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DETERMINATION OF TORQUE TENO VIRUS' INTESTINAL LOADS IN IMMUNOSUPPRESSED PATIENTS

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Index

Determination of Torque Teno Virus' Intestinal Loads in Immunosuppressed Patients

Abstract

Torque Teno Virus (TTV, Anelloviridae family) is a ubiquitous virus that can be found in most of the human population without a clear link to pathogenesis. Due to its characteristics, it has been studied as a possible biomarker in immune monitoring and its correlation with other viruses' viral loads. One of these viruses is the human Cytomegalovirus (HCMV, Orthoherpesviridae family). For these studies, qPCR in blood samples is the preferred method for detection and quantification of TTV. However, in cases where blood is a scarce resource (such as in pediatric patients), an alternative sample such as rectal swabs could be useful. In this project, the ability to detect TTV by qPCR in rectal swabs as well as its relationship with HCMV are studied in a cohort of 143 samples from different patients.

Key words

Torque Teno Virus, Rectal swabs, Immunossuppression, qPCR

Resumen

El Torque Teno Virus (TTV, familia *Anelloviridae*) es un virus ubicuo presente en la mayoría de la población humana, sin una clara asociación patogénica. Debido a sus características, se ha investigado como un posible biomarcador en la monitorización inmunológica y su correlación con las cargas virales de otros virus. Uno de estos virus es el Citomegalovirus humano (HCMV, familia *Orthoherpesviridae*). Para estos estudios, la qPCR en muestras de sangre es el método preferido para la detección y cuantificación del TTV. Sin embargo, en situaciones donde la sangre es un recurso limitado (como en pacientes pediátricos), una muestra alternativa como los hisopados rectales podría ser de utilidad. En este proyecto, se estudia la capacidad de detectar TTV mediante qPCR en hisopados rectales, así como su relación con el HCMV, en una cohorte de 143 muestras de diferentes pacientes.

Palabras clave

Torque Teno Virus, Hisopados rectales, Imunosupresión, qPCR.

Introduction

Viruses present in human blood presence include a wide variety that cause different clinical presentations, including immune status and latent infections. Among them, Torque Teno Virus (TTV) is a virus that can exist in human blood cells in its latent or active phases, but one that is not directly related to infections. This non-enveloped virus belongs to the *Alphatorquevirus* genus from the Anelloviridae family(Biagini *et al*., n.d.). It has a circular, negative sense and single stranded DNA genome (Focosi *et al*., 2016). The Anelloviridae family englobes a high number of species with up to 30 genera to date (Biagini *et al*., n.d.; Varsani *et al*., 2021). Viruses belonging to the *Alphatorquevirus* genus have genomes that range between 3,6 kb to 3,9 kb. TTV was first discovered in 1997 in posttransfusion non A to E hepatitis patients with rising ALT levels (caused after blood transfusion but not by any of the known hepatitis viruses). It was isolated through cloning and sequencing the DNA obtained from patients' sera (Nishizawa *et al*., 1997). TTV can be found in most of the healthy human population with different ages, sexes and immune statuses, with no sign of clinical disease (Haloschan *et al*., 2014). There are two main proteins conserved in the *Alphatorquevirus* and *Betatorquevirus* genera encoded by one large open reading frame (ORF1) and a smaller one (ORF2) (Figure 1), as well as other proteins from additional ORFs specific to the sequence (Biagini *et al*., n.d.; Varsani *et al*., 2021). Its pathogenicity was first theorized to be related to a viral etiological cause of hepatitis (Nishizawa *et al*., 1997). However, its ubiquitous prevalence among the general population does not endorse this initial theory. Some studies remark its possible importance in acting as a biological blood marker for the evaluation of the immune system's status in immunocompromised patients. These patients include those that undergo allogenic hematopoietic stem cell transplantation (replacement of damaged bone marrow cells with healthy stem cells), solid organ transplantation (SOT) or patients with Human Immunodeficiency Virus (HIV) infection (Gilles *et al*., 2017; Mouton *et al*., 2020; Rezahosseini *et al*., 2019). In these cases, the patients receive immunomodulatory treatments that affect immune competence and can lead to complications such as opportunistic infections or graft rejection. Thus, a reliable biological marker could be helpful in controlling possible complications related to immune status.

