Quantitative Real-Time Polymerase Chain Reaction for Evaluating DNAemia due to Cytomegalovirus, Epstein-Barr Virus, and BK Virus in Solid-Organ Transplant Recipients

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Testing for cytomegalovirus-, Epstein-Barr virus–, and BK virus–specific gene targets in specimens from solid-organ transplant recipients for DNA by quantitative real-time polymerase chain reaction has been implemented in many diagnostic facilities. This technology provides rapid, accurate, and reproducible results for early detection, monitoring, and medical management of patients with these infections. Because these assays are becoming commonly used in clinical practice, the technical variables associated with specimen processing (e.g., nucleic acid extraction, gene target, and result reporting), amplification, and unique patient characteristics (e.g., age, sex, underlying diseases, immune status, and immunosuppressive regimens received) are factors that may influence the understanding and interpretation of test results. We emphasize the need for standardization of existing variables through parallel comparative and proficiency testing, uniform units for expressing results, to provide for clinical correlation with the results of these molecular assays.

Cytomegalovirus (CMV), Epstein-Barr virus (EBV), and BK virus (BKV) are common pathogens and major causes of morbidity and mortality in patients who have received a solid-organ transplant [1–3]. Infection with these viruses is indicated by demonstrating the presence of the virus in tissue and/or blood specimens. Because all of these viruses (i.e., CMV, EBV, and BKV) contain DNA, the term “DNAemia” certainly applies. The more general “viremia” can apply to a bloodstream infection caused by a DNA- or RNA-containing virus. BKV and EBV are not detected in cell cultures by routine diagnostic virology methods. Detection of CMV in blood specimens can be qualitatively recognized rapidly (16 h after inoculation) in shell vial cultures, but quantitation of the CMV load with this technology is cumbersome and impractical.

The first laboratory method for quantitation of CMV load was the antigenemia test [4]. Widespread implementation of this test in clinical laboratories has been somewhat limited by the restrictive technical aspects of sample processing, manual performance of the test, limited quantitative range, and subjective interpretation of the test results [5–7]. Importantly, both shell vial cell cultures (qualitative) and antigenemia tests (quantitative) have been shown in comparative studies to be less sensitive than PCR. In addition, DNAemia was detected in an earlier stage of nucleic acid amplification [6–10].

As an alternative to cell culture and the antigenemia test, implementation of molecular techniques for the rapid and sensitive detection of nucleic acid targets has yielded new levels of capabilities for laboratory diagnosis of many microbial infections [11]. Implementation of these tests has been facilitated by the availability of real-time PCR instrumentation with automation of nucleic acid–target amplification and amplicon-detection steps in a “closed system” (i.e., reaction tubes never opened during or after amplification). Therefore, for both qualitative and quantitative detection of viruses, automated real-time PCR has generally replaced the time-consuming and labor-intensive conventional amplification and detection of products by gel electrophoresis and Southern blot or ELISA methods that frequently cause contamination events and false-positive results because of the inadvertent transfer of high-copy nucleic acid products to other specimens.

REAL-TIME PCR

The first applications of real-time PCR (7 years ago at The Mayo Clinic; Rochester, MN) were the routine, sensitive, and specific assays for herpes simplex virus and varicella-zoster virus.
as replacements for cell-culture methods; the menu of assays has now been extensively expanded to include the laboratory detection of many microbial targets [11–14]. More recently, quantitative real-time PCR has been developed for assessing copy levels of DNA of CMV, EBV, and BKV in blood specimens obtained from patients receiving solid-organ transplants.

For target amplification, oligonucleotide primers and probes for amplification and detection, respectively, of nucleic acid are selected from conserved nucleotide sequences within a viral gene; these products constitute the first level of sensitivity and specificity for quantitative real-time PCR. Together with other components, the assay is subsequently adjusted to permit the polymerase enzyme to function optimally and to produce sensitive and specific signals from labeled probes that are proportional to the amount of target DNA present in the blood sample [11, 15]. Three to 5 commercial quantitative standards are included in the quantitative test. The software for the real-time PCR instrument generates a standard curve with use of these quantitative standards. This plot relates the cycle number in which the amplified nucleic acid target from the standards is detected (by measuring fluorescence) to the amount of target present in the standards. The quantitative level of viral nucleic acid in a test specimen is then determined by comparing the cycle number (crossover point) of the specimen with the standard curve generated with the known levels of the target nucleic acid [11].

