; digital droplet PCR; methods comparison

1. INTRODUCTION

Soon after its discovery,[1] it became clear that Torquetenovirus (TTV) was just one of a vast group of related, previously unrecognized viral agents, all of which were characterized by small, circular single-stranded DNA genomes with negative polarity.[2] Presently, all these viruses are classified within the family Anelloviridae.[3] TTV is currently attracting considerable interest due to a number of specific features. Among these the most intriguing is probably the extremely high prevalence of chronic plasma viremia in more than 80% of the general population, regardless of disease status, age, gender, socioeconomic conditions, geographic location, risk factors, and other variables.[4,5] Individual viremia levels have been shown to vary between 10^1 to 10^9 genome copies per ml of blood, with kinetics of virus replication that are highly dependent upon the immune status of infected hosts.[6] Based on this viro-immunological relationship, recently the measure of the total load of TTV viremia has been used as a marker of the immune status of the subject; namely high TTV levels are associated with immunosuppression.[6-10] Currently, there is no assay for the diagnosis of immunosuppression that can be used universally and, in some clinical situations, it can be difficult to precisely assess the level of immunosuppressive status of a subject.[11] Thus, during the last year it has been proposed that the measure of TTV viremia could represent an innovative, rapid, and reliable test to use for the assessment of immunosuppression.[12-15]

The laboratory diagnosis of TTV infection is limited since there are neither virus culture systems of sufficient sensitivity nor reliable serological assays that may permit the

demonstration of viral products or antiviral antibodies. Thus, TTV detection in humans is exclusively based on molecular methods.[16,17] Due to the great genetic variability of TTV (at least 29 human species are currently identified, previously classified in five main genogroups),[18] the choice of the viral DNA segment targeted for amplification has an enormous impact on PCR assay sensitivity, possibly more so than for most viruses. For historical reasons, the N22 tract of the open reading frame (ORF) 1 has been extensively targeted in nested or heminested PCR protocols.[2] The primers designed on this region have been considerably improved over time (also by using multiple sets of primers), but nonetheless, they still may fail to amplify most TTV species, especially if the virus content in test samples is low. Due to its higher conservation, the 5' untranslated region (UTR) of the TTV genome is much more suitable for PCR primer design. UTR PCRs currently in use are in the format of in-house quantitative PCRs and have the potential for sensitive and specific detection of most, if not all, the species of human TTV hitherto identified.[19,20] For this reason, they are also called 'universal' or 'consensus' PCRs. Uncertainties about the clinical relevance of TTV infection have discouraged commercial companies from developing and marketing adequate reagents and diagnostic kits. Thus, only in 2017 a UTR quantitative PCR assay has been commercialized for detection and quantification of TTV genome simultaneously. However, there has been no extended comparison study between the TTV PCR methods, which is crucial for a reliable and rigorous inter-laboratory sharing of TTV measurements. The latter is an indispensable step before assuming the TTV viremia as an immunological marker.

In this study, we analyzed for TTV DNA levels in blood samples from 327 subjects and the results obtained by an in-house real-time PCR (rtPCR), previously developed in our laboratories, were compared with those obtained by a recently marketed PCR assay. An

in-house digital droplet PCR (ddPCR) was also developed for TTV quantification and used as an alternative method of comparison.

2. MATERIALS AND METHODS

2.1 Samples

A total of 327 randomly selected samples (209 plasma and 118 whole blood samples) were studied. Two hundred thirty-nine were samples from diseased patients who were referred to our laboratories for routine virological analyses; the remaining 88 included healthy blood donors. The diseased patients were 136 solid organ transplant patients, 56 hematopoietic stem cell transplant patients, and 47 hepatitis B or hepatitis C positive patients. Blood samples were obtained by venipuncture, aliquots were immediately prepared, stored and kept under sterile conditions at –80°C until use. The study was approved by the ethics committee at Pisa University Hospital, Pisa (protocol study number 1130).