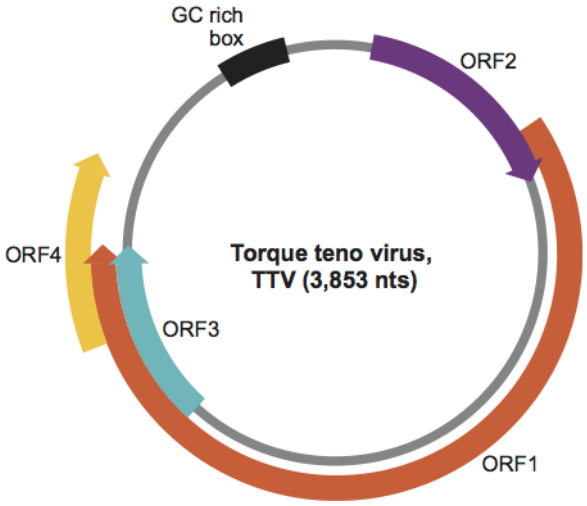

Figure 1. Genome scheme of TTV. (From Biagini et al., n.d.).

Along with TTV, human cytomegalovirus (HCMV) is one of the latent viruses present in most of the population, with up to 80% the whole world population being seropositive for immunoglobulin G as a result of a previous HCMV infection (Zuhair *et al*., 2019). This virus has an enveloped icosahedral structure containing a linear, dsDNA genome of about 200 kb. It belongs to the Orthoherpesviridae family, Betaherpesvirinae subfamily and, specifically, *Cytomegalovirus* genus (Hulo *et al*., 2011). The importance of HCMV in immunocompromised patients comes from the reactivation of a latent infection when the immune system is suppressed due to factors such as immunomodulators and HIV infections. Thus, HCMV viraemia is correlated with a worse clinical outcome as it affects the morbidity and mortality of both SOT and HIV patients (Atabani *et al*., 2012; Deayton *et al*., 2004). Its connection to immunosuppression established itself as a target to study its relationship with TTV replication in these patients.

In terms of viral detection, one of the most common techniques used is the quantitative Polymerase Chain Reaction (qPCR), a molecular technique used in the quantification and determination of genetic material in a sample. This procedure is divided into three stages within a cyclical state repeating itself for a number of cycles established beforehand. The cycles consist of denaturalization of the double stranded DNA at a temperature of 95ºC to allow the primers involved to bind to the single stranded DNA molecule once the temperature lowers. This is followed by the annealing phase, when the temperature goes down to an adequate level as determined by the primers' characteristics (usually between 50ºC to 60ºC). Lastly is the elongation phase or polymerization of the new chain using the deoxynucleotides triphosphates (dNTPs) provided for the reaction, with a temperature of 72ºC (optimal functional temperature for the Taq DNA polymerase). This process is illustrated in Figure 2 . At the end of each cycle,

the fluorescence emitted by a DNA intercalant, or other emission molecules, is measured. qPCRs result in a threshold (below which background fluorescence is detected) and the threshold cycle (Ct) where the first fluorescence value above the background fluorescence (Clewley JP, 1994; Qiagen, 2006; San Segundo-Val & Sanz-Lozano, 2016).

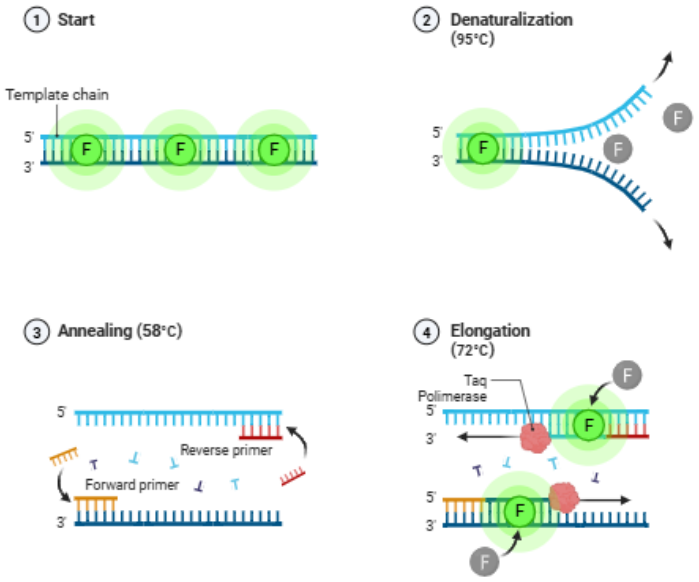

Figure 2. Illustration of qPCR process. Created by BioRender.

