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VALIDATION OF A COMMERCIAL REAL-TIME QUANTITATIVE PCR ASSAY FOR DETECTION OF CMV VIRAL LOAD IN PLASMA OF TRANSPLANTATION RECIPIENTS AND ASSESSMENT OF WHO CMV INTERNATIONAL PANEL FOR IN-HOUSE CMV ASSAY CALIBRATION

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STUDY BACKGROUND AND AIM

Monitoring cytomegalovirus (CMV) viral load in the plasma provides essential information for the management of CMV disease in post-transplant patients. An in house real time PCR assay (LOD-QPCR) has been implemented at ProvLab for detection of CMV viral load in plasma (reporting in copies/ml) since 2003. Several commercial assays are now available for measuring CMV viral load with improved QC. The variability between the molecular methods employed, and the lack of traceability to a reference system, make it difficult to compare assay performance and to develop uniform treatment strategies. The first World Health Organization (WHO) International Standard for human cytomegalovirus (HCMV), NIBSC code 09/162, is now available to be used in the standardization of nucleic acid amplification technique (NAT)-based assays for HCMV. The purposes of this study were:

- to validate the RealStar™ CMV QPCR Kit 1.0 (Altona Diagnostics) against the LOD-QPCR assay
- to use the WHO CMV international standard (IU/ml) to assess the LOD-QPCR results for calibration of CMV viral load (VL).

METHODS

- Sample preparation: Plasma (PL) samples from organ transplant recipients participating in the ProvLab's CMV monitoring program were included. Other samples were virus suspensions from cell culture, or in BaseMatrix™ (SeraCare), or reconstituted WHO HCMV 09/162 as per the product insert.
- DNA Extraction: Viral DNA was extracted from 200ul sample and was eluted in 100ul using the QIAcube with the Qiagen blood mini kit according to the manufacture's instructions.
- Quantitation of viral load: LOD-QPCR with hybridization probes was performed using the LightCycler™ 1.0/2.0 (Roche) and the commercially available RealStar™ QPCR (Altona Diagnostics) using hydrolysis probes was performed on a 7500 platform (Applied Biosystems).

- **Objective 1: RealStar™ Validation against LOD-QPCR**
  1. To determine the linearity, a series of 10-fold dilutions of CMV Merlin strain (Sample 1), AD169 strain (Sample 2) and a clinical PL sample (Sample 3) were sent to the two molecular diagnostic labs in ProvLab and run on both assays.
  2. The specificity of each assay was confirmed with Epstein-Barr virus (EBV), varicella-zoster virus (VZV), herpes simplex virus (HSV), BK virus, and adenovirus. The DNA of each virus was run in duplicate. Both assays were specific and no cross-reaction was observed between CMV and the other viruses (Data not shown).
  3. To investigate assay to assay correlation and to look at inter-lab and inter-instrument variation, 50 DNA PL specimens were run using both assays at both ProvLab sites.
  4. Precision determination was performed using 3 DNA samples run in triplicate over 5 days using the same instruments.
  5. To determine the accuracy of the quantitation, 100 DNA PL specimens were run using both assays at both molecular diagnostic labs in ProvLab.

- **Objective 2: Calibration of LOD-QPCR with the WHO CMV International Standard**
  This objective was performed in two steps:
  1. For the calibration of the in-house assay, a serial 10-fold dilution using BaseMatrix™ as a substitute for a PL specimen was performed on the WHO HCMV and extracted as above. The extracts were run in triplicate over 3 days using the LOD-QPCR assay.
  2. For the conversion of the LOD-QPCR to the commercial RealStar™ QPCR, serial 10-fold dilutions were performed on extracts: WHO HCMV that was reconstituted as per the product insert, a clinical PL specimen of high VL, and AD169 strain from cell culture. Each DNA dilution series was run in triplicate with each assay.

Acknowledgements

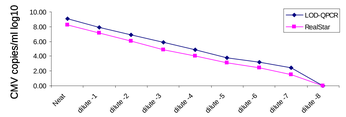
We thank all the staff in the molecular diagnostic laboratory, ProvLab for all their contributions for this study.

RESULTS AND DISCUSSION

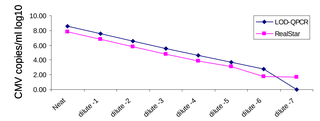
OBJECTIVE 1: RealStarTM Validation against LOD-QPCR

- Comparable limit and linearity of CMV detection were observed between LOD-QPCR and RealStar™ QPCR. The variation was greater for both assays when the VL was low. The VL for the LOD-QPCR was greater in absolute number than the RealStar™ QPCR. However, the RealStar™ QPCR assay was more sensitive and detected approximately 1.0 log more than the LOD-QPCR.

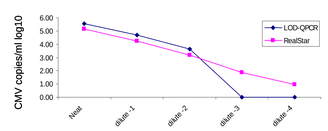
Sample 1



Sample 2

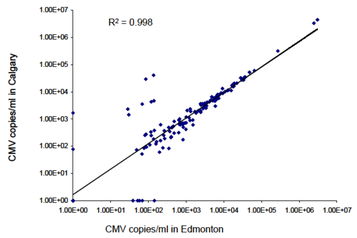


Sample 3

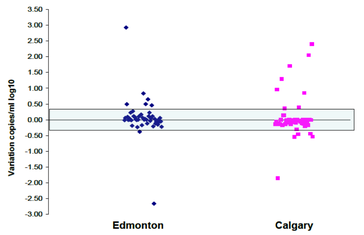


- Inter-laboratories (A:) and –instruments (B:) variations were evaluated using 50 DNA samples. The correlation between LOD-QPCR and RealStar™ QPCR was R^2=0.99. Increased detection of low VL samples was achieved with the RealStar™ QPCR assay compared to the LOD-QPCR assay. VL levels +/- 0.5 $\log_{10}$ was considered as an acceptable standard of variation in clinical samples. The greatest variation of quantitative results was seen with low VL.

