Multicenter Comparison of Current Practices in CMV and EBV Viral Load Testing using WHO International Standards

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Running Title

CMV and EBV Viral Load Testing using WHO Standards

Keywords

Cytomegalovirus, CMV, Epstein-Barr virus, EBV, viral load, quantitative nucleic acid testing, WHO

Abbreviations

Cytomegalovirus – CMV

Epstein-Barr virus – EBV

Real-Time quantitative polymerase chain reaction - qPCR

Clinical Trials in Organ Transplant - CTOT

Intermediate Early – IE

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Abstract

Infections with Cytomegalovirus (CMV) and Epstein Barr Virus (EBV) remain important sources of morbidity in solid organ transplantation. Quantitative viral nucleic acid testing has been a major advance in management of these patients. A limitation of these assays has been that they are not standardized, resulting in viral load measurements that can differ significantly among clinical laboratories. Furthermore, the inherent variability in viral load measurements makes interpretation of clinical trials data difficult. This study was designed to compare the current practices in CMV and EBV viral load testing at four large transplant centers participating in multicenter Clinical Trials in Organ Transplantation (CTOT/CTOTC). Viral load testing was performed on well-defined viral preparations according to standard operating procedures at each site. Among the different centers, CMV viral load testing was accurate compared to the WHO International Standard and within the acceptable degree of variation for this testing method. EBV viral load data were more variable and less accurate. These data suggest that comparison of CMV measurements, but not EBV viral load data, at these study sites is possible using the current assays. Standardizaton of these assays is becoming increasingly easier with the recently released WHO International Standards and will likely allow for comparison of viral load results among transplant centers.

Introduction

In transplant recipients, viral load testing has become the primary modality for diagnosing active disease due to Cytomegalovirus (CMV) and Epstein Barr Virus (EBV) infections and monitoring response to therapy [1-7]. Using whole blood or plasma, viral nucleic acid is extracted and subjected to amplification using quantitative, real-time polymerase chain reaction (qPCR) based assays. The number of viral copies present in the initial sample is determined by comparison to a set of standards with known copy number. Although the methodologies are similar from one laboratory to another, the reagents, extraction method, primers, and amplification platform may vary from one clinical laboratory to another [8-14]. Until recently, there were no standardized and well characterized viral preparations to assess the accuracy of these assays. Thus, laboratories have developed and validated testing protocols using calibrators that may or may not be equivalent. Previous studies have demonstrated that this lack of standardization has made the comparison of viral load results among laboratories problematic [10, 11].

Quantitative viral load testing for CMV and EBV provides a method to assess the intensity of immunosuppression and protocol safety in the setting of clinical trials, notably for studies of new immunosuppressive regimens or antiviral therapies. Given the lack of assay standardization, the interpretation of study data in terms of development of viral load cutoffs to predict tissue-invasive CMV infection or EBV-associated post-transplant lymphoproliferative disorders, and for diagnostic and therapeutic interventions, has been challenging. Recently the World Heath Organization (WHO) developed standards for both CMV and EBV [15, 16]. These viral preparations are intended to be used by laboratories

and manufacturers to calibrate secondary reference materials, such that the concentration of virus in a sample can be expressed in international units and compared. The Clinical Trials in Organ Transplantation (CTOT) and the Clinical Trials in Organ Transplantation in Children (CTOT-C) are research consortia sponsored by the National Institute of Allergy and Infectious Diseases (NIAID) that conduct clinical trials and associated mechanistic studies to improve outcomes in adult and pediatric organ transplantation. Given that viral monitoring is a routine component in the care of transplant recipients, this study was designed to compare the accuracy and variability of CMV and EBV viral load testing using the WHO viral standards at four of the CTOT transplantation centers.

Materials and Methods

Study Design

CMV and EBV viral load testing was performed at four independent clinical laboratories from academic medical centers that are members of the Clinical Trials in Organ Transplant (CTOT) Mechanistic Studies Working Group (Cleveland Clinic, Emory Transplant Center, Massachusetts General Hospital, and Washington University School of Medicine). Each laboratory performed the assays according to center-specific standard operating procedures. All samples were tested blindly. Results were reported in copies per milliliter (ml). The procedural characteristics of the assay(s) used at each site are shown in Table 1 and described below. One site used two separate protocols for testing.

