Assessment of prevalence and load of torquetenovirus viremia in a large cohort of healthy blood donors

Daniele Focosi[1], Pietro Giorgio Spezia[2], Lisa Macera[2,3], Stefano Salvadori[4], David Navarro[5], Maria Lanza[1], Guido Antonelli[6], Mauro Pistello[2,3], Fabrizio Maggi[2,3 #]

[1] North-Western Tuscany Blood Bank, Pisa University Hospital, Pisa, Italy.

[2] Retrovirus Center and Virology Section, Department of Translational Research, University of Pisa, Pisa, Italy.

[3] Division of Virology, Pisa University Hospital, Pisa, Italy.

[4] National Research Council, Pisa, Italy.

[5] Department of Microbiology, School of Medicine, University of Valencia, Valencia, Spain.

[6] Department of Molecular Medicine, Laboratory of Virology and Pasteur Institute-Cenci Bolognetti Foundation, Sapienza University of Rome, Rome, Italy.

[#] Corresponding author: Prof. Fabrizio Maggi, Department of Translational Research, University of Pisa, and Virology Division, Pisa University Hospital, Pisa, Italy, Via Paradisa 2, I-56127 Pisa, Italy. Phone: +39 050 993756. E-mail: fabrizio.maggi@unipi.it

Abstract words: 250

Text words: 2500

Abstract

Objectives: Torquetenovirus (TTV) is an emerging marker of functional immune competence with the potential to predict transplant-related adverse events. A large-scale epidemiological study was performed to understand how basal values vary in healthy subjects according to age and gender.

Methods: We tested plasma from 1017 healthy blood donors aged 18-69 years. The presence and load of TTV were determined by a real-time PCR assay. A sub-cohort of 384 donors was tested for anti-cytomegalovirus IgG antibodies, and 100 subjects were also tested for TTV viremia on a paired whole blood sample.

Results: The overall prevalence of TTV was 65% (657/1017) with a mean (± standard deviation, SD) growth of 5 ± 4% every 10 years of age increase, but stably higher in males (465/690, 67%) than in females (192/327, 59%). Mean (± SD) TTV load was 2.3 ± 0.7 Log copies/ml with no sex difference. TTV viremia showed modest increases along 10-years age intervals (mean ± SD: 0.3 ± 0.1). TTV viremia in donors sampled two years later remained stable (mean ± SD: 2.3 ± 0.8 versus 2.2 ± 0.7 Log copies between samples). Twenty-six percent (9/34) of blood donors with TTV-negative plasma scored positive when whole blood was tested, and the donors with positive plasma showed a mean (± SD) 1.4 ± 0.5 Log increase in copy numbers when whole blood was tested.

Conclusions: This study establishes the mean value of TTV viremia in plasma in healthy blood subjects and suggests that ageing causes only minimal increases in TTV viremia.

Keywords: TTV, torquetenovirus, anelloviridae, viremia, prevalence, healthy controls, blood donors.

Introduction

Torquetenovirus (TTV), first identified in 1997 [1], is the prototype of the Anelloviridae family [2, 3]. TTV exhibits a number of remarkable properties, including a high prevalence in the general population and an ability to induce chronic infections with no clearly associated clinical manifestations [3]. Increasing evidence has been recently reported regarding the successful interplay of TTV with its host, and the control of TTV replication exerted by the immune system [4, 5]. Accordingly, plasma TTV loads tend to be higher in patients with immune system dysfunction compared to healthy controls [4] and these loads undergo moderate and transient changes following perturbations in immunity due to, e.g., immunosuppressive therapy, transplantation, and chemotherapy [6-13]. A precise understanding of how and how much immunity modulates TTV replication is of utmost importance, for using PCR monitoring of TTV viremia as a way to assess global immune function [14, 15]. TTV PCR already has been successfully used in retrospective and prospective studies, and standardization is ongoing [16]. Detailing prevalence in healthy blood donors could prove useful both for basic virology (e.g. suggesting transmission routes) and clinical applications (e.g. understanding how universally TTV could be used as a biomarker). Defining stability over time, as long as a healthy state is preserved, could help to increase confidence in TTV viremia as an immune status marker not affected by other variables.