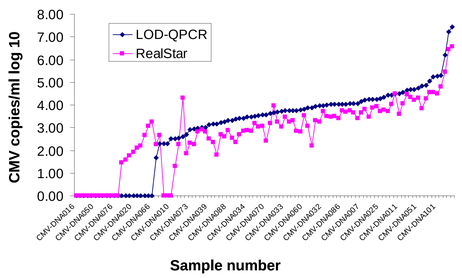
A: Correlation of the CMV VL using the Commercial QPCR between two sites



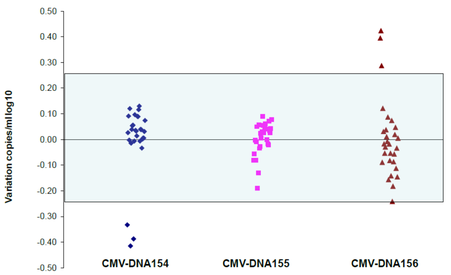
B: Variation between two ABI 7500 instruments relative to expected value assigned as 0



- 100 DNA samples extracted from clinical samples with different VLs were used for comparison of detection and quantification. The 10.6% discordance showed that the RealStar™ QPCR assay was more sensitive compared to the LOD-QPCR assay. The most significant variation with both assays was observed with low level VLs 2.07-2.50 copies/ml $\log_{10}$. The mean difference in VL between the two assays was 0.52 ± 0.40 copies/ml $\log_{10}$. The difference in quantification values was considered to be the result of the use of different calibrators used in each assay. With the new WHO CMV International Standard, this could now be addressed.



- The precision was evaluated using three DNA samples tested in triplicate over five different days using the same instrument to minimize inter-instrument variation. A precision of CV = 1.44 ± 0.55 % and a narrow variation of inter-laboratories and instruments were observed in RealStar™ QPCR.



OBJECTIVE 2: Calibration of LOD-QPCR with the WHO CMV International Standard

- Using WHO HCMV (IU/ml) that was diluted in BaseMatrix™ and extracted, the calibration of the LOD-QPCR (copies/ml) was determined as 5.65 copies/ml to 1 IU/ml.

CMV LOD-QPCR on the LightCycler

Expected	Actual	
WHO in BM IU / ml	Mean	SD
1.00E+06	6.33E+06	1.45E+06
1.00E+05	5.63E+05	1.11E+05
1.00E+04	5.09E+04	4.52E+03
1.00E+03	5.57E+03	1.68E+03
1.00E+02	9.13E+02	6.65E+02
1.00E+01	----	----
1.00E+00	----	----

- A direct comparison of the two assays using the WHO HCMV reconstituted as per the product insert and extracted, AD169 DNA, and extracted clinical PL samples confirmed an overall conversion of LOD-QPCR/RealStar™ by a factor of about 2-fold. Note that the LOD-QPCR primers/probe set match the sequence in AD169 so there is a higher efficiency with this strain. After parallel testing with clinical PL samples for a month, the discordant results all originated from samples with low VL.

LOD-QPCR			RealStar™	
Mean Copies/ml	SD	WHO IU/ml	Mean IU/ml	SD
2.52E+07	1.18E+06	$5x10^6$ IU/ml	1.58E+07	2.38E+06
3.77E+06	2.60E+05	$5x10^5$ IU/ml	1.09E+06	1.67E+05
4.87E+05	1.54E+04	$5x10^4$ IU/ml	1.64E+05	5.20E+03
5.21E+04	8.62E+03	$5x10^3$ IU/ml	1.81E+04	2.87E+03
4.85E+03	1.61E+03	$5x10^2$ IU/ml	2.12E+03	1.03E+03
		Specimen		
3.55E+06	9.10E+04	Neat	3.07E+06	2.71E+05
3.44E+05	7.85E+03	Diln -1	2.11E+05	2.79E+04
3.03E+04	2.80E+03	Diln -2	3.37E+04	8.22E+03
4.03E+03	2.42E+03	Diln -3	5.08E+03	6.71E+02
		Diln -4	2.56E+02	1.17E+02
		AD169		
5.82E+06	9.74E+05	Neat	2.28E+06	1.68E+05
6.60E+05	7.00E+04	Diln -1	2.66E+05	1.47E+04
7.48E+04	6.51E+03	Diln -2	3.17E+04	3.64E+03
7.70E+03	9.84E+02	Diln -3	3.45E+03	8.40E+02

CONCLUSIONS

The precision, sensitivity, linearity and the use of an internal control make the RealStar™ QPCR a very good alternative assay for monitoring CMV VL in post-transplant patients. However, even with a commercial kit, quality control and monitoring are still essential as any introduced error will have a significant effect. A WHO international reference standard for CMV VL assay calibration is an important step in quality improvement and inter-lab communication. In our lab, the LOD-QPCR calibration is 5.65 copies/ml to 1 IU/ml. The conversion factor of LOD-QPCR to RealStar™ assay was approximately 2-fold.