For quantification SYBR Green-based qPCR is commonly used, in contrast with the more sensitive TaqMan method, classified as the gold standard method in diagnosis in the detection of several viruses including SARS-Cov2 (WHO, 2020). However, SYBR Green's lower price as well as its ability to dismiss possible unspecific replication via melting curve analysis makes it a suitable option for this study. Despite the greater sensitivity of the TaqMan qPCR, the false positives from the replication of the union of the primers to other than the target sequence of DNA can be left out via. Melting curve analysis measures the temperature at which the double stranded DNA separates and the fluorescent emission stops. This point of temperature is called melting point, and it depends on the longitude and composition of the double stranded DNA, so by comparing it with a positive control, false positive samples that have different melting temperatures can be determined (Gudnason *et al*., 2007; Hanna *et al*., 2006).

Nowadays, evaluation of immune status depends on the patient treated. Targeting just the pathogenic agent of interest in cases like viral infections (viruses like SARS-CoV-2, HPV, HCMV) is related to via specific antigen tests (enzyme-linked immunosorbent assay, ELISA); or using other immunosorbent assays (Joshi *et al*., 2023; Nickel *et al*., 2022). Nevertheless, an accurate and common method between SOT, HIV or hematological transplant patients is not established, an essential tool in cases where a constant evaluation of immune competence is required.

There have been several techniques used for immune monitoring in SOT, mainly focusing on graft rejection measuring circulating donor-specific antibodies (DSA) or donor-derived cell-free DNA (Hachem *et al*., 2010; Oellerich *et al*., 2021; Roux *et al*., 2019). There are other specific methods used that depend on the case being treated, like kidney biopsies in kidney transplant patients, a common and gold-standard but invasive method used for identifying allograft rejection; or detecting a rise in serum creatinine or proteinuria as a less specific and less invasive method (Han & Lubetzky, 2023; Laroche & Engen, 2024). Those techniques are not adequate for predicting excess or lack of immunosuppression, useful in the regulation of immunosuppressors or in the development of latent viruses' reactivations.

Levels of CD4+ T-cells along with HIV viral load measurements through qPCR are the main techniques used for monitoring the immune status of HIV patients. Monitoring immune recovery during antiretroviral treatments facilitates the personalization of the treatment for each case treated, something that has been studied using TTV plasma level to predict the course of the recovery (Tarancon-Diez *et al*., 2024).

An important aspect to consider when talking about immunocompromised patients is viral monitoring, since viral infections are a common complication in these individuals. HCMV monitoring is one of the most studied as its infection is one of the most common causes of viral clinical presentations in immunocompromised patients (Atabani *et al*., 2012; Deayton *et al*., 2004). qPCR in blood has been commonly used as the best option for HCMV diagnostic and monitoring (Kotton *et al*., 2018). For measuring specific immunity against HCMV, an enzyme-linked immunosorbent assay has been developed, QuantiFERON-CMV. It evaluates the capability of the patient to respond against HCMV infection via measuring interferon- γ (INF- γ) produced by T-cells in response to HCMV antigens in blood. However, it is not used in clinical routine diagnostic procedures due to its poorly defined thresholds for interpreting results (Kumar & Humar, 2020; Limaye *et al*., 2020; Walker *et al*., 2007).

Therefore, when it comes to immunocompromised patients and their viral determination, the qPCR is the preferred method. As stated before, in HIV patients it can be seen an example of this, as its viral determination is commonly measured by the qPCR assay in blood samples, with several studies assessing the different quantification assays (Borrego *et al*., 2017; Damond *et al*., 2008). Different viruses have been studied to monitor patients' response to transplantation, since their reactivation can cause infections associated with immunosuppression. One example is the polyomavirus BK virus, responsible for nephropathy and common among renal transplant patients where a qPCR in plasma and urine samples is used to monitor its replication (Mischitelli *et al*., 2007). Another example is monitoring a group of three viruses (BK virus, Epstein-Barr virus and HCMV) in candidates for transplant surgeries using a multiplex qPCR (Hwang *et al*., 2018). TTV

has been studied as a potential immunological state's marker, as its detectable replication in blood does not imply pathological or clinical diseases, in contrast with the previous viruses. However, TTV viral load determination via qPCR for predicting immune recovery has been studied, finding concordance with CD4 T-cell levels and the patient's disease state (Tarancon-Diez *et al*., 2024). Furthermore, in kidney transplantation, the importance of TTV as a possible guideline in the regulation of immunosuppressive medication was treated, finding a strong association between TTV load in blood (measured via qPCR) and graft rejection (van Rijn *et al*., 2021). Another study analyzes TTV loads in relation to immune recovery after hematopoietic stem cell transplantations and found it to be correlated to opportunistic infections (Gilles *et al*., 2017).