Materials and methods

Study population and specimens

We collected plasma from 1017 healthy blood donors, normally distributed by age, and whose biological samples were discarded after routine molecular screening for blood donation. As shown in Table 1, the mean age of the population was 44 years (median: 46 years, range: 18-69 years) and the male: female ratio was 2.1:1, as expected by average Italian blood donor population. Plasma was used for both TTV detection and quantification, and, on a statistically representative sub-cohort of 384 subjects (median: 47 years, range: 18-67 years; male: female ratio: 2.2:1), also for anti-

cytomegalovirus (CMV) IgG titration. A sub-cohort of 100 individuals was selected using computer-aided randomization (median: 46 years, range: 18-63 years; male: female ratio: 2.1:1), paired plasma and whole blood aliquots were collected from the same tube before and after centrifugation, respectively. Finally, for 46 donors a follow-up sample (same origin and type) collected within 2 years was available for retesting. The study was run after ethical approval (protocol number 63409).

TTV DNA detection and quantification

Viral DNA was extracted from 200 µl plasma and whole blood samples using QIAamp DNA minikit® (Qiagen, Chatsworth, CA, USA), as specified by the manufacturer. The presence and load of TTV genome were determined by using the recently commercialised TTV R-GENE® kit (bioMérieux, Marcy-l'Etoile, 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 allows the amplification of a 128-base pairs fragment of the untranslated region of the TTV genome. An extraction and inhibition internal control, as well as a range of 4 quantification standards, is included in the TTV R-GENE® kit. Real-time PCR amplification was performed using a 7500 Fast instrument (Applied Biosystems). The method boasts high sensitivity, measuring as low as 1.1 Log TTV DNA copies/ml of plasma or whole blood, and specificity since it does not detect the other human anelloviruses, such as torquetenominivirus and torquetenomidivirus. The procedures used to quantitate the copy numbers and assess specificity, sensitivity, intra- and inter-assay precision, and reproducibility have been previously described [17]. Importantly, when aliquots from independent DNA extractions of a reference serum were processed separately, the overall variations were less than 0.5 Log.

CMV IgG titration

The quantitative determination of IgG antibodies to CMV was performed on available residual plasma samples for a total of 384 subjects (stratified according to TTV viremia). The test was

performed on the fully automated random-access Liaison XL platform with LIAISON® CMV IgG II (Saluggia, VC, Italy). The results were interpreted according to the manufacturer's instructions and expressed as UI/ml.

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. The Chi-square test, the Chi-square test for trends, and Fisher's exact test were applied to evaluate the heterogeneity of contingency tables. Differences between distributions were calculated by using non-parametric the Mann-Whitney U test. The association among variables was evaluated by a nonparametric the ANOVA using the Kruskal-Wallis test. Correlations between variables were assessed using Spearman rho correlation coefficient and Student's *t*-test. Regression analyses were conducted to evaluate the association between the dependent variable TTV viremia and 3 independent variables (age, sex and CMV serostatus). All *p* values presented are based on two-tailed tests, and $p < 0.05$ was considered statistically significant.

Results

TTV prevalence and loads in plasma samples

The presence of TTV DNA was investigated in plasma samples from 1017 blood donors. The overall prevalence was 65% (657/1017), as 360 samples were below the detection limit of the real-time PCR assay and 657 yielded a positive detection signal. Statistically significant differences were noted when the subjects were grouped by age and sex. TTV prevalence was highly related to age as shown in Figure 1 ($p < 0.001$; Chi-square test for trends). TTV prevalence was of 45% (23/51) in the youngest 5-years group (aged from 18 to 22 years) and 75% (52/69) in the over 60 aged group with an overall increase in virus prevalence of 30% in the 50 years span within the study cohort. Table 1 shows that total TTV prevalence was also significantly higher in males versus

females (465/690, 67% and 192/327, 59%, respectively; p = 0.0068, Chi-square test), and this difference tended to persist also when subjects were grouped by 10-years age intervals (Figure 1).

TTV viremia was precisely quantified in the plasma samples of 657 subjects. Total mean (± standard deviation, SD) was 2.3 ± 0.7 Log TTV DNA copies/ml (median: 2.3 Log; range: 1.1 – 4.9 Log copies/ml), with no sex difference (Table 1). Supplementary Figure S1 shows the distribution of TTV loads in the infected individuals. TTV viremia was below 3.0 Log copies/ml in 81% (533/657) of TTV positive samples, and levels higher than 4.0 Log were seen only in 1.7% (11/657) of subjects. In order to investigate whether the range of TTV loads detected in the different age groups was comparable, the Kruskal-Wallis analysis of variance was used. As shown in Supplementary Figure S2, although the increase was relatively modest (mean ± SD: 0.3 ± 0.1), there was a significant difference among distributions of TTV load (p = 0.006). Spearman's rho correlation showed also a positive correlation between age and TTV viremia (r = 0.142, p < 0.001; data not shown).

