Abstract

Background: Quantitative Cytomegalovirus (CMV) polymerase chain reactions are increasingly being used for monitoring CMV DNAemia in haematopoietic stem cell transplants and solid organ transplants. Objective: In this study, a commercial CMV viral load assay was compared with an in-house viral load assay. Materials and Methods: A total of 176 whole-blood samples were tested for CMV DNAemia using both assays. Results: Our evaluation showed a difference of 1 log_{10} copies/ml between the two assay systems in determining CMV viral loads in the clinical samples. Conclusion: The in-house viral load assay had a better correlation with clinical findings compared to the commercial assay. Quality assessment of these assays was done by the United Kingdom National External Quality Assessment Scheme (UKNEQAS), an external proficiency testing programme, and by the National Institute for Biological Standard and Control (NIBSC) standard. For UKNEQAS and NIBSC standards, the bias between the assays was 0.73 log_{10} and 0.85 log_{10}, respectively. This difference is well within the acceptable range already reported in the literature.

Keywords: Commercial assay, Cytomegalovirus, in-house, quantitative, real-time PCR

Introduction

Cytomegalovirus (CMV) infection remains a major cause of morbidity and mortality among immunocompromised persons such as stem cell transplant recipients and solid organ transplants.\(^1\) High CMV load in the course of viraemia is a major risk factor associated with CMV disease in bone marrow, renal and liver transplant individuals.\(^1\) Quantitative polymerase chain reaction (PCR) assays are being used to identify individuals at risk for developing CMV disease, enable early detection of confirmed CMV disease and monitor response to antiviral therapy. It is also used to anticipate those at risk of virologic and clinic relapses as well as indicate possible antiviral resistance.\(^2\)\(^-\)\(^4\) Real-time-PCR (RT-PCR) for quantitative detection of CMV DNA has become an archetype of post-transplant care.\(^1\)

There are numerous commercial and in-house CMV quantitative RT-PCR (CMVQRT-PCR) assays available, each of which targets different CMV genes employing different chemistries, with the majority being in-house assays.\(^6\)

Since in-house-developed PCRs are standardised in various laboratories, they vary in their assay performance and validation. Furthermore, their lower limits of detection and linear ranges differ, thus making the comparison of results across studies difficult.\(^7\)

There is a paucity of data in the Indian literature on the comparison of CMVQRT-PCRs for the detection of CMV DNA. The aim of this study was to compare the performance characteristics of an in-house RT-PCR assay based on Taqman chemistry for the quantification of CMV viral load in whole-blood specimens with the commercial assay system Artus CMV RG Real-Time PCR assay (Qiagen, GmbH, Hilden, Germany).
Materials and Methods

Clinical specimens
A total of 176 whole-blood samples were received from 42 bone marrow transplant and 33 renal transplant patients from haematology and nephrology departments, respectively, of a tertiary care hospital in South India, to screen for CMV reactivation. Approximately 8–10 ml of blood was collected in dipotassium ethylenediaminetetraacetic acid tubes (BD vacutainer, UK) from each patient at the laboratory collection site or from the ward. To maintain integrity of nucleic acid, all the samples collected from the ward were transported between 2°C and 8°C within 30–45 min to the virology laboratory where it was aliquoted and stored at −20°C until testing.

World Health Organization international standard
The 1st World Health Organization (WHO) international standard (IS) for human CMV for nucleic acid amplification techniques (National Institute for Biological Standard and Control [NIBSC] code 09/162, NIBSC, Hertfordshire, Great Britain) was obtained as a lyophilised whole-virus preparation of the CMV Merlin strain. After reconstitution in 1 ml of water, the WHO IS has a concentration of 5 × 10^6 IU/ml, i.e., 6.7 log_{10} IU/ml. The NIBSC standard was prepared by diluting the freshly prepared stock to obtain final concentrations (IU/ml) of 5 × 10^6, 5 × 10^5, 5 × 10^4, 5 × 10^3, 5 × 10^2, 5 × 10^1 and 5 × 10^0 and tested in parallel using the quantitative CMV in-house PCR and the Artus assay.

United Kingdom National External Quality Assessment Scheme samples
Ten samples (freeze-dried plasma) for CMV were received on the United Kingdom National External Quality Assessment Scheme (UKNEQAS) programme during the study period and tested in parallel using both the quantitative CMV in-house PCR and the Artus assay.

Viral nucleic acid extraction
Whole-blood samples, stored at −20°C prior to extraction, were thawed at room temperature following which DNA was extracted from 200 µl. Also tested were UKNEQAS and NIBSC standards obtained as lyophilised powder and reconstituted with nuclease-free water. DNA was extracted using QIAamp DNA blood Mini kit (Qiagen, GMBH, Germany); the final DNA extract was eluted in 200 µl of elution buffer for each sample. DNA extracts were immediately tested by PCR, after which extracts were stored at −20°C until needed for further use.