The main kind of sample used in the prior studies is blood sample, because of its reproducibility and accessibility. However, blood is a scarce resource in pediatric or neonatal patients, to whom blood sampling could not exceed 3% of the total blood volume in a period of 4 weeks and never exceeding 1% on a single withdrawal (EMA, 2009). Besides, in anemia patients the importance of blood conservation rises, and blood sampling could worsen the pathology (Salisbury & Kosiborod, 2012). In consequence, alternative samples were studied to replace whole blood or plasma sampling such as a new use of dried blood spots in drug studies (Patel *et al*., 2010) or the evaluation of microneedle arrays, 20 000 tiny projections capable of extracting blood without bleed nor pain, prioritizing children's perspective on traditional needles (Bhargav *et al*., 2012; Kaushik *et al*., 2001; Mooney *et al*., 2014). Though scarce literature can be found, in one study the use of qPCR in fecal samples as diagnostic method for HCMV intestinal disease was performed in contrast with the more invasive actual method, via tissue biopsies. The study reports that finding HCMV DNA in feces is poorly correlated to HCMV detection in the tissue biopsy (Ganzenmueller *et al*., 2014a; Reddy & Wilcox, 2007). Nevertheless, more in-depth studies for viral detection through fecal or rectal samples are needed as a possible alternative to blood sampling.

The importance of TTV resides in its potential as an immunological marker in less invasive and poorly studied samples such as rectal swabs. Therefore, the capability to detect this virus in this type of specimen is one of the key points towards a deeper understanding of its clinical relevance.

Objectives

- To determine Torque Teno Virus' presence in rectal swabs.
- To correlate Torque Teno Virus' presence with previous or current Cytomegalovirus' infection.
- To determine whether qPCR is adequate for the detection of TTV in rectal swabs.

Methodology

To achieve the objectives of this study, we used two principal techniques, DNA extraction via magnetic beads and quantitative PCR in a series of rectal samples obtained from selected patients.

Clinical samples

One hundred and forty-three patients were included in the study. The selected samples were from rectal swabs used in epidemiological studies of the Hospital Universitario La Paz (HULP), as part of the routine surveillance procedures performed at the hospital. The selection criteria were based on whether a HCMV diagnostic technique (quantitative and qualitative) was performed for the respective patients during the past 2 years. Both PCR, targeting HCMV's viral load in blood (quantitative), and ELISA, detecting HCMV's specific IgG (qualitative), from blood samples were evaluated. This was done via an institutional platform (Microb®) where the medical data related to microorganisms' diagnostic techniques performed by various sections of La Paz healthcare system is recorded. Any value corresponding to HCMV viral load recorded in Microb® was considered as positive, as well as for any value corresponding to HCMV's specific IgG recorded from ELISA. Samples that were negative and samples that were positive were selected for the study.

Moreover, patients' age, sex, type of sample and ward were recorded. Rectal swabs from these patients were reused after the routine clinical epidemiology tests were performed by the hospital's microbiology department. The collected swabs' contents were dissolved in Tris-EDTA (1x) (TE) and conserved in a temperature of 0-4ºC until DNA extraction, which was performed less than 3 days after the collection the samples.

DNA extraction

DNA extraction first required a dilution of 1:10 in distilled water of the rectal sample dissolved in TE in a final volume of 1ml. The reason for this dilution was to prevent PCR inactivation due to excess in genetic material. From this dilution, 100 µl were separated to follow the next processes, beginning with the lysis of the cellular membrane through a thermal shock at 95ºC for 10 minutes. This was followed by a mechanical lysis using the Omni International™ Bead Ruptor Elite™ Bead Mill Homogenizer which uses 2 ml tubes containing ceramic beads (MagMAX™ Microbiome tubes). This lyses helps in breaking down cell walls through a specific protocol of two cycles of 4.2 m/s for one minute each and 15 seconds of rest between cycles.