2.2 TTV quantification by rtPCRs

Viral DNA was extracted from 200 μl of plasma or whole blood samples by using QIAamp DNA Mini kit (QIAGEN, Chatsworth, CA) manually or associated with a QIAsymphony SP/AS instrument (QIAGEN, Chatsworth, CA), respectively. rtPCR amplification was performed using 7500 Fast instrument (Applied Biosystems). Presence and load of TTV were determined by using two rtPCR assays, both targeted on a highly conserved 5'UTR of the TTV genome. The first one was a single step TaqMan in-house rtPCR, previously developed in our laboratories. The procedures used to quantitate the copy numbers and assess specificity, sensitivity, intra- and inter-assay precision, and reproducibility have been previously described.[19] The method amplifies a UTR fragment

of 63 nucleotides and it boasts high sensitivity, up to 10 viral genomes per ml of plasma or whole blood. Inhibition risk was determined in the testing process. A number of extracted DNA samples were spectrophotometrically analysed for determining the A260/280 ratio and excluding the presence of certain inhibitors. In addition, inhibition controls were added pre-extraction by spiking TTV negative plasma or whole blood samples with a target DNA incorporated into a plasmid at a level of 100 target copies/µl (approximately 10 times the limit of detection) and then subjecting it to the complete procedure (extraction and amplification) as described. Inhibition rates were calculated on the basis of lack of detection of the target DNA-containing plasmid.

The second rtPCR assay was the recently commercialized TTV R-GENE® kit (BioMerieux, Marcy-l'Etolle, France). This assay is able to detect and/or quantify TTV DNA in plasma and whole blood samples by using the 5'nuclease TaqMan technology, it is commercialized in the format of the ready-to-use amplification mixture, and it allows the amplification of a UTR fragment of 128 base pairs in size. An extraction and inhibition internal control, as well as a range of 4 quantification standards, is included in the TTV R-GENE® kit.

2.3 ddPCR for TTV DNA quantification

TTV DNA was quantified by the QX200 Droplet Digital PCR system (Bio-Rad, Hercules, CA, USA) using the same primers and probe that are currently used in the in-house rtPCR that target the UTR region of TTV genome. PCR reactions were performed in the 20-µl final reaction containing ≤ 66 ng of DNA template (4 µl), 1 µl of 20X AMTS primer assay, 1 µl of 20X AMTAS primer assay, 1 µl of 20X AMTP probe assay (FAM/TAMRA), 10 µl of 2X ddPCR Super Mix and 3 µl of DNAse/RNAse free water. Droplet generation oil (70 µl) was added, and the eight-well cartridge was placed into the

droplet generator; 50 µl of the droplet solution was then transferred into a 96-well PCR plate. The amplification protocol was standardized to the following conditions: 95°C for 10 minutes followed by 94°C for 30 seconds and 58°C for 60 seconds for a total of 39 cycles, and finally a step at 98°C for 10 minutes. The droplet fluorescence signal was determined by droplet reader. The number analysis of positive versus negative droplets for both fluorophores (FAM/VIC) was done by using the Quanta Soft software (Bio-Rad); their ratio was then fitted to a Poisson distribution to determine the copy number of the target molecule, as copies per µl in the input reaction. Droplets with a fluorescence intensity threshold higher than 3,000 were considered positive and results were given as copies of TTV DNA per ml of plasma or whole blood.

2.4 TTV genetic characterization

Selected specimens found TTV positive by both rtPCR assays were characterized using 5 different nested or heminested PCR protocols, each specific for one of the 5 groups in which the different TTV species are gathered. PCR assays are targeted on either the UTR (groups 4 and 5) or ORF1 genes (groups 1, 2, and 3) of the viral genome. Specificity and sensitivity of each group-specific assay have been previously described.[19,21] All samples were tested at least in duplicate.

2.5 Statistical analysis

SPSS software version 23 (IBM, Chicago, IL, USA) and MedCalc statistical software version 18.2.1 (Ostend, Belgium) were used for statistical analysis. Transformed TTV load in Log format was used for analysis. For the methods comparison analysis, correlations were analysed using Passing-Bablok regression and Kendall rank correlation tests. The coefficient of determination R^2 (Spearman rho coefficient) was used to

measure overall correlation between methods. Bland-Altman analysis was used to analyse the concordance and mean difference between assays. Overall agreement between assays was measured by weighted Cohen's kappa (κ). The association among variables was evaluated by a nonparametric ANOVA using the Kruskal Wallis test. Tests were two sided and P-values <0.05 were considered statistically significant.