Real-time quantitative Cytomegalovirus polymerase chain reaction
DNA extracts were simultaneously tested using the Artus RG PCR kit and the in-house assay on the Rotor gene RT PCR machine (Corbett Research pvt limited, Sydney, Australia).

In-house polymerase chain reaction assay
The in-house RT quantitation PCR for CMV was performed using an already published protocol from our centre.[9] The in-house assay was developed using Quantitect Multiplex PCR Norox master mix (Qiagen, Gmbh, Germany) and a pair of primer-probes targeting a 76 bp region of the major immediate early (MIE) gene of CMV. The sequences of primers and probe used are as follows: forward AACTCAGCTTCCCTAAGACCA; reverse GGGAGCACTGAGGCA AGTTC; probe Fam-CAATGGCTGAGTCAGGCCATGG-BHQ1. Ten microlitres of the extracted DNA was added to 15 µl of PCR master mix with the following cycling conditions: 95°C for 10 min followed by 50 cycles of 95°C for 45 s and 60°C for 75 s. Specificity of the PCR was determined by testing samples with high viral loads of the following viruses: herpes simplex virus (HSV)-1, HSV-2, Epstein–Barr virus, varicella zoster virus, human herpes virus (HHV)-6, HHV-8, adenovirus, JC and BK viruses. None of these viruses showed any cross-reactivity in the above PCR reactions. Quantitative plasmids for in-house assay were prepared by amplifying a 1000 bp segment of CMV MIE gene spanning the target region. The lower limit of detection was estimated at 150 copies/ml.

Artus RG polymerase chain reaction assay
The Artus CMV RG PCR (Qiagen, GmBH, Germany) assay is a ready-to-use master mix assay that contains reagents and enzymes amplifying a 105 bp region of the MIE gene of the CMV genome and an internal control. The manufacturers claim to have 100% sensitivity and 84.8% specificity with a lower limit of detection of 57.1 copies/ml. The range of the quantitative plasmids provided by the manufacturer was between 10^7 and 10^6 copies/ml.[9] The assay was carried out as per manufacturer’s instructions.

Statistical analysis
Data were presented as log_{10} of the viral load values. The correlation between the two systems was analysed using SPSS software 16.0, IBM SPSS Inc, New York, USA on log-transformed results by using Bland–Altman and scatter plots.

Results
We obtained clinical information of 42 patients who underwent bone marrow transplantation in the year 2011 and from whom serial blood samples were collected for CMV DNA estimation. The mean age for patients was 20.5 years (range: 1–55 years); the majority (52.4%) were diagnosed with leukaemia. Among these transplant recipients, 67.5% underwent matched sibling donor transplant. Among the 37 in whom details were available, 35 were CMV seropositive. Their clinical characteristics are summarised in Table 1. We also obtained clinical information of 33 patients who underwent renal transplantation, the majority (64%) between 2008 and 2011. The mean age of the patients was 41 years (range: 15–58 years). Of these renal transplants, 85% had live-related donors, 6% had live-unrelated donors and 3% had cadaveric donors.

Of 176 samples tested by both assays, there were 58 (32.95%) with detectable DNA samples (positive samples) by
either or both assays, with CMV DNA being detected in 48/176 samples (27.27%) using the in-house assay compared to 47/176 (26.70%) by the commercial assay.

Both in-house and commercial assays were congruent in 37/176 (21.02%) of positive samples. Of the 21 samples showing discordant positivity, 11 samples were positive by the in-house assay alone (log_{10} 1.78–3.23), while 10 were positive only by Artus (log_{10} 1–2.43) as shown in Table 2.

The majority of samples (118/176, 67%) were negative both by in-house and Artus assays as shown in Table 2. All samples were tested for an internal housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase) before reporting. The discrepant positivity was found when the viral loads were below log_{10} 3.2. In the 21 discrepant-positive samples, the 11 samples positive by the in-house PCR were repeated and viral load values were obtained. The mean of the values obtained was also included in the statistical analysis. Seven of the 10 samples positive by the commercial assay gave negative results on retesting and hence were concordant with the in-house assay results (there was thus a discrepancy on repeat testing with the commercial assay).

The median viral load obtained using the in-house assay was 1580.5 copies/ml (log_{10} 3.20), while for the commercial assay, it was 206 (log_{10} 2.48) copies/ml as mentioned in Table 3. Mean (standard deviation [SD]) viral loads of the assays were significantly different (paired t-test, P < 0.001). The correlation between the two methods is presented as a scatter plot and Bland–Altman analysis in Figure 1 and 2.