In a randomly selected sub-cohort of 384 healthy donors (63% of which were TTV-positive with a mean ± SD load of 2.3 ± 0.7 Log copies/ml), anti-CMV IgG prevalence was 71% (272/384). TTV presence and load did not differ between CMV IgG positive versus negative patients (data not shown). Again, TTV load did not correlate with anti-CMV IgG level (r = 0.089, p = 0.162), while anti-CMV IgG level significantly correlated with the subjects age (r = 0.299, p < 0.0001; Spearman's rho).

Of interest, when a linear regression analysis model was applied using TTV viremia as the dependent variable and age, sex, and anti-CMV IgG serostatus as predictors, a predominant effect of age on TTV levels was revealed (Table 2).

TTV loads in longitudinal plasma samples

Supplementary Figure S3 shows the sequential kinetics of plasma TTV levels of the 46 healthy donors for whom a follow-up sample was available two years later. TTV viremia remained

essentially stable in the two samples (mean ± SD: 2.3 ± 0.8 versus 2.2 ± 0.7 TTV DNA Log copies between samples, respectively). Fifteen samples, which were TTV negative at the first test, remained all with viremia levels under the detection limit at the follow-up sampling point. It is noteworthy that most plasma samples (27/31, 87%) showed a non-significant, e.g. < 0.5 Log, variation of viremia (mean ± SD: 0.3 ± 0.2 Log) and the 2 samples that differed most, the change was 0.7 Log between the two sampling points.

TTV loads in simultaneous plasma and whole blood samples

In 100 subjects, we also determined the loads of TTV DNA found in simultaneous whole blood samples. Seventy-five samples tested TTV positive at copy numbers with a mean ± SD of 3.6 ± 1.0 Log copies/ml of whole blood (median: 3.8 Log; range: 1.2 – 6.0 Log copies/ml). Viral loads in plasma and whole blood were highly correlated in individual subjects (r = 0.937; p < 0.0001) (Figure 2). It should be noted that 9 whole blood samples tested TTV positive (in one case at 3.1 Log copies/ml; total mean ± SD: 2.0 ± 0.7 Log) in spite of the fact that the corresponding plasma samples were below the detection threshold (Table 3). The 66 donors with TTV-positive plasma showed a mean of 1.4 Log increase (range: 1.1-2.8 Log) in copy numbers when whole blood was tested (p < 0.0001, Mann-Whitney test).

Discussion

Our study confirms a linear increase in TTV prevalence with ageing and establishes the mean value of TTV viremia in healthy subjects (2.3 ± 0.7 Log copies/ml). Our results suggest that healthy ageing causes only minimal increases in TTV viremia and proof stability of TTV viremia under healthy conditions in the short-term. The findings also elect whole blood as a more sensitive sample for TTV detection.

This study is the largest in the field (n.= 1017) and shows an overall prevalence of 65%. Spandole-Dinu et al. [18] conducted the former largest study on healthy Romanian controls (n.=701) using a 5'UTR hemi-nested PCR and reported a 58.4% prevalence. A much higher 76% prevalence was

found in a study using TaqMan real-time PCR on 313 Austrian subjects undergoing routine screening [19]. Prevalence linearly correlates with the mean cohort age (38 years and 58% in the Romanian study, 44 years and 65% in our study, 53 years and 76% in the Austrian study). The lower limit of detection of the methods used in those studies was similar to the one used in the current study, excluding it as cause for differences.

Our study confirms previous findings that whole blood TTV PCR results in higher TTV loads than when using plasma, which has implications for epidemiological studies. Takahashi et al. [20] reported that the TTV load was 6.9 fold higher in the whole blood than in the plasma samples. The authors attributed the difference to the high viral loads found in blood cells, specifically in granulocytes. Only one additional small study (n= 25) compared TTV loads in different samples (plasma vs. exosomes [21]).