3. RESULTS

3.1 Comparative evaluation in TTV DNA detection and quantification by rtPCR methods

While the performances of in-house rtPCR have been evaluated and validated previously,[19] those of TTV R-GENE® assay were determined in this study. Intra – and inter – assay precision and reproducibility of the assay were investigated by testing, in three independent experiments, a panel of 20 nominal TTV DNA concentration levels (ranging from 8.5 to 2.0 Log copies per ml) by diluting 3 highly positive TTV DNA clinical samples. The differences between input and calculated copy numbers were small, the latter ranging between 8.3 and 8.8 and between 1.3 and 2.1 Log copies per ml. The results of TTV R-GENE® assay linearity are shown in Figure 1, which also indicates the performances of the in-house rtPCR when used for testing the same panel of nominal TTV DNA concentration levels. Again, 5 aliquots from independent DNA extractions of a reference serum were also processed separately. Overall variations were less than 0.2 Log. A plasma sample at well-known TTV DNA concentration was diluted at different copy numbers and used to evaluate the sensitivity of the assay. The lower limit of sensitivity was found to be 12 copies of TTV DNA per ml of plasma, thus demonstrating that the TTV R-GENE® assay showed the same sensitivity level of the in-house rtPCR in revealing the presence of TTV in blood samples.

After having established the performances of the TTV R-GENE®, a total of 327 samples were tested by both rtPCR methods. Considering all of the specimens, 236 (72%) and 227 (69%) samples were detected as positive when TTV R-GENE® or in-house rtPCR assays were applied, respectively. TTV quantification of the 236 and 227 positive samples revealed a mean of 4.1 (95% CI: 3.9 - 4.1) and 4.2 (95% CI: 3.8 - 4.1) Log copies per ml of plasma/whole blood, respectively.

When the results obtained by both methods were qualitatively compared, 308 out of 327 (94%) samples were identified concordantly: 222 were TTV positive and quantified by both assays, and 86 were below the sensitivity limit on both tests. In contrast, the remaining 19 samples (6%) gave discordant results, being quantified with one test but below the sensitivity limit of the other assay (Table 1). Since inter-assay variation may be influenced by the number of TTV copies present in each sample, the mean load of TTV DNA between the group of samples that gave concordant and discordant results was determined. As evaluated by the TTV R-GENE® assay, the mean number of copies in concordant results (222 of 227 samples, excluding the 86 TTV negative results) was 4.2 Log copies per ml (95% CI: 3.9-4.1 Log copies/ml), while in discordant results (14 of 19 samples) was 1.6 (95% CI: 1.5-1.7 Log copies/ml), with the difference that was statistically significant ($p < 0.0001$). Of these discordant samples, 14 were quantified with TTV R-GENE® assay (mean: 1.6; 95% CI: 1.5-1.7 Log copies/ml) but not with in-house rtPCR, and 5 were quantified with in-house rtPCR (mean: 2.4; 95% CI: 1.5-3.2 Log copies/ml) but not with TTV R-GENE® assay. No sample quantified with TTV R-GENE® assay only had results above 2.0 Log copies/ml, while TTV DNA values above 2.0 Log copies/ml were in 3 of 5 discordant samples quantified with in-house rtPCR but virus negative with TTV R-GENE® assay.

Overall, the assays concordance was 94% with an excellent agreement in TTV detection as revealed by Cohen's kappa value of 0.88. For the 222 paired quantified samples, the overall concordance between results as assessed by Passing-Bablok linear regression analysis was excellent (R = 0.903; Spearman rho correlation, $p < 0.0001$) (Figure 2). The Bland-Altman analysis showed that the mean difference between the two methods was -0.3 Log copies /ml, with the ±2 standard deviation limits of agreement of 0.9 and −1.4 (Figure 3). Importantly, 65% (145 of 222) of the results differed by less than 0.5 Log copies/ml (mean: -0.05; CI 95%: -0.08 – 0.04 Log copies/ml) and 35% (77 of 222) were within 0.5 and 1.0 Log copies/ml (mean: 0.6; CI 95%: 0.5 – 0.7 Log copies/ml) of each other.