Quantitation Panels

For each virus and study site, one commercial panel and one panel created from the WHO International Standards were tested. These panels were chosen to represent the control materials that were available and in use for verifying CMV and EBV test systems. They also allow the entire process from nucleic acid extraction to quantitation to be assessed since they consist of viral particles suspended in a plasma matrix. The CMV commercial panel consisted of a single replicate of five members from the OptiQuant CMV_{tc} Panel from Acrometrix (Life Technologies, Grand Island, NY) and a negative control consisting of dialyzed, defibrinated human plasma (BaseMatrix; SeraCare, Milford, MA). The OptiQuant CMV_{tc} panel consists of human plasma containing CMV strain AD169. The manufacturer specified that the concentration of CMV DNA in each of the panel members was 500, 5000, 50,000 and 500,000 copies/ml. The CMV International Standard panel consisted of triplicate ten fold serial dilutions of the 1st WHO International Standard for Human Cytomegalovirus obtained from NIBSC (code 09/162; Hertfordshire, England). This preparation consists of lyophilized CMV Merlin strain and was assigned a potency of $5x10^6$ IU/ml based on a worldwide collaborative evaluation in which the consensus value was $5x10^6$ copies/ml [15]. The lyophilized standard was reconstituted in 1 ml of nuclease free water and dilutions were prepared in dialyzed, defibrinated human plasma (BaseMatrix; SeraCare, Milford, MA) to achieve panel members spanning 50 to 500,000 copies/ml. A negative control consisting of BaseMatrix alone was also included.

The EBV commercial panel consisted of a single replicate of all six members of the OptiQuant EBV Plasma Panel obtained from Acrometrix (now part of Life Technologies, Grand Island, NY). This panel includes human plasma containing EBV ranging in

concentration from 1000 to 10,000,000 copies/ml and a negative control. The EBV International Standard panel consisted of triplicate ten fold serial dilutions of the 1st WHO International Standard for EBV obtained from NIBSC (code 09/260; Hertfordshire, England). This preparation contains lyophilized EBV strain B95-8 and was assigned a potency of $5x10^6$ IU/ml based on a worldwide collaborative evaluation in which the consensus value was $5x10^6$ copies/ml [16]. This standard was prepared in the same manner as the CMV International Standard and included panel members spanning 10 to 1,000,000 copies/ml and a negative control.

For all four panels, each panel member was assigned a code and relabeled to anonymize the source and remove any indication of the expected copy number. The quantitation panels were frozen at -80˚C and shipped overnight on dry ice to each study site.

Viral Load Assays

Each laboratory performed CMV and EBV viral load testing on each of the panels as per institutional standard protocols for plasma as indicated in Table 1. One site performed two separate assays for both CMV and EBV testing including one standard and one multiplex assay system.

All of the extraction protocols involve sample lysis under denaturing conditions in the presence of protease. For protocols 2 and 4 the initial sample volume was 200ul. For protocol 1 it was 263ul. For protocol 3 it was 500ul for CMV and 1000ul for EBV. For protocol 5 it was 200ul. In protocols 1, 2, and 4, nucleic acids are adsorbed onto a silica

membrane, washed, and eluted into buffer. In protocols 3 and 5, magnetic beads rather than a silica membrane are used. The exact makeup of the reagent buffers is proprietary, but differs among the protocols. The elution volumes for protocols 3 and 4 were 60ul. For protocol 1, 2, and 5 the elution volume was 83ul, 50ul, and 100ul respectively.

The amplification and detection protocols are all based on real-time quantitative detection using fluorescent dyes linked to oligonucleotide probes. Protocols 1, 2, and 3 all used the same amplification and detection kit, although on different thermocycling instruments (Table 1). Protocol 4 used the IcePlex system, which is a multiplex real-time PCR and capillary electrophoresis instrument. Protocol 5 used a lab-developed protocol and reagents on an ABI 7500 Real Time System. The volume of nucleic acid used in protocols 1, 2, and 3 was 20ul. Protocol 4 and 5 used an input volume of 10ul and 5ul for amplification.

Statistical Methods

Each viral load result was $\log_{10}$ transformed before analysis. Negative results and any result reported as positive but below the reportable range were not included in the calculation of mean and range.