Using a clinical threshold of 1000 copies/ml, 16/176 samples had a viral load <1000 copies/ml. In this group, the mean difference in viral load values was 0.7 log_{10} copies/ml between the in-house and commercial assays. In the samples with >1000 copies/ml by both assays (32/176), the mean difference in viral load was 1.06 log_{10} copies/ml between the in-house and commercial assays. The maximum number of viral load values obtained was in the range of 1000–10,000 copies/ml (21/176).

The UKNEQAS samples tested in parallel by both assays showed a bias from the original median of log_{10} 0.36 and -log_{10} 0.37 for the in-house and commercial viral load assays, respectively. Thus, there is a 0.73 log difference between the two assays. However, both assays have given results which are within the acceptable range provided by the UKNEQAS. Similarly, by using the NIBSC standard also, we observed the same bias between the commercial and in-house viral load assays (mean = −0.85) (range: 0.74–0.95).

Among the type of transplants, although matched unrelated donors had higher viral loads in comparison with matched sibling donors (P = 0.235), it was not statistically significant which may be due to the small sample size in the respective groups as shown in Table 3.

Table 1: Baseline characteristics of patients who underwent bone marrow transplant (n=42)

| Parameter                      | Number of patients (%) |
|--------------------------------|------------------------|
| Sex                            |                        |
| Male                           | 26 (61.9)              |
| Female                         | 16 (38.1)              |
| Underlying disease             |                        |
| Acute myeloid leukaemia        | 7 (16.7)               |
| Acute lymphoblastic leukaemia  | 10 (23.8)              |
| Aplastic anaemia               | 7 (16.7)               |
| Chronic myeloid leukaemia      | 5 (11.9)               |
| Thalassaemia                   | 9 (21.4)               |
| Others*                        | 4 (9.5)                |
| Treatment choice               |                        |
| Chemotherapy                   | 2 (4.8)                |
| Stem cell transplant           | 40 (95.2)              |
| Conditioning (n=40)            |                        |
| Myeloablative                  | 32 (80)                |
| Reduced intensity conditioning  | 8 (20)                 |
| Type of transplant             |                        |
| Autologous                     | 2 (5)                  |
| Matched sibling donor          | 27 (67.5)              |
| Matched unrelated donor        | 11 (27.5)              |
| CMV serostatus (n=37)          |                        |
| R+                             | 35 (94.6)              |
| R-                             | 2 (5.4)                |

*Indicates other underlying disease conditions (n=4): multiple myeloma(2), myelodysplastic syndrome(1), juvenile myelomonocytic leukemia(1). CMV: Cytomegalovirus

Table 2: Comparison of the qualitative results of the in-house and commercial assays

|                     | Commercial assay positive | Commercial assay negative |
|---------------------|---------------------------|----------------------------|
| In-house assay positive | 37                        | 11                         |
| In-house assay negative | 10*                       | 118                        |

*On repeat testing, 7 were negative

Figure 1: The scatter plot of log_{10} -transformed Artus and in-house viral load values are presented (overall). Though there is a good correlation between the two methods (R^2 = 0.739) P < 0.001, there is a systematic difference between them.
To determine if there was a difference in clinical correlation between the assays, we scrutinised the clinical details of the patients in whom there was a discrepancy between the two assays. These patients with discrepant viral load were from haematology department ($n=14$) and the clinical details of CMV disease were available for 13 patients. Ten patients who were positive for CMV DNA with $<1000$ copies/ml by the in-house assay but negative by the commercial assay had clinical features suggestive of CMV disease such as gastroenteritis, pneumonia, oesophagitis and febrile illness. However, the two patients with samples which were commercial assay positive with $<1000$ copies/ml but were in-house assay negative did not have any CMV disease manifestation. Of these, one sample was negative for CMV by biopsy.

On serial follow-up of the blood samples of these patients ($n=42$) for CMV viral load monitoring, the following three case scenarios were significant. In the first case, a high viral load of $5.42 \log_{10}$ copies/ml, detected by the in-house assay, was underestimated by the commercial assay which detected a viral load of $3.60 \log_{10}$ copies/ml. Subsequently after 1 week, CMV was detected in bronchoalveolar lavage in this patient, indicating CMV-invasive disease, which correlates with the viral load estimation of the in-house assay. In the second case, a CMV viral load of $2.87 \log_{10}$ copies/ml by the in-house assay was negative by the commercial assay. On subsequent follow-up, the patient had a viraemia of $4.41 \log_{10}$ copies/ml (in-house assay) and $3.52 \log_{10}$ copies/ml (commercial assay) after 2 weeks. Preemptive treatment was initiated in this case, only because of the follow-up due to the early detection of CMV by the in-house assay. In the third case, 6 months following transplant, relapse occurred which correlated with the high viral load obtained by the in-house assay (in the range of $>4 \log_{10}$ copies/ml) in comparison with the commercial assay (in the range of $<4 \log_{10}$ copies/ml).