DNA extraction was performed using the MagCore® HF16 Automated Nucleic Acid Extractor with MagCore® Viral Nucleic Acid Extraction Kit using a predetermined program for whole blood DNA extraction, ending up with a 60 µl of purified DNA elution from a 500 µl sample volume. This program is designed to purify DNA through a first chemical lysis with chaotropic salt (lysis buffer, 500 µl), then the addition of magnetics beads and binding buffer. This leads the

negatively charged DNA to bind to the beads to get rid of the debris resulting from the lyses through two washes with 70% ethanol, and elimination of the supernatants. After this, DNA is eluted due to a low salt elution buffer (DNAase-free or ultrapure H2O) that breaks the electrostatic bonds between the purified DNA and the magnetic beads. This entire extraction takes 58 minutes.

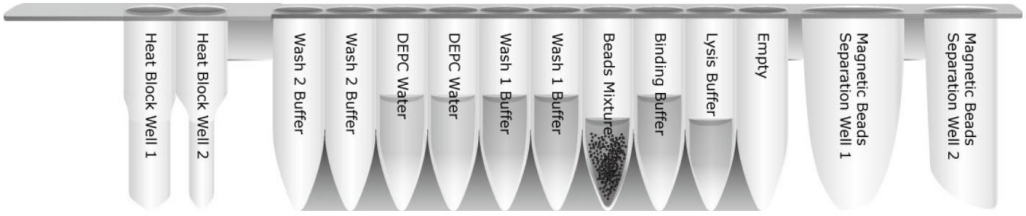

Figure 3. MagCore® Viral Nucleic Acid Extraction Kit. (From RBCBioscience, n.d.)

All the purified nucleic acids were preserved at -20ºC until the procedure of qPCR.

qPCR

The samples conserved at -20ºC needed 30 minutes at a constant temperature of 4ºC in ice-containing racks to defrost without damaging the nucleic acids. The primers used for the qPCR (Table 1), were dissolved in distilled water following manufacturer's instructions in order to get a concentration of 100 µM. This was considered as the stock dilution, from which a 1:10 dilution in distilled water was performed and the reverse and forward primers were mixed. This serial dilution resulted in a final concentration of 1 µM for each reaction of qPCR according to the concentrations prepared in the master mix.

Table 1. Primers' sequence

Primers	GC%	Sequence 5' ➡ 3'
TTV-Forward	50	GTGCCGIAGGTGAGTTTA
TTV-Reverse	78.5	AGCCCGGCCAGTCC

PowerUp™ SYBR™ Green Master Mix from Sigma Aldrich was used, which is a mix containing dNTPs, $MgCl_2$ as the polymerase cofactor, a Taq polymerase, SYBR Green I colorant and a stabilizing pH buffer.

For each sample, the proportions were 10 µl of 2 X PowerUp™ SYBR™ Green Master Mix, 1 µl of the mix of primers and 7 µl of distilled water to complete a final volume of 18 µl of this master mix for each reaction. At the moment of preparing this master mix, the pipetting error was always considered by adding at least two more units in the calculations over the real number of samples. Along with the 18 µl from the master mix, 2 µl of sample was disposed for each reaction, ending up with a final volume for reaction of 20 µl.

Table 2. Table of contents and their proportions in the protocol of qPCR

Content	Per sample	Para 16 (18 para error)
SYBR Green	10 µl	180 µl
Primers	1 µl	18 µl
H20	7 µl	126 µl
PCR (20 µl)		
Master Mix	18 µl	
Sample	2 µl	

Plasma samples positive for different viruses (Adenovirus, HCMV…) were tested for TTV using the same methodology described before. A positive control was selected from this group of samples with a Ct of 23.77, to ensure the reproducibility and efficacy between all qPCR reactions carried out during the study.

The qPCR thermal cycler used was BIO-RAD CFX Connect™ Real Time System, with a protocol of 45 cycles with the following temperatures for the different stages in the polymerase chain reaction:

- 95 ºC for denaturalization.
- 58ºC for annealing the primers to the template chain.
- 72ºC for the elongation of the new DNA chain.

The reactions were prepared in 8-strip opaque tubes with translucent 8-strip lids to minimize interference with reading the fluorescence.

Another parameter measured by the thermocycler was the melting curve, at the end of the qPCR. This was done by increasing the temperatures from 65ºC to 85ºC while reading the fluorescent signal after every increase of 0.5ºC.