Results

Testing Protocols

For CMV and EBV viral load testing, two quantitation panels for each virus were sent to each of the four transplantation sites for testing. All five protocols used automated nucleic acid extraction systems with either silica-membrane or magnetic bead based isolation of

the viral DNA. However, the sample volume, elution volume, reagents, and extraction platforms differ for each protocol (Table 1). For CMV, three of the protocols targeted the Major Intermediate Early (IE) gene using the Qiagen artus CMV reagent kit, and amplification and detection was performed on three separate thermocycler platforms. The other two protocols (Primera Dx ViraQuant and a laboratory developed assay protocol) utilized primers targeting the US28 and UL54 genes. The reportable range for these protocols varied considerably among sites. For one assay, the lower limit for quantitation was 50 copies/ml while another assay had a lower limit of 2000 copies/ml. Similarly for EBV, three protocols used the same Qiagen artus TM EBV reagent kit with primers that target the EBNA1 protein. Protocol 5 also targeted the EBNA1 gene, while Protocol 4 targeted EBNA-LP. The reportable ranges for these assays also varied with a limit of detection for one assay of 25 copies/ml while another produced quantitative data above 4,000 copies/ml. This information shows the absence of standardization among these sites in terms of the protocols, reagents, and extraction, and amplification systems being used.

CMV

The qualitative and quantitative performance of CMV viral load testing was compared using two different sample panels covering the typical range of concentrations assessed in clinical laboratories using human plasma containing known amounts of CMV or serial dilutions of the WHO International CMV Standard. There were no false positive results reported for the negative control from either panel (Table 2). The results from the commercial panel included a single result using Protocol 5 (see Table 1), in which no viral DNA was detected in the specimen expected to contain 2.7 $\log_{10}$ copies/ml (500 copies/ml)

which is below the expected lower limit for Protocol 5. CMV was detected using all five protocols in samples expected to contain greater than 3.7 $\log_{10}$ copies/ml (5000 copies/ml), although one result was not quantifiable. All five protocols provided quantitative results for the commercial panel samples expected to contain 4.7 and 5.7 log10 copies/ml (50,000 and 500,000 copies/ml). The mean viral load measured at each of these concentrations was lower than the expected value. The difference between the mean reported value and the expected value at each concentration varied from 0.44 to 0.54 $\log_{10}$ copies/ml. As shown in Figure 1, individual results for the commercial panel were all below the expected value. Eight of the 20 samples (40%) fell within ±0.5 $\log_{10}$ copies/ml of the expected value, which is considered the acceptable degree of variation for quantitative nucleic acid tests [10]. Only two of the five assays gave results within 0.5 $\log_{10}$ of the expected value at every concentration tested. At those concentrations where quantitative results were reported, the difference between the highest viral load result and the lowest viral load result was within or close to 0.5 $\log_{10}$ copies/ml (Table 2).

For the CMV WHO International Standard panel, virus was not detected in the majority of samples expected to contain 0.7 or 1.7 $\log_{10}$ copies/ml (5 or 50 copies/ml). CMV was detected in all of the samples expected to contain 2.7 $\log_{10}$ copies/ml (500 Copies/ml). Quantitative results were obtained on all but one sample expected to contain 3.7 $\log_{10}$ copies/ml (5000 copies/ml). For those replicates with quantitative data, the mean viral load was lower than the expected value at all concentrations and varied from 0.09 to 0.4 log10 copies/ml. As shown in Figure 1, the majority (39 of 60, 65%) of the individual results were below the expected value and all fell within ±0.5 $\log_{10}$ copies/ml. When data

were compared amongprotocols, the difference between the highest quantitative result and the lowest quantitative result was within or close to the acceptable degree of variation of 0.5 $\log_{10}$ copies/ml (Table 2).

EBV

The qualitative and quantitative performance of EBV viral load testing was also performed using the two sample panels: a commercial preparation and the WHO International EBV Standard. As shown in Table 3, there were no false positive results reported on the negative control samples for either panel. There were discrepant results for the samples from the commercial panel expected to contain 3 or 4 $\log_{10}$ copies/ml (1000 or 10,000 copies/ml). At these concentrations, no virus was detected using Protocol 5, while the other protocols were able to detect but not necessarily quantitate virus. Quantitative results were reported from all five protocols for the commercially prepared samples expected to contain at least 5 $\log_{10}$ copies/ml (100,000 copies/ml). The mean EBV viral load on these samples was lower than the expected value at each of the concentrations tested (Table 3). The difference between the mean and the expected value at each concentration ranged between 0.71 and 0.96 $\log_{10}$ copies/ml. The individual results from each protocol were also all less than the expected value, with 8 of the 20 samples falling within 0.5 $\log_{10}$ copies/ml of the expected result (Figure 2). When compared, the difference between the highest result and the lowest result was more than 1.5 $\log_{10}$ copies/ml at every concentration.