3.2 Quantitative performance of rtPCR methods according to infecting TTV groups

TTV was successfully typed by using 5 distinct group-specific PCRs in 60 randomly selected samples. Most TTV-positives carried out two distinct groups, followed by three, one, and four. TTV group 3 was the most prevalent (53 samples), followed by groups 1 (41 samples) and 4 (36 samples), while group 5 was rather infrequent (12 samples). No group 2 infection was found, and group 1 and group 5 isolates were only found in mixed infections with one or more other genogroups. Mean TTV loads of the 60 typed subjects were then stratified according to the number of TTV groups they harbored. As shown in Figure 4, similar trends in their values were obtained when measured by the two quantitative rtPCR methods. Furthermore, the mean TTV loads of different combinations of single, dual, triple and quadruple group infections were measured similarly by in-house rtPCR and TTV R-GENE® assay (Figure 5).

3.3 TTV detection and quantification by ddPCR

A new molecular method for TTV quantification in clinical samples was developed using ddPCR technology which performance was evaluated for the first time. Specifically, in order to estimate the reproducibility of the assay, three reference TTV-positive plasma were repeated in four independent experiments, and the coefficients of variation (CV) was calculated. The CVs for sample ranged between 0.1 to 0.8%, with the maximum variation in TTV copy number lower than 0.5 Log, thus indicating a good reproducibility of the ddPCR assay. For evaluating the lower limit of sensitivity of the complete procedure (extraction and ddPCR amplification), four plasma samples containing from 6.0 to 9.0 Log copies per ml of TTV DNA and different mixes of TTV groups were serially diluted to 1 copy per ml. With the standard procedure used, i.e. amplification of extracted DNA corresponding to 200 ul of plasma or whole blood, the limit of sensitivity was found to be 10 copies of TTV DNA per ml. Taken together, these results indicated that the ddPCR assay was a sensitive and accurate method for the detection of templates ranging from 10^1 to 10^9 copies of TTV DNA.

Having established the reliability of the method, a group of 94 samples were tested independently by ddPCR and the results were compared with those obtained by the two rtPCR methods. Of these 94 samples, 46 had previously reacted TTV-positive by both rtPCR assays, 37 samples were below the sensitivity limit on both tests, and 11 samples were quantified with one test but below the sensitivity limit of the other assay. The comparison results are summarized in Table 2. All TTV positive samples and 36 out of 37 TTV negative samples were confirmed by ddPCR, thus demonstrating a very similar efficiency of the three methods for demonstrating TTV DNA and an assay concordance of 99%. One sample that reacted TTV negative by in-house rtPCR and TTV R-GENE®

assays was found positive by ddPCR at level of 2.2 Log copies per ml. Considering the 11 discordant samples, 10 samples gave a positive result (which 9 were negative by in-house rtPCR and positive by TTV R-GENE® assay, and 1 was the opposite), and 1 sample tested ddPCR negative according to the result obtained by in-house rtPCR but not to one with TTV R-GENE® test.

The correlation between TTV levels detected by each quantitative assay was also calculated. TTV levels measured by ddPCR were strongly correlated with those detected by both in-house rtPCR and TTV R-GENE® assays ($r = 0.819$, $p < 0.001$, and $r = 0.903$, $p < 0001$, respectively; Kendall tau test). In order to investigate whether the range of TTV loads detected by each quantitative PCR was comparable to each other, the Kruskal - Wallis analysis of variance was used. As shown in Figure 6, there was no significant differences among distributions of TTV viremia when tested by the various quantitative PCRs, thus revealing that the ability of these methods to quantify the levels of TTV was not statistically different.