Statistical analysis

A chi-square test of independence was performed to assess a potential association between the presence of presence and HCMV status in the selected samples. TTV's qPCR results and recollected data related to HCMV status were used to create a contingency table to conduct the analysis. Chi-square was executed through Python programming language (3.13.2 version) within the development environment PyCharm Community Edition (JetBrains®). "spicy.stats", a module for statistical analyses was used. Within this module, the "chi2_contingency()" function was employed for the Chi-squared test. A 95% confidence interval (p-value < 0.05) was used to ensure statistical and scientific robustness, along with 1 degree of freedom (= (number of columns-1) x (number of rows-1)) according to the contingency table (Table 4. Contingency table for TTV and HCMV's observed frequencies). If the observed frequencies differ significantly from the expected values of the null hypothesis (no association between TTV and HCMV status) with p-values of < 0.05, the null hypothesis was rejected.

For visual purposes, data from the contingency table were displayed in a stacked bar chart designed with Python using the module pyplot from the package matplotlib to display images from plotting data.

Chronology

Table 3. Chronology table

Period	Task developed
Weeks 1-2	Familiarization to the laboratory environment
Week 3	Selection of samples for the study using Microb
Week 4	First trials with TTV primers to ensure their functionality
Week 5	Optimization of qPCR and DNA extraction procedures
Weeks 6-9	Production of qPCR results
Week 10	Data curation and descriptive analyses
Week 11-15	Development of the theoretical framework, discussion and statistical analyses of the data obtained.

Results

A total of 143 rectal samples (102 (71.32%) were positive and 41 (28.67%) were negative for HCMV) from patients receiving treatment at the Hospital Universitario La Paz were included in this study. Sixteen (11.18%) of the positive results were quantitative, and 127 (88.81%) were qualitative.

The most common sections from which the swabs were received were the nursing unit (51, 35.66%), neonatal and pediatric intensive care units (18, 12.58%), and intensive care unit (16, 11.18%).

Within the data recorded from the patients, sex in the sample was distributed as 93 males (65.03%) and 50 females (34.96%). Fifty-three were under the age of 18 (37.06%) while 90 were above the age of majority (62.93%), overall age had a very diverse distribution (mean = 40.44; standard deviation = 30.51; maximum = 87; minimum = 0; median = 46). Results recorded in the database for HCMV diagnostic tests carried out in patients from the sample (both quantitative and qualitative) were 102 (71.32%) for positive results and 43 for negative ones (28.67%). It is important to highlight the proportion between quantitative results, 16 (11.18%), and qualitative ones, 127 (88.81%).

qPCR was performed with TTV's primers on all these samples, resulting in 31 (27.67%) in which TTV was identified with Ct values between 24.81-40.78 (mean = 34.79; standard deviation =

4.42; maximum = 40.78; minimum = 24.81). TTV's prevalence in these samples was 21.7%, discarding two false positive results that had a melting temperature differing in over two degrees Celsius compared to the positive control. Among the HCMV positive samples, 22 (21.15%) of them were conclusively positive for TTV, while among the HCMV negative ones there were 9 (21.95%) TTV positive results. Figure 4 shows a representation of some samples where TTV was amplified.

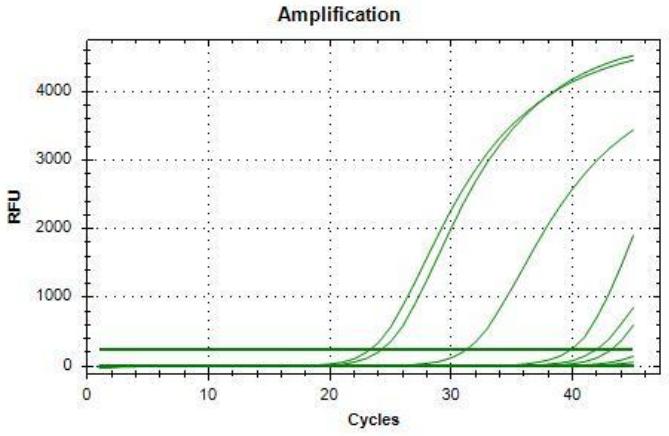

Figure 4. TTV targeted qPCR curves obtained from HCMV positive patients' rectal swabs

To determine a significative association between TTV's presence and HCMV's status a contingency table was constructed through the frequencies of the results obtained for both organisms (Table 4).

Table 4. Contingency table for TTV and HCMV's observed frequencies

	CMV+	CMV-	TOTAL
TTV+	22	9	31
TTV-	80	32	112
TOTAL	102	41	143

A chi-square test of independence was conducted and had a chi-square statistic of 0.0016 and a p-value of 0.967. Therefore, H0 (that the difference in results is due to chance) could not be rejected, leading to no significant association between TTV in rectal swabs and HCMV in blood samples in the set of swabs that were included in this study.