As shown in Table 3, for the WHO International EBV Standard panel, EBV was detected in the majority of the replicates expected to contain 2 $\log_{10}$ copies/ml (100 Copies/ml). All of the protocols were able to detect, but not necessarily quantitate, virus in the samples expected to contain 3 $\log_{10}$ copies/ml (1000 copies/ml). For samples expected to contain at least 4 $\log_{10}$ copies/ml (10,000 copies/ml), all five protocols gave quantifiable results. In these cases, the mean viral load was higher than the expected value, with the difference ranging from 0.16 to 0.44 $\log_{10}$ copies/ml. However, when considering data from each laboratory separately, the results were distributed above and below the expected value with the results obtained using protocols 2, 3, 5, and the majority of replicates from protocol 2 above the expected value, and the results from protocol 4 all below the expected value. Of the 42 samples containing at least 4 $\log_{10}$ copies/ml (10,000 copies/ml), 26 (62%) were within 0.5 $\log_{10}$ copies/ml of the expected result. When compared to each other, the difference between the highest result and the lowest result was more than 1.0 $\log_{10}$ copies/ml at each concentration.

Discussion

International guidelines recommend the use of viral load testing for the diagnosis and management of both CMV and EBV infections in organ transplant recipients [1, 2, 5, 17, 18]. Additionally, these assays provide valuable data for the evaluation of new immunosuppressive regimens or antiviral therapies in the clinical trial setting. The purpose of this study was to compare the current practices in CMV and EBV viral load testing performed at four large transplantation centers. Similar to previous studies [10, 11], we found considerable differences among the CMV and EBV viral load values when

commercially available viral panels were tested. In all cases, the observed viral load was lower than the expected viral load, suggesting that either the expected concentration of virus in the viral panels was not accurate, or the assays were under calibrated. However, the CMV and EBV viral loads were relatively accurate (±0.5 log10 copies/ml of the expected value) when compared to the WHO Standards. At the time this study was performed, the commercial reagents were not traceable to any official standard, whereas the assigned value for the concentration of the WHO standards was established in a large multisite study. This may explain why the assays appeared to be more accurate using the WHO standards as the reference material.

Each component of the testing method can contribute to variation in viral load results, including differences in the extraction method, amplification reagents, genes targeted, and calibrators used [19]. In one study comparing EBV viral load results obtained using the same amplification system, viral loads were in close agreement when the same extraction method was used, but varied 2.3 fold when different extraction methods were used [9]. Others have shown that automated extraction and commercially available amplification systems tend to perform better than laboratory developed "home brew" assays [10]. However, even among commercial systems, considerable differences among viral load values have been reported [8, 10]. Our data suggests that the assays used for CMV viral load testing at these transplant centers provided results that were within the normal range of variation for this type of assay and thus may be compared from one center to the next. This was not true for the EBV assays. It is not clear why this is the case; however, the small number of replicates we tested in this study may have contributed. Alternatively, there may

be something inherent to EBV that contributes to the larger degree of variation that we encountered [20].

The WHO International Standards were designed to facilitate the standardization of CMV and EBV viral load testing. Manufacturers and individual laboratories now have the resources to recalibrate assays to conform to these standards. However, there are other issues that should also be considered before comparison between sites becomes commonplace. First, collaborating laboratories may need to standardize the reportable ranges. In our small sampling of protocols, the lower limit of the reportable range varied widely, as did the apparent sensitivity of each assay. Given that CMV and EBV can replicate at a low-level even in a normal host, it is not necessary for these assays to be overly sensitive. However, it may be helpful to establish a reportable range with a clinically relevant cutoff to help distinguish intermittent low-level viremia in latently infected individuals from clinically relevant infection. Second, it is important to acknowledge that some clinical laboratories use plasma as the specimen of choice, whereas others use whole blood. It is not acceptable to compare viral load values from these different specimen types given that both EBV and CMV DNA may be present in peripheral blood cells that are found in whole blood but not plasma. Third, laboratories should begin the process of converting viral load values from copies to international units. This involves designing an experiment to determine a conversion factor. There are currently no readily available consensus documents to guide the laboratory in designing this type of experiment and the volume of calibrator necessary for obtaining a robust data set may be cost prohibitive. Finally, even when these assays become standardized, collaborating centers need to establish a

proficiency testing program using the WHO standards or other traceable materials to ensure the reliability and comparability of the assays over time.