4. DISCUSSION

To our knowledge, this is the first comparative evaluation of three available tests for the qualitative and quantitative detection of TTV viremia in blood samples. The ability to precisely detect TTV viremia is becoming more and more important for the assessment of immune suppression in patients receiving solid organ or bone marrow transplantations.[22] In fact, although many aspects of TTV replication remain to be clarified, there is already convincing evidence that TTV levels can be significantly influenced by imbalances of immunity, increasing in response to an immunodepression status.[23-26] TTV viremia quantification has therefore been suggested as an important criterion for transplant clinicians in deciding therapeutic strategies, modifying the doses

of immunosuppressants, and/or changing the types of drugs used. All in order to reduce the risk of infectious and immunological complications after organ transplantation and for optimizing an appropriate and efficient use of personalized therapy. Determining the best threshold of TTV viremia able to predict the post-transplant risk is the challenge of many studies. TTV predictive cutoffs from the many single-center investigations have been proposed in different transplantation settings, but differences in the type of transplantation, immunosuppressive drug regimens used, and transplant timepoints in which TTV viremia has been measured account for most of the observed cutoff differences.[6,8-10,12-15,22,23,25,27] To greater extent, calculated TTV cutoffs are often not transferable from laboratory to laboratory given the use of different molecular methods used for virus quantification and the lack of comparative data on the performances of these diverse assays. This is true despite the recent availability of a TTV-specific independent external quality assessment (EQA) program that might make easier the comparability of results among laboratories (29). Then, comparing the performances of available PCR based tests for the measure of the TTV viremia could improve interoperability, reproducibility, and reliability among different worldwide laboratories.

Several formats of PCR methods have been developed in a number of laboratories for the qualitative and quantitative detection of the TTV DNA in plasma or other clinical specimens.[2,16,17,19,20,30,31] The methods described include several formats of PCR targeted to different regions of the viral genome; however, the one potentially capable of amplifying all of the genetic forms of TTV hitherto recognized is based on a small, highly conserved segment of the 5' UTR. Used in real-time format, this "universal PCR" has led to a correct appreciation of the pervasiveness of TTV and revealed that, in individual subjects, its loads may vary between 10^1 and 10^9 DNA copies/ml of plasma.[5,21]

In this study we compared the performances of three UTR PCR methods using a panel of clinical samples. Two methods, an in-house PCR and the commercial TTV R-GENE® assay (BioMerieux) rely on the rtPCR technology, while the third method is in the format of ddPCR. ddPCR is a quantitative PCR method that provides a sensitive and reproducible way of measuring the amount of viral DNA present in a sample. It does not use an external curve to extrapolate the number of templates and, therefore, is not affected by the range of linearity. Further, quantitation is very precise even at very low template copy and for viral variants exhibiting high sequence diversity. Overall, the results of this study suggest some considerations. The first one is that all the PCR assays demonstrated comparable lower limits of TTV DNA detection in blood samples, around 10-12 copies per ml of blood. The comparison of qualitative PCR results revealed an overall observed agreement of more than 90%, indicating strong assays reliability. Again, when the numbers of copies of TTV DNA detected by the different assays were compared, a strong correlation in the ability to quantify the virus among different assays was found, being within 0.5 Log copies/ml the viral load differences for the most samples. Finally, the assays were found to quantify similarly TTVs belonging to genogroup 1, 3, 4 and 5 (no isolate of TTV group 2 was included in the panel). Although their performances with single TTV species were not investigated in this study, all tests readily quantitated the array of TTV species included in the panel examined.

Of interest, the composition of this panel largely reflects the prevalence of TTV groups in the general population with TTV infection, where groups 1 and 3 represent approximately the 70% of TTV.[19,21]