These results are illustrated in a stacked bar chart confirming no visual differences of samples positive for HCMV and those negatives for this virus in samples that were positive for TTV and those that were negative. Although having more samples analyzed for HCMV positive samples, the same proportion of TTV positive results in HCMV's positive samples can be seen in the chart, as well as in HCMV's negative ones. Proportionally, TTV positive results in HCMV positive samples represent the same space in the chart, according to the results obtained (21.15% and 21.95%, respectively).

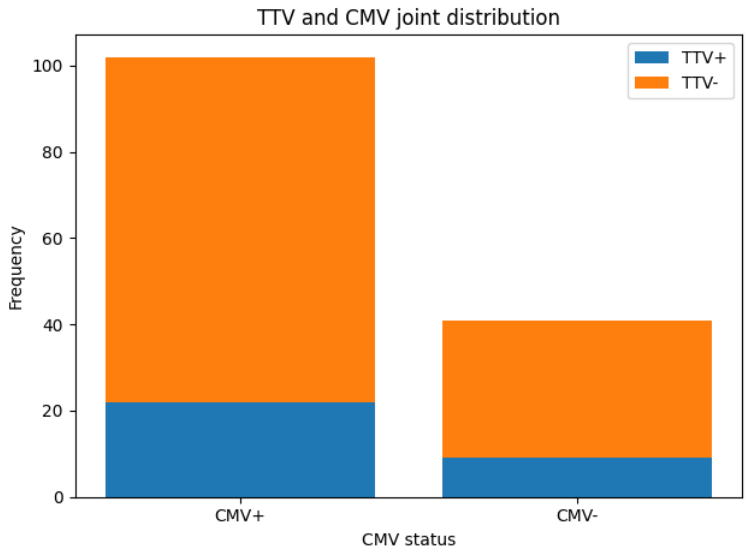

Figure 5. TTV results' proportion related to HCMV status from the same patients

Discussion

The development of a specimen alternative to whole blood or plasma samples in immune monitoring is of vital importance for the management of immunocompromised cases such as SOT or HIV patients. TTV as an important tool in immune monitoring has been studied in recent studies as well as its correlation with HCMV load in the same cases. In this study, TTV detectability through qPCR in rectal swabs and its correlation with HCMV presence in blood have been studied.

TTV's presence was determined in 31 out of the 143 samples (21.6%) selected from rectal swabs. In contrast, in other studies using blood samples a greater proportion of TTV detection in relation to HCMV was observed. For instance, TTV in blood samples in different cohorts of patients showed a range of prevalence between 60-80% (Focosi *et al*., 2020; Parreira *et al*., 2004; Wohlfarth *et al*., 2018) and up to 99.4% in patients undergoing hemodialysis (Rinonce *et al*., 2017). A 91.1% prevalence of TTV in fecal samples was detected in gastroenteritis patients combining the results from distinct qPCR methodologies (Pinho-Nascimento *et al*., 2011). In other study, a 52.7% detection in cervical smears along with 50% in serum samples from healthy women were detected, showing similar results in both types of samples (Saláková *et al*., 2009). The type of sample, different PCR procedures, study design or different primers' targets in the genome can be the reasons for the variability in the results of detecting TTV. Results obtained in this current study suggest rectal swabs as a possible alternative to blood-based sampling for detecting TTV. However, to properly assess significant correlation in the capacity to detect TTV in rectal swabs, the same needs to be done for blood samples from the same patients, which is beyond the scope of this study. Nevertheless, the fact that this virus is detected in these samples shows a promising avenue of research for future studies.

Other studies comparing viral detection between fecal and blood samples for other viruses showed interesting results. For example, the monkeypox virus was determined in rectal swabs and whole blood samples using the same qPCR with 100% agreement (Mills *et al*., 2023). Additionally, a good correlation between HCMV viraemia or active infection and detection of HCMV in fecal samples through qPCR was found in a study (Ganzenmueller *et al*., 2014). Along with this study, other fecal-related samples have been studied as an alternative to blood-related samples, suggesting a possible future research line of TTV detection in rectal swabs as an alternative to any blood sampling for immune monitoring SOT, hematological transplant or HIV patients. However, as stated before, further studies need to be accomplished addressing this specific matter, considering viral load agreement as well.