Former studies on TTV prevalence in large (n. > 300) healthy donor cohorts worldwide are very limited [18, 19, 22, 23]. The current study represents the largest investigation in healthy blood donors to date. Blood donors are not representative of the general population: in other words, a selection bias implies that frail people (e.g. with mild anaemia, underweight, drug addicted, etc.) are excluded from blood donation, resulting in a cohort healthier than the general population. This study excludes healthy subjects aged less than 18 years and more than 70 years because of current laws regulating blood donation in Italy: similar age limits apply to blood donors worldwide, so it is not easy to procure a cohort healthier than the general population in both age extremes. Nevertheless, dynamics of TTV prevalence during the first years of life has been investigated in at least three small-scale clinical studies [24-26], and our results in the adult cohort fit the previously known prevalence growth curve.

It cannot be excluded that the study population includes a probably low rate of individuals infected with TTV strains non-detectable by the PCR assay used. TTV exhibits an extremely high level of genetic heterogeneity and to validate the PCR assays used for demonstrating TTV DNA for their

ability to detect *in vivo* the entire spectrum of TTV variants is much complicated. Finally, due to specimens' limitation, we tested sequential plasma samples only from a limited number of healthy donors (n. 46).

This study shows that the overall prevalence in males is higher than in females and also tends to be present at any age group, which suggests that sexual transmission may be another route how TTV is transmitted. Nevertheless, further studies are required to confirm this hypothesis. The mean load in males versus females is not different, excluding the impact of sex hormones on replication competence or other steps of the viral life cycle. A former study in Qatar found no difference in prevalence between genders but the sample population was not powered to show differences (488 males versus 12 females) [22]. Another study in Russian athletes using a different qPCR [23] found a much higher prevalence (94%) than the one found in our study: in that unusual setting it was reasonable that the authors could not find a difference in prevalence between the 205 males and the 232 females.

Former studies have proven different TTV viremia in CMV-positive versus CMV-negative subjects. Haloschan et al. [19] reported that subjects undergoing routine virological screening had lower TTV viremia in women than in men in the age range 20-30 (but not in the 50-93 age range), and higher TTV load in CMV IgG-seropositive than seronegative persons in the age range 20-60 (but not in 50-93). The authors suggested that the host's immune control of TTV replication decreases with age and is gender-specific. In accordance with other former studies on cohorts healthier than the general population [23], we were not able to replicate those findings in our cohort.

Importantly, the high prevalence in serum, and the even higher prevalence in whole blood, raise doubts as to whether true TTV negativity exists in adulthood or whether the lower limit of sensitivity of current methods and specimens represent a hurdle to detection of TTV viremia in immunocompetent patients. The close to 100% prevalence in immunocompromised patients having not received blood derivatives [27] rather suggest a model whether iatrogenic immunosuppression

220 weakens immune surveillance against TTV, causing viremia to grow above the lower limit of
221 sensitivity of PCR.

222 This study sets the baseline median TTV viremia values in Caucasians that could be used for
223 prospective interventional trials. The results also highlight that healthy ageing (i.e. ageing while
224 preserving fitness to donate blood) is associated with a modest increase in TTV viremia, which
225 could be related to physiological immune senescence [19].

226 Our results suggest whole blood is the best sample to avoid false negatives. These findings
227 stimulate the intriguing hypothesis that TTV is almost ubiquitous in adults, and that negativity is
228 just a laboratory artefact due to poor sensitivity of the methods [28-30]. So, in addition to PCR
229 upgrades (e.g. digital PCR [17]), employment of alternative biological specimens could increase the
230 detection rate and broaden applicability of TTV as a status marker to more subjects.

231

232

233

234

235

Conflict of interest disclosure

F.M. has received a non-financial support by QCMD as scientific expert for TTV panel, and a research support by BioMerieux as consultant for TTV test development.

Funding

This work was supported by a grant from Fondazione "Istituto di Ricerca Virologica Oretta Bartolomei Corsi". BioMerieux, the manufacturer of the TTV R-GENE® assay, provided the TTV test kits used in this study, and had no whatsoever role in study design, data collection and analyses, decision to publish, and preparation of the manuscript.

Author Contributions

D.F. and F.M. designed the study and wrote the manuscript with support from D.N., M.L., G.A., M.P. that also contributed to the interpretation of the results. P.G.S, L.M, and S.S. contributed to the sample preparation, statistical analyses and carried out all the experiments. All authors contributed to revisions of the manuscript, participated in the decision to submit, and had full access to all of the data in the study.