Acknowledgements

This research was performed as part of an American Recovery and Reinvestment (ARRA) funded project under Award Number U0163594 (Peter Heeger, PI) from the National Institute of Allergy and Infectious Diseases. The work was carried out by members of the Clinical Trials in Organ Transplantation (CTOT) and Clinical Trials in Organ Transplantation in Children (CTOT-C) consortia. These studies were also supported by awards from the NIH/NIAID: U01-AI077816-01 and U01AI063623-04 (JAF), 1U01AI77810-01 (GS, LDI), and U01 AI084150-01 (AM, CL). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy And Infectious Diseases or the National Institutes of Health.

Disclosures

JAF of MGH and GS of Washington University were members of the Scientific Advisory Board of PrimeraDx, Inc. Assay systems developed by that company were not used at either site as part of this study. GS is a member of the Roche Diagnostics Advisory Board.

Figure Legends

Figure 1. Accuracy of CMV viral loads

Each point represents an individual replicate from the protocol as indicated in the legend. Negative results and viral load values below the reportable range of the assay for each protocol are not included. The horizontal dotted lines correspond to the acceptable range of variation of ±0.5 $\log_{10}$ copies/ml.

Figure 2. Accuracy of EBV viral loads

Each point represents an individual replicate from the protocol as indicated in the legend. Negative results and viral load values below the reportable range of the assay for each protocol are not included. The horizontal dotted lines correspond to the acceptable range of variation of ±0.5 $\log_{10}$ copies/ml.

References

1. Andrews, P.A., V.C. Emery, and C. Newstead, *Summary of the British Transplantation Society Guidelines for the Prevention and Management of CMV Disease After Solid Organ Transplantation.* Transplantation, 2011. **92**(11): p. 1181-7.
2. de la Torre-Cisneros, J., et al., *GESITRA-SEIMC/REIPI recommendations for the management of cytomegalovirus infection in solid-organ transplant patients.* Enferm Infecc Microbiol Clin, 2011. **29**(10): p. 735-58.
3. Hadaya, K., et al., *Monitoring of cytomegalovirus infection in solid-organ transplant recipients by an ultrasensitive plasma PCR assay.* J Clin Microbiol, 2003. **41**(8): p. 3757-64.
4. Humar, A., et al., *Clinical utility of quantitative cytomegalovirus viral load determination for predicting cytomegalovirus disease in liver transplant recipients.* Transplantation, 1999. **68**(9): p. 1305-11.
5. Humar, A., D. Snydman, and A.S.T.I.D.C.o. Practice, *Cytomegalovirus in solid organ transplant recipients.* Am J Transplant, 2009. **9 Suppl 4**: p. S78-86.
6. Kraft, C.S., W.S. Armstrong, and A.M. Caliendo, *Interpreting quantitative cytomegalovirus DNA testing: understanding the laboratory perspective.* Clin Infect Dis, 2012. **54**(12): p. 1793-7.
7. Le Page, A.K., et al., *International Survey of Cytomegalovirus Management in Solid Organ Transplantation After the Publication of Consensus Guidelines.* Transplantation, 2013.
8. Bravo, D., et al., *Comparative evaluation of three automated systems for DNA extraction in conjunction with three commercially available real-time PCR assays for*

quantitation of plasma Cytomegalovirus DNAemia in allogeneic stem cell transplant recipients. J Clin Microbiol, 2011. **49**(8): p. 2899-904.

9. Caliendo, A.M., et al., *Evaluation of real-time PCR laboratory-developed tests using analyte-specific reagents for cytomegalovirus quantification.* J Clin Microbiol, 2007. **45**(6): p. 1723-7.
10. Pang, X.L., et al., *Interlaboratory comparison of cytomegalovirus viral load assays.* Am J Transplant, 2009. **9**(2): p. 258-68.
11. Preiksaitis, J.K., et al., *Interlaboratory comparison of epstein-barr virus viral load assays.* Am J Transplant, 2009. **9**(2): p. 269-79.
12. Hayden, R.T., et al., *Multicenter comparison of different real-time PCR assays for quantitative detection of Epstein-Barr virus.* J Clin Microbiol, 2008. **46**(1): p. 157-63.
13. Ito, Y., et al., *Multicenter evaluation of prototype real-time PCR assays for Epstein-Barr virus and cytomegalovirus DNA in whole blood samples from transplant recipients.* Microbiol Immunol, 2010. **54**(9): p. 516-22.
14. Gouarin, S., et al., *Multicentric evaluation of a new commercial cytomegalovirus real-time PCR quantitation assay.* J Virol Methods, 2007. **146**(1-2): p. 147-54.
15. Fryer, J.F., Heath A.B., Anderson, R, Minor, P.D., and the Collaborative Study Group, *Collaborative Study to Evaluate the Proposed 1st WHO International Standard for Human Cytomegalovirus (HCMV) for Nucleic Acid Amplification (NAT)-Based Assays*, E.C.o.B. Standardization, Editor. 2010: Geneva, Switzerland.
16. Fryer JF, H.A., Wilkinson DE, Minor PD and the collaborative study group., *Collaborative study to evaluate the proposed 1st WHO International Standard for Epstein-Barr virus (EBV) for nucleic acid amplification (NAT)-based assays.* 2011.