Although a high level of correlation between the PCR assays was observed, there were discrepancies that deserve discussion. The majority of discordant results were due to

samples with a positive result by an individual PCR assay and negative result by the other tests. Since the detection ability of each assay and, consequently, the level of agreement among assays may be influenced by the number of TTV copies present in each sample, we compared the levels of TTV DNA from samples that gave concordant and discordant results. This analysis showed that samples that gave discordant results had significantly lower numbers of TTV copies than samples that gave concordant results. Then, the discrepancies might have been due to the small amount of target nucleic acid present in the clinical sample rather than to false-positive results by the individual PCR assays. The final proof for the hypothetical better sensitivity of one PCR method could come from serial measurements of patients undergoing immunosuppression without new infection from exogenous transfusion/transplantation. Exclusion of false positives concurrently identifies the most sensitive method, and at the same time increases TTV prevalence in the general population. The latter consequence is reasonable given the already extremely high prevalence in adulthood, making avoidance of contagion extremely unlikely. It is also important to emphasize that the performance of these assays could vary depending on the type of PCR platform that is used (i.e. rtPCR versus ddPCR).

The study is limited by the absence of characterization of the single TTV species in the samples tested. Genetic data were obtained directly by gel analysis of the group-specific PCR products, a method that doesn't allow discrimination of a single TTV species infecting. Then, it cannot be excluded that our samples harboured TTV species that may be differently detected by the different PCR assays. Because the number of TTV species is high and growing,[32] and their prevalences could also vary in different populations and geographical regions, future comparative studies should incorporate samples containing all the TTV species. In fact, it might be necessary that the PCR primers used in these

assays are updated continuously and improved as the understanding of TTV genetic diversity and species distribution progresses. Again, the need for the assays to be tailored based on the epidemiological situation of the specific populations and regions where the methods are to be used cannot be excluded.

In conclusion, since the interest in TTV is rapidly increasing, harmonization of the diagnostic methods across laboratories worldwide appears to be a key requirement; in this context, the present study is a first attempt toward the ability to globally compare results. To this end, the comparison of different protocols for TTV detection has highlighted the comparable performances of the PCR methods actually in use and their ability to properly detect and quantify TTV. However, in order to monitor detection capabilities of TTV assays, it is important that efforts should be done to develop standards for improving standardization and comparability of quantitative results between different assays and laboratories and that attempts be made to extend the number of TTV species included in the future trials.

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FIGURES

Figure 1.

Linearity of TTV R-GENE® and in-house rtPCR, as determined by dilutions of three highly positive TTV DNA plasma samples (A, B, and C). In parenthesis, are also reported the TTV groups infecting each plasma sample.

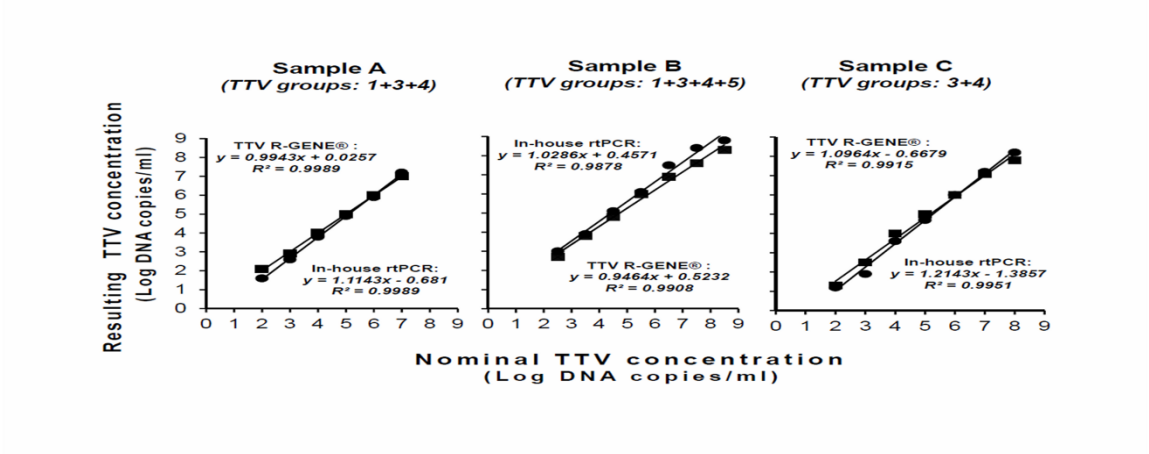

Figure 2.

Linear regression analysis of TTV levels of 222 paired quantified samples using Passing-Bablok regression analysis.