**Discussion**

Quantitative RT PCR assays are progressively utilised for monitoring active CMV infection in allogeneic stem cell transplant recipients. There is very little data available from India on the performance characteristics of the quantitative CMV DNA assays in comparison to each other.

Quantitation of CMV DNA in whole-blood samples has been found to be the most sensitive method for monitoring CMV infection in immunosuppressed patients, as it yields higher DNA loads than serum or plasma.$^{[10]}$

In the present study, the in-house assay was developed with primers and probes targeting a 76 bp region of the MIE gene of CMV, a conserved region.$^{[11]}$ It had a specificity of 91% and has been established to be very efficient for clinical strain detection.$^{[11,12]}$

Of the 176 samples, both systems detected CMV in 37 samples and 118 were negative by both assays. The total number of discrepant results was 21, of which 11 samples were detected by the in-house assay and negative by the commercial assay. Thus, the in-house PCR detected 30% more positive cases than the commercial assay. In these discrepant samples, the viral load values were $\leq 1000$ copies/ml, indicating a higher sensitivity of the in-house assay. This characteristic of higher sensitivity of the in-house assay allows early diagnosis of CMV infection and institution of earlier preemptive antiviral therapy. This is in agreement with earlier published literature.$^{[13]}$
Another important finding we observed was the reproducibility of the in-house assay (100%) at the lower level of viral load values when compared to the commercial assay (30%).

Of the 14 patients where the CMV viral load values were below 1000 copies/ml, medical reports were obtained for 13 patients. Of these 13 patients, the majority (n = 10, 76.9%) had clinically suspected CMV infection for which CMV levels were monitored serially. Preemptive antiviral therapy among bone marrow transplant patients in many centres is set at a CMV viral load of 500–1000 copies/ml. Our in-house PCR’s limit of detection is 150 copies/ml. In our experience, this in-house PCR seems to be more sensitive even though the commercial assay claims a limit of detection of 58 copies/ml. The superior sensitivity of the in-house assay over the commercial assay is highlighted by the three case scenarios as mentioned earlier.

In comparison to earlier published literature using the Artus assay, most samples (except for 3) showed results within 2 SDs of the mean difference using the Bland–Altman analysis (the bias being 1). Of the three samples, the results of the two samples were >+2 SDs, while for one sample, it was <-2 SDs from the mean difference.

Ten samples were obtained from UKNEQAS while participating in their external proficiency testing programme in 2014–2015. The samples showed a bias of log10+0.36 by the in-house assay and log10−0.37 by the commercial assay when compared with the median values resulting in a 0.73 log difference between the two systems, similar to another published study. The difference between these two systems (+0.36 and −0.37) is probably indicative of the inherent characteristics of the two different assays.

The bias obtained between the in-house and the commercial assays with the NIBSC standard was found to be 0.85 log. The performance of the in-house RT PCR assay is on par with the commercial assay on external proficiency testing with UKNEQAS and with the NIBSC standard (R2 = 0.98) as shown in Figure 3.

Differences in the results of testing standards have been noted previously. For example, a dilution panel of CMV-derived reference material that ranged from 0 to 500,000 copies/ml distributed to 23 different laboratories showed a mean coefficient of variance of 2%–5% on a log scale. The authors suggest that viral load differences of <3–5 folds may not be significantly different. Furthermore, when the NIBSC standard (NIBSC code 09/162) was tested by 32 laboratories from 14 different countries, the variability in the individual mean estimates of the whole virus samples was 2 log10 (100 folds). This was attributed to the differences in the extraction and amplification methodologies used between laboratories. Thus, studies on quantitation of CMV standards have reported a difference ranging from a minimum of 2 log10 to a maximum of 7 log10.

Our evaluation showed a maximum of 1 log10 difference between the two assay systems, which is below the reported difference in detection of the NIBSC standard.

CMV reactivation will be increasingly detected with the increasing numbers of solid organ and bone marrow transplants being performed every year in India. It is therefore necessary that tertiary care centres here have well-validated sensitive assays that can detect CMV DNAemia early so that the appropriate intervention can be provided.

Conclusions
The in-house CMV viral load assay reported in this study is robust and clinically useful for early specific detection of CMV DNAemia in a transplant set-up, provided it is used consistently and can be used interchangeably with other commercial assays.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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