17. Kotton, C.N., *Management of cytomegalovirus infection in solid organ transplantation.* Nat Rev Nephrol, 2010. **6**(12): p. 711-21.

18. Preiksaitis, J.K., et al., *Canadian society of transplantation consensus workshop on cytomegalovirus management in solid organ transplantation final report.* Am J Transplant, 2005. **5**(2): p. 218-27.

19. Caliendo, A.M., et al., *A commutable cytomegalovirus calibrator is required to improve the agreement of viral load values between laboratories.* Clin Chem, 2009. **55**(9): p. 1701-10.

20. Hayden, R.T., et al., *Factors contributing to variability of quantitative viral PCR results in proficiency testing samples: a multivariate analysis.* J Clin Microbiol, 2012. **50**(2): p. 337-45.

Table 1. Testing Protocols

	Nucleic Acid Extraction	*Amplification and Detection*	*Target*	*Reportable Range*
1	QiAmp Virus on Qiagen BioRobot MDX	Qiagen artus TM EBV/CMV on Aplied Biosystems 7500 Real-Time PCR System	EBV EBNA1 CMV Major IE	EBV 500-5,000,000 cp/ml CMV 313 – 3,130,000 cp/ml
2	QiAmp DNA Blood Mini Kit on QiaCube	Qiagen artus TM EBV/CMV on Applied Biosystems QuantStudio 12K Flex	EBV EBNA1 CMV Major IE	EBV >25 cp/ml CMV >50 cp/ml
3	Qiagen Virus/Bacteria Mini/Midi kit on QiaSymphony	Qiagen artus TM EBV/CMV on Qiagen RotorGene Q	EBV EBNA1 CMV Major IE	EBV 300-1,500,000 cp/ml CMV 1000-5,000,000
4	Qiagen MinElute kit on QiaCube	Primera Dx ViraQuant on ICEPlex	EBV EBNA-LP CMV US28	EBV 750-15,000,000 cp/ml CMV 750-15,000,000 cp/ml
5	MagNA Pure Compact Nucleic Acid Isolation Kit 1 on Roche MagNA Pure Compact	Lab developed assays on ABI 7500 Real-Time System (EBV) and ABI 7300 Real-Time PCR System (CMV)	EBNA1 CMV UL54	EBV 4,000-40,000,000 cp/ml CMV 2,000 – 1,250,000 cp/ml

Table 2. Summary of Qualitative Performance for CMV Viral Load Testing

CMV - ***Single replicate of each dilution at each site***					
Expected copies/ml (log10 copies/ml)	***Negative***	***Positive****	***Quantifiable***	***Mean (log_{10} copies/ml)***	***Range (log_{10} copies/ml)***
0	5				
500 (2.7)	1	4			
5000 (3.7)		1	4	3.34	3.07-3.52
50,000 (4.7)			5	4.26	4.01-4.49
500,000 (5.7)			5	5.19	4.94-5.53
CMV – WHO International Standard, ***three replicates of each dilution at each site***					
Expected copies/ml (log10 copies/ml)	***Negative***	***Positive****	***Quantifiable***	***Mean (log_{10} copies/ml)***	***Range (log_{10} copies/ml)***
0	15				
5 (0.7)	13	2			
50 (1.7)	10	5			
500 (2.7)		12	3	2.42	2.27-2.52
5000 (3.7)		1	14	3.46	3.23-3.81
50,000 (4.7)			15	4.54	4.33-4.85
500,000 (5.7)			15	5.61	5.36-5.81