Legend to Figures:

Figure 1. TTV prevalence grouped by 10-years age intervals in total study population and in males versus females.

Figure 2. Correlation between plasma and whole blood TTV loads in 100 paired samples.

Supplementary Figure S1. Distribution of plasma TTV loads in the positive sub-cohort (n.= 586).

Supplementary Figure S2. Mean plasma TTV loads in the positive sub-cohort (n.= 586), grouped by 10-years age intervals.

Supplementary Figure S3. Plasma TTV loads in a sub-cohort of 46 blood donors at 2-year follow-up.

References

1. Nishizawa T., Okamoto, H., Konishi, K., Yoshizawa, H., Miyakawa, Y., Mayumi M. **A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology.** *Biochem Biophys Res Commun.* 1997; 241:92-97.
2. Biagini, P. **Classification of TTV and related viruses (anelloviruses).** *Curr Top Microbiol Immunol.* 2009; 331:21-33.
3. Focosi, D., Antonelli, G., Pistello, M., Maggi, F. **Torquetenovirus: the human virome from bench to bedside.** *Clin Microbiol Infect.* 2016; 22:589-593.
4. Maggi, F., Bendinelli, M. **Immunobiology of the Torque teno viruses and other anelloviruses.** *Curr Top Microbiol Immunol.* 2009; 331:65-90.
5. Simmonds, P. **TT virus infection: a novel virus-host relationship.** *J Med Microbiol.* 2002; 51:455-458.
6. Jaksch, P., Kundi, M., Gorzer, I., Murakozy, G., Lambers, C., Benazzo, A., et al. **Torquetenovirus as a novel biomarker targeting the efficacy of immunosuppression after lung transplantation.** *J Infect Dis.* 2018; 218:1922-1928.
7. Ruiz, P., Martínez-Picola, M., Santana, M., Muñoz, J., Pérez-Del-Pulgar, S., Koutsoudakis, G., et al. **Torquetenovirus is associated with the state of immune suppression early after liver transplantation.** *Liver Transpl.* 2019; 25:302-310.
8. Strassl, R., Schiemann, M., Doberer, K., Görzer, I., Puchhammer-Stöckl, E., Eskandary, F., et al. **Quantification of torquetenovirus viremia as a prospective biomarker for infectious disease in kidney allograft recipients.** *J Infect Dis.* 2018; 218:1191-1199.
9. Frye, B.C., Bierbaum, S., Falcone, V., Köhler, T.C., Gasplmayr, M., Hettich, I., et al. **Kinetics of torquetenovirus-DNA plasma load predict rejection in lung transplant recipients.** *Transplantation.* 2019; 103:815-822.

10. Rezahosseini, O., Drabe, C.H., Sørensen, S.S., Rasmussen, A., Perch, M., Ostrowski, S.R., et al. **Torquetenovirus viral load as a potential endogenous marker of immune function in solid organ transplantation.** *Transplant Rev.* 2019; 33:137-144.
11. Gilles, R., Herling, M., Holtick, U., Heger, E., Awerkiew, S., Fish, I., et al. **Dynamics of torquetenovirus viremia could predict risk of complications after allogeneic hematopoietic stem cell transplantation.** *Med Microbiol Immunol.* 2017; 206:355-362.
12. Wohlfarth, P., Leiner, M., Schoergenhofer, C., Hopfinger, G., Goerzer, I., Puchhammer-Stoeckl, E., et al. **Torquetenovirus dynamics and immune marker properties in patients following allogeneic hematopoietic stem cell transplantation: a prospective longitudinal study.** *Biol Blood Marrow Transplant.* 2018; 24:194-199.
13. Martín-López, M., Albert, E., Fernández-Ruiz, M., González-Álvaro, I., Rodríguez. E., Aguado, J.M., et al. **Torquetenovirus viremia in patients with chronic arthritis: Influence of biologic therapies.** *Semin Arthritis Rheum.* 2019 Jun 29. pii: S0049-0172(19)30278-1.
14. Mitchell, A.B., Glanville, A.R. **Kinetics of TTV-DNA plasma load: a global measure of immune suppression?** *Transplantation.* 2019; 103:660-661.
15. Kotton, C.N. **Torquetenovirus: predictor of infection after solid organ transplant?** *J Infect Dis.* 2018; 218:1185-1187.
16. Focosi, D., Maggi, F. **Torquetenovirus monitoring in transplantation: the quest for standardization.** *Am J Transplant.* 2019; 19:1599-1601.
17. Macera, L., Spezia, P.G., Medici, C., Rofi, E., Del Re, M., Focosi, D., et al. **Comparative evaluation of molecular methods for the quantitative measure of torquetenovirus viremia, the new surrogate marker of immune competence.** *J Med Virol.* 2019 Apr 19.
18. Spandole-Dinu, S., Cimponeriu, D.G., Crăciun, A.M., Radu, I., Nica, S., Toma, M. et al. **Prevalence of human anelloviruses in Romanian healthy subjects and patients with common pathologies.** *BMC Infect Dis.* 2018; 18:334.