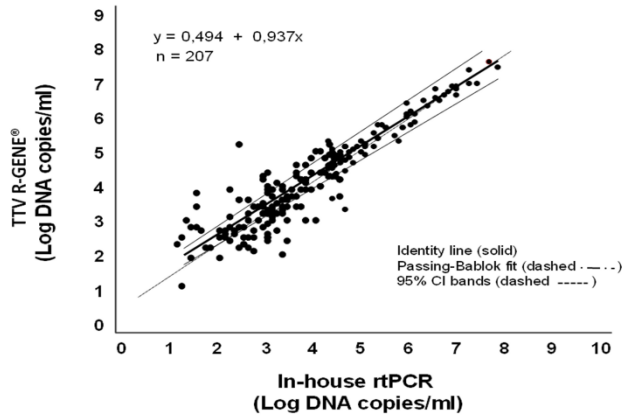

Figure 3.

A Bland-Altman difference plot to show the differences in TTV levels between the two assays. Horizontal lines are drawn at the mean difference (solid line), and at the upper and lower limits of agreement (dashed lines).

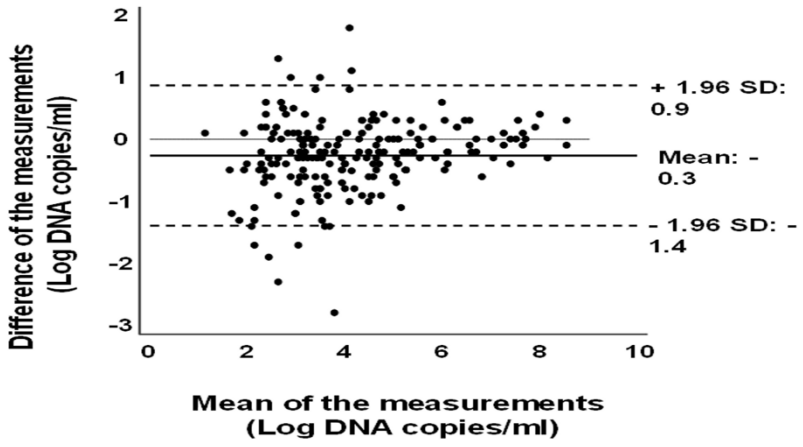

Figure 4.

Comparison of TTV loads measured by TTV R-GENE® and in-house rtPCR in 60 subjects whose TTV infections proved typeable, stratified by the number of viral groups

carried. TTV load is expressed as the mean Log DNA copies per ml of plasma ± 95% confidence intervals (CI). Solid and dashed lines represent TTV values measured with in-house rtPCR and TTV R-GENE®, respectively. The numbers in parentheses are numbers of samples in the respective groups.

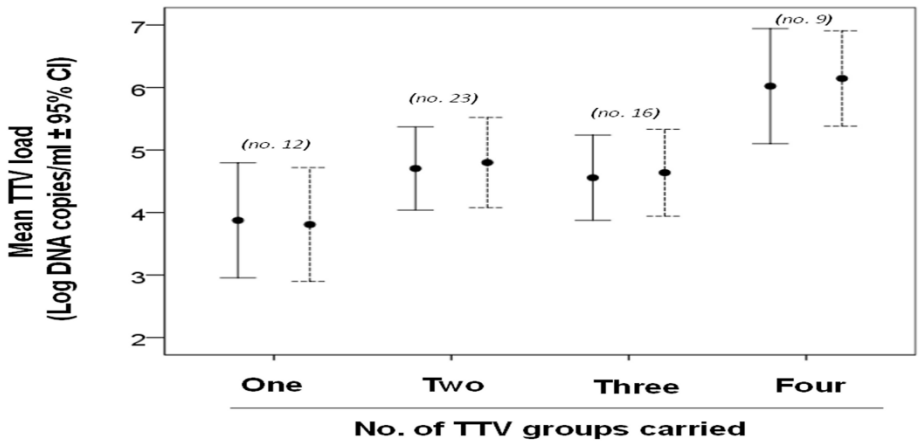

Figure 5.