*Positive but below the reportable range

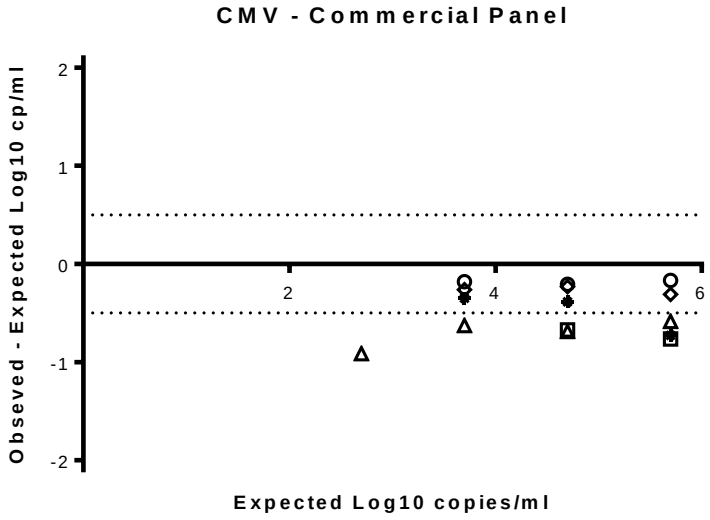
Table 3. Summary of Qualitative Performance for EBV Viral Load Testing

EBV - ***Single replicate of each dilution at each site***					
	Number of replicates				
Expected (log10 copies/ml)	***Negative***	***Positive****	***Quantifiable***	***Mean (log_{10} copies/ml)***	***Range (log10 copies/ml)***
0	5				
3	1	3	1	2.27	
4	1		4	3.17	2.00-3.76
5			5	4.04	3.07-4.88
6			5	5.27	4.23-5.77
7			5	6.29	5.29-6.75
EBV – WHO International Standard, ***Three replicates of each dilution at each site****					
Expected copies/ml (log10 copies/ml)	***Negative***	***Positive****	***Quantifiable***	***Mean (log_{10} copies/ml)***	***Range (log_{10} copies/ml)***
0	14				
10 (1)	10	4			
100 (2)	4	7	3	2.44	2.01-3.16
1000 (3)		4	10	3.38	2.37-3.75
10,000 (4)			14	4.16	3.45-4.69
100,000 (5)			14	5.21	4.55-5.73
1,000,000 (6)			14	6.18	5.46-6.67

**Protocol 4 only 2 replicates; *Positive but below the reportable range

Figure 1

A


CMV - Commercial Panel
Obseved - Expected Log10 cp/ml
2
1
0
-1
-2
2
4
6
Expected Log10 copies/ml