On the other hand, qPCR as the methodology of choice to determine TTV presence has been discussed in other studies. A study on 22 serum samples from children with leukemia aimed at comparing qPCR with shotgun metagenomic sequencing for detection of TTV was performed (Leijonhufvud *et al*., 2022). In that study, TTV reads (via Illumina in cDNA and DNA whole-genome libraries) were found in serum samples having negative qPCR results. In addition, sequencing TTV resulted in the identification of various strains and mutations in the priming sites, possible reasons of the lower efficacy of the detection via qPCR. However, the complex procedure and laborious bioinformatic analysis of the shotgun metagenomic sequencing in contrast with the simple, fast and low-cost pipeline of qPCR make it difficult to replace it as the most promising detection method of TTV. A better correlation between TTV viral load calculations from metagenomic next generation sequencing (mNGS) and qPCR viral load obtained is suggested by another study, but with semiquantitative TTV results due to a lack of information from the database used for calibration curves (Carbo *et al*., 2022). Finally, 83% agreement between single-target qPCR assays and a shotgun mNGS platform was found for TTV detection, but with discrepancies in TTV quantification (Shah *et al*., 2022). Although qPCR remains the most accessible option for TTV detection and possible immune monitoring through this procedure, mNGS and other sequencing tools give an interesting and, possibly, more accurate point of view for characterization and detection of TTV. Nevertheless, due to these techniques being expensive and laborious, they might not be easily implemented in all diagnostic laboratories.

No significant correlation between the presence of TTV in rectal swabs and detection of HCMV in blood samples within the collection included in this study was found. Conversely, a strong correlation in plasma viral loads from both viruses in hematological (mainly allogenic) and solid transplant patients was demonstrated (Roberto *et al*., 2024). TTV loads were found to increase over time in patients to whom HCMV tested negative at first evaluation but had a later reactivation/infection of the virus. Moreover, a strong association between peak HCMV loads and TTV viral loads was found, but only in SOT patients. This was not observed among hematological

patients. No correlation between the detection of HCMV and TTV in plasma among HIV patients was detected, in line with previous research on hematological transplant patients (Schmidt *et al*., 2021). In another study, similar high rates of HCMV and TTV detection in plasma were found in a large cohort of hematological patients, specifically in the post-transplant population, suggesting a similar role played by both in immunocompromised patients (Ma *et al*., 2024). Several contradictions between different studies about the correlation between the increase in HCMV viral load and the following increase in TTV levels have been identified. Generally speaking, in SOT patients, to whom continuous immunosuppressive treatment is administered for a long period of time results in high TTV and HCMV loads, whereas this relationship does not seem to exist among hematological transplant nor HIV patients. This might be due to the lower degree of immunosuppression among the latter type of patients as compared to SOT patients, but this requires further studies on large cohorts to be verified.

This study is aligned with the 2030 Agenda for Sustainable Development adopted by the United Nations in 2015 and is part of some of their established goals (Sustainable Development Goals, SDGs). Namely, the third goal (Good Health and Well-Being) that aims at ensuring healthy lives and promoting well-being for people at all ages aligns with this study. This is because the study of a possible biological marker for immune monitoring (such as TTV) supports the idea of an improvement in healthcare as it encourages further investigations into this matter, raising awareness in the research of other, less invasive, alternatives to blood sampling. As this study was developed in collaboration with a public healthcare institution (IdiPaz-HULP), its results, as well as future applications, are in line with the idea of a cutting-edge public healthcare system as the goal 3 remarks. The promotion of industry, innovation and infrastructure (9th objective) is supported too as one of the SDGs. This is also in line with this study as it contributes to increasing and developing the scientific investigation via innovative plans with a future prospect. Finally, quality education (4th goal) was promoted via the development of competences needed in a scientific environment, with the purpose of encouraging an adequate growth of up-coming investigators (*i.e.* the student performing this study and the lab members who were informed of it).

Conclusions

- TTV can be detected via qPCR in rectal swabs.
- Correlation between TTV and HCMV was not detected in this study but might need further studies to determine a clearer perspective of the relationship between these 2 viruses.
- qPCR assessment in the detection of TTV in rectal swabs needs investigation by further studies that include comparisons with blood samples.

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Title: DETERMINATION OF TORQUE TENO VIRUS' INTESTINAL LOADS IN IMMUNOSUPPRESSED PATIENTS

This project was developed in Instituto de Investigación Hospital Universitario La Paz, grupo de Microbiología.

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