Comparison of TTV loads measured by TTV R-GENE® (black columns) and in-house rtPCR (dots columns) in 60 subjects whose TTV infections proved typeable, stratified by the specific viral group(s) carried. Single, dual, triple, and quadruple genogroup infections are reported. TTV load is expressed as described in the legend to Figure 4. The numbers in parentheses are numbers of samples in the respective groups.

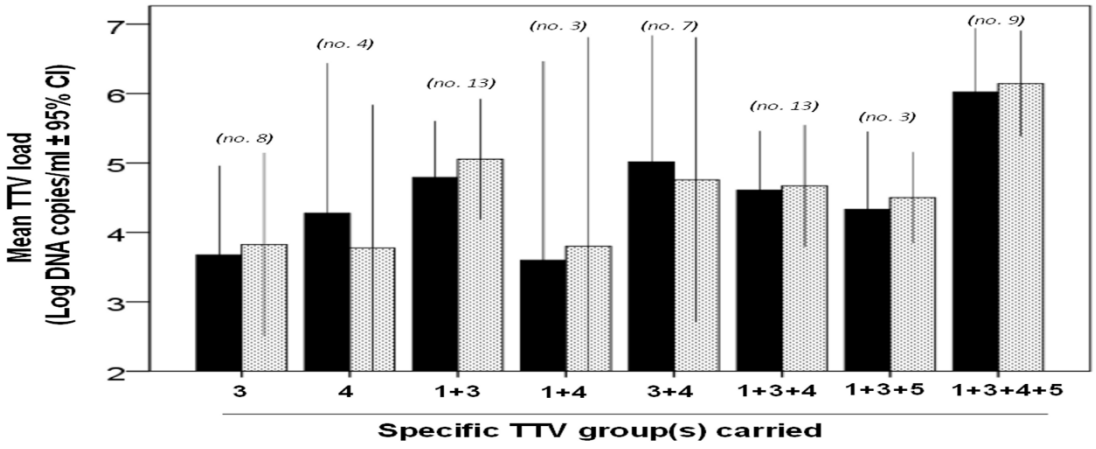

Figure 6.

Comparison of TTV loads among three testing methods. Blox plot of results from the Kruskal – Wallis analysis. TTV levels for 94 samples, determined independently by each of the 3 PCR methods, were used to the variance analysis. In the graph, median, 25%-75%, min-max, and outgroups are reported.

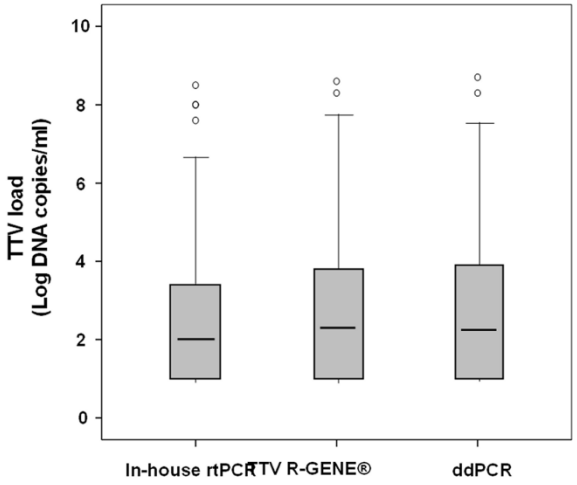

TABLE 1 Concordance of TTV load results between in-house rtPCR and TTV R-GENE® assays

TTV DNA with in-house rtPCR	No. examined	TTV DNA with TTV R-GENE®	
		Negative	Positive
Negative	100	86 (86) [a]	14 (14)
Positive	227	5 (2)	222 (98)

[a] Number (percentage).

TABLE 2 Concordance of TTV load results among the 3 PCR methods

TTV DNA with in-house rtPCR and TTV R-GENE® assays	No. examined	TTV DNA with ddPCR	
		Negative	Positive
Concordant results			

Negative	37	36 (97) [a]	1 (3)
Positive	46	0 (0)	46 (100)
Discordant results	11	1 (9)	10 (91)

[a] Number (percentage).