B

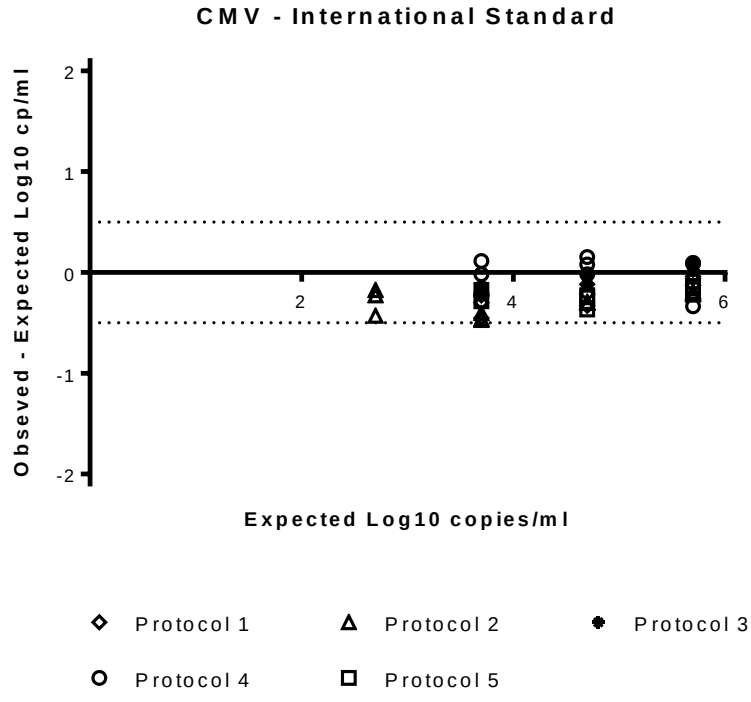
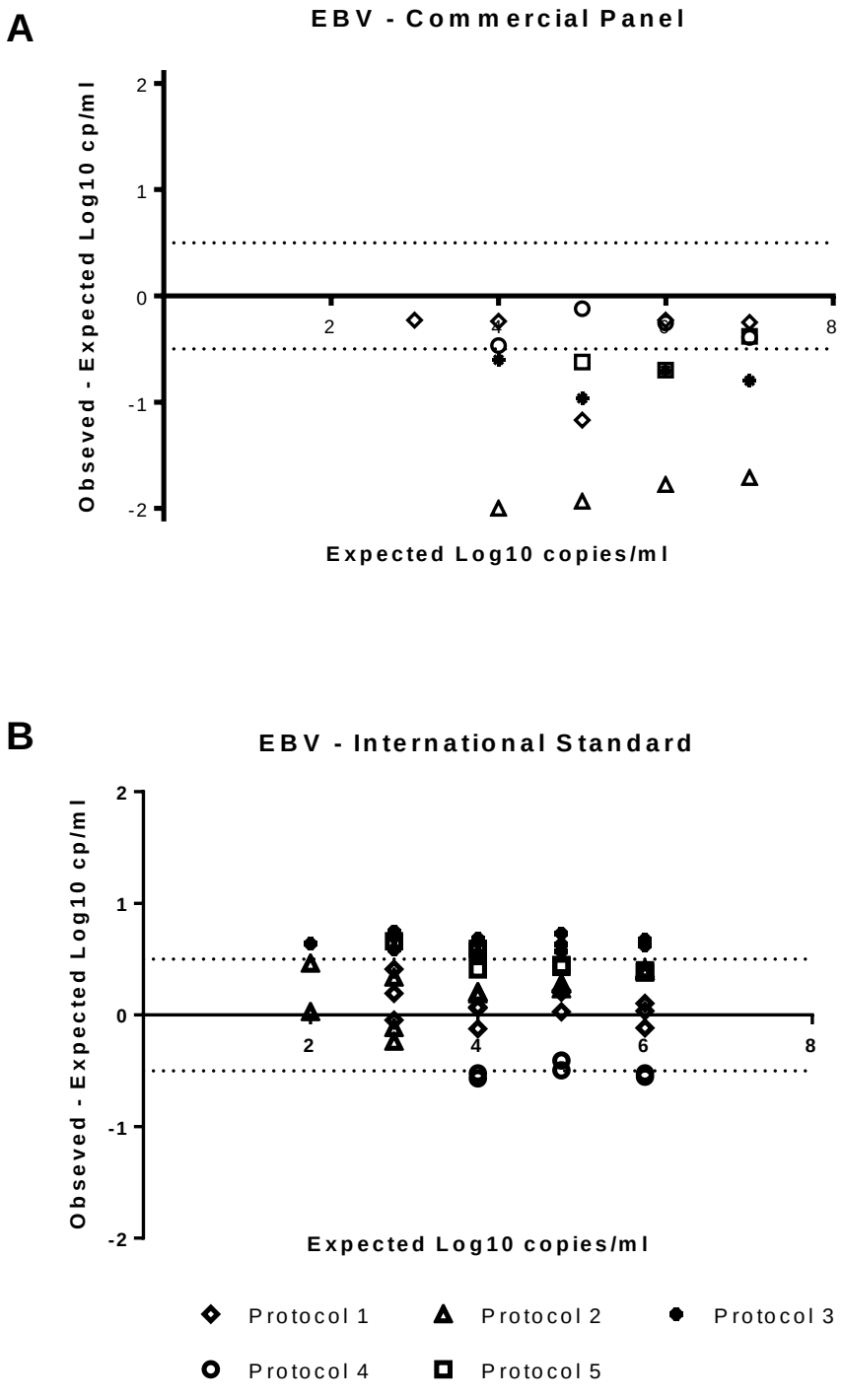

CMV - International Standard
Obseved - Expected Log10 cp/ml
2
1
0
-1
-2
2
4
6
Expected Log10 copies/ml
Protocol 1
Protocol 2
Protocol 3
Protocol 4
Protocol 5

Figure 2


A
EBV - Commercial Panel
Obseved - Expected Log10 cp/ml
2
1
0
-1
-2
2
4
6
8
Expected Log10 copies/ml
B
EBV - International Standard
Obseved - Expected Log10 cp/ml
2
1
0
-1
-2
2
4
6
8
Expected Log10 copies/ml
Protocol 1
Protocol 2
Protocol 3
Protocol 4
Protocol 5