NUCLEIC ACID EXTRACTION

The preanalytical extraction of nucleic acid from a blood specimen is a critical step for the determination of the viral load [11]. Commercial kits are available for manual and automated extraction of nucleic acids from specimens. Automated extraction platforms vary regarding the number of specimens processed, cost, and time requirements for sample throughput. Specimen extraction must achieve effective recovery of the target nucleic acid from the specimen, but the process should also remove inhibitory substances (i.e., heme present in blood samples). Differences in technical aspects of manual and automated extraction methods may be significant variables that affect downstream generation of results of a PCR assay. For practicality, consistency, and reproducibility of results, most laboratories that perform high-volume molecular testing process specimens with automated systems that reduce manual operational procedures and may reduce ergonomic repetitive-motion injuries among laboratory personnel. Overall, the preanalytical nucleic acid–extraction step, with use of manual or automated methods, represents a major challenge for standardization of PCR assays among laboratories.

RESULT REPORTING

Result reporting of quantitative PCR assays varies widely among laboratories. For quantitative PCR, the lower limit of detection is determined by probit analysis. For example, both the laboratory and the clinician need to know the lowest level for detecting target DNA that is of sufficient copy level to obtain a positive result if the specimen is retested. If a 50-μL extract has only a single copy of target DNA and 10 reactions are performed, each 5-μL aliquot of the sample would yield a result in only 1 test vessel; the other nine 5-μL aliquots would yield negative results (figure 1A). Similarly, if 2 copies of target DNA were present in the original extract, a positive result may be expected in a maximum of 2 of 10 test vessels (figure 1B). Finally, high copy levels of target DNA would be expected to produce positive results in each 5-μL aliquot of an extract (figure 1C).

Standardization of result information is critical for each laboratory, so that derived values can be compared and be relatively uniform among institutions that perform quantitative PCR testing. An essential component to this goal is the use of commercially available quantitative standards prepared in units of, for example, CMV DNA copies per microliter (as used in our laboratory). Subsequent amplification results are then generated as the number of copies per microliter. For report purposes, these values are converted from microliter to milliliter by multiplying the value by 1000. Because of a 2-fold concentration of the sample during extraction, the resultant value is divided by 2. Therefore, the copy number of CMV DNA/μL is multiplied by a factor of 500 to convert microliter to copies per milliliter. Some laboratories report results as CMV DNA copies/ml but use different conversion constants to convert microliter to milliliter. This may vary depending on the nucleic acid–extraction method, volume of sample tested, and the automated platform used for real-time amplification [11, 16, 17].

Unfortunately, in a review of articles relating to real-time PCR, the units of measurement were not standardized among laboratories, with quantitative PCR values expressed as CMV DNA copies per microliter, CMV DNA copies per 10^5 peripheral

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**Figure 1.** Illustration of sampling distribution of target nucleic acid and subsequent amplification by PCR.
eral-blood leukocytes, and CMV DNA copies per microgram of human DNA [11].

**INTERPRETATION OF RESULTS**

Interpretation of quantitative PCR report values can be misleading (e.g., ≤5000 CMV DNA copies/mL), because low copy levels of target nucleic acid (i.e., 500–5000 CMV copies/mL) may not be reproducible if detected in repeated assays from the same or different specimens. In these instances, a laboratory report such as “2500 CMV DNA copies/mL” with an appended message such as “Results may not be reproducible due to low copy levels” is appropriate. Nevertheless, such low copy level results may be significant in certain clinical situations.

**CMV**

CMV is a herpesvirus; other viruses within this group are herpes simplex virus, varicella-zoster virus, EBV, and human herpesviruses 6–8. CMV infection is the most frequent cause of infection in patients who have undergone solid-organ transplantation; in varying series, its incidence ranges from 25% to 85% [1, 6].

Home-brew (i.e., in-house developed tests) molecular assays are unique for every laboratory. In a recent review of quantitative real-time PCR for CMV, at least 9 different target regions of the genome were used in various home-brew quantitative tests [11]. Importantly, all primer-probe combinations are not equally efficient in amplifying CMV DNA. For example, among 148 specimens, primer pairs directed at the HindIII-X fragment region of CMV detected target DNA with 94% sensitivity, compared with 87% sensitivity with primer pairs directed to EcoR1 fragment D, 32% sensitivity with primer pairs directed at immediate early 1 gene (IEA1), and 20% sensitivity with primer pairs directed at major immediate early (MIE) gene [18].