19. Haloschan, M., Bettesch, R., Görzer, I., Weseslindtner, L., Kundi, M., Puchhammer-Stöckl, E. **TTV DNA plasma load and its association with age, gender, and HCMV IgG serostatus in healthy adults.** *Age (Dordr).* 2014; 36:9716.

20. Takahashi, M., Asabe, S., Gotanda, Y., Kishimoto, J., Tsuda, F., Okamoto, H. **TT virus is distributed in various leukocyte subpopulations at distinct levels, with the highest viral load in granulocytes.** *Biochem Biophys Res Commun.* 2002: 290:242-248

21. Martelli, F., Macera, L., Spezia, P.G., Medici, C., Pistello, M., Guasti, D., et al. **Torquetenovirus detection in exosomes enriched vesicles circulating in human plasma samples.** *Virol J.* 2018; 15:145.

22. Al-Qahtani, A.A., Alabsi, E.S., AbuOdeh, R., Thalib, L., El Zowalaty, M.E., Nasrallah, G.K. **Prevalence of anelloviruses (TTV, TTMDV, and TTMV) in healthy blood donors and in patients infected with HBV or HCV in Qatar.** *Virol J.* 2016; 13:208.

23. Vasilyev, E.V., Trofimov, D.Y., Tonevitsky, A.G., Ilinsky, V.V., Korostin, D.O., Rebrikov, D.V. **Torquetenovirus (TTV) distribution in healthy Russian population.** *Virol J.* 2009; 6:134.

24. Bagaglio, S., Sitia, G., Prati, D., Cella, D., Hasson, H., Novati, R., et al. **Mother-to-child transmission of TT virus: sequence analysis of non-coding region of TT virus in infected mother-infant pairs.** *Arch Virol.* 2002; 147:803-812.

25. Komatsu, H., Inui, A., Sogo, T., Kuroda, K., Tanaka, T., Fujisawa, T. **TTV infection in children born to mothers infected with TTV but not with HBV, HCV, or HIV.** *J Med Virol.* 2004; 74:499-506

26. Tyschik, E.A., Rasskazova, A.S., Degtyareva, A.V., Rebrikov, D.V., Sukhikh, G.T. **Torquetenovirus dynamics during the first year of life.** *Virol J.* 2018; 15:96.

.

27. Maggi, F., Focosi, D., Statzu, M., Bianco, G., Costa, C., Macera, L., et al. **Early post-transplant torquetenovirus viremia predicts cytomegalovirus reactivations in solid organ transplant recipients.** *Sci Rep.* 2018; 8:15490

28. Béland, K., Dore-Nguyen, M., Gagné, M.J., Patey, N., Brassard, J., Alvarez, F., Halac, U. **Torquetenovirus in children who underwent orthotopic liver transplantation: new insights about a common pathogen.** *J Infect Dis.* 2014; 209:247-254.

29. Kulifaj, D., Durgueil-Lariviere, B., Meynier, F., Munteanu, E., Pichon, N., Dubé, M., et al. **Development of a standardized real time PCR for Torquetenoviruses (TTV) viral load detection and quantification: A new tool for immune monitoring.** *J Clin Virol.* 2018; 105:118-127.

30. Biagini, P., Gallian, P., Touinssi, M., Cantaloube, J.F., Zapitelli, J.P., de Lamballerie, X., et al. **High prevalence of TT virus infection in French blood donors revealed by the use of three PCRsystems.** *Transfusion.* 2000; 40:590-595.