The specimen type is critical for obtaining optimal results. The compartment of blood (serum or plasma, whole blood, or concentrated leukocytes) most productive for detection of CMV DNA may depend on the posttransplantation serostatus of the donor and recipient. For example, primary CMV infection (seronegative donor and recipient) may result in viral replication that is associated with the cellular components of blood. Therefore, assays of concentrated leukocytes may be more productive for detecting CMV DNA than acellular specimens. Conversely, reactivated CMV infection in the transplant recipient may result in predominately cell-free (e.g., plasma) virus [19].

Blood specimens from immunocompetent, CMV-seropositive, asymptomatic patients are rarely positive for CMV DNA [20]. Importantly, once active CMV infection has occurred in the immunosuppressed patient, the optimal compartment of blood (serum or plasma, anticoagulated whole blood, or concentrated leukocytes) in which to detect CMV DNA has not been established or standardized among laboratories. In one study of 286 samples from immunosuppressed patients, CMV DNA was detected more frequently in whole blood than in peripheral blood leukocytes or plasma [21]. In another comparison of 558 whole-blood and plasma specimens from 50 patients with allogenic bone marrow, no difference was obtained between the 2 sample types [9]. Ideally, comparative study designs should focus on collection of serial blood specimens from individual patients rather than testing fractionated specimens (cells or plasma) from several patients at random times after transplantation. For standardization among laboratories, an obvious compromise for optimal detection of DNAemia would be the universal use of anticoagulated whole blood (i.e., a blend of cell and cell-free associated blood components) [22, 23]. In our laboratory, the typical linear dynamic range of the real-time assay is $5.0 \times 10^2$ to $1.0 \times 10^4$ CMV DNA copies/mL (figure 2). The reportable level for specimen 1 is $1.8 \times 10^2$ CMV DNA copies/μL or $90,000$ CMV DNA copies/mL. Specimen 2 contains $6500$ CMV DNA copies/mL. Among $705$ positive blood specimens tested at our institution, $50\%$ of the values were $\leq 4.5 \times 10^4$ CMV DNA copies/mL (figure 3).

Ideally, a single positive quantitative PCR result (copy level or viral load) can be established as a “threshold” viral load, to be able to implement treatment and/or predict symptomatic infection with CMV. Unfortunately, the complex variables of many subpopulations (e.g., age, sex, underlying diseases, immunosuppression drug regimens, and serostatus to the virus) of transplant recipients do not permit establishment of accurate and meaningful threshold viral loads for prediction of symptomatic infections [16]. At our institution, we generally use a cutoff of $\geq 5000$ copies/mL for preemptive therapy of asymptomatic CMV reactivation in solid-organ transplant recipients and $\geq 1000$ copies/mL for preemptive therapy of asymptomatic CMV DNAemia in allogenic peripheral blood stem cell or bone marrow transplant recipients, although neither of these cutoffs has been formally validated. Alternatively, serially monitoring of patients by quantitative PCR for CMV viremia may provide quantitative viral loads, so that the clinician will know whether the copy level of virus nucleic acid in the blood is increasing, decreasing, or remaining constant over a period of several days. The critical question here is whether one value is significantly different from the preceding value in the same patient. For this purpose, in our practice, we established 95% CIs using inverse prediction methodology for the reported result (figure 4). Known viral loads were treated as a predictor variable, and the results from the PCR were treated as the response variable. Inverse prediction methods, as illustrated in the work by Sokal [24], were used to predict the true known concentration on the basis of the value obtained from real-time PCR. Thus, if the range of the confidence levels overlap (lower 95% and upper 95%) with those from a previous result, the apparent viral load cannot be interpreted as significantly different.
Figure 2. Real-time PCR amplification of commercial standards and specimens for the quantitative detection of cytomegalovirus DNA (top). Standard curve generated using known amounts of standard cytomegalovirus DNA relating log concentration to cycle number of amplification (bottom).

EBV

EBV is associated with posttransplantation lymphoproliferative disease (PTLD); this complication most commonly occurs in organ transplant recipients who are seronegative for the virus before transplantation [7, 25–28]. Accordingly, the highest incidence of PTLD occurs in children. However, in a review of adults who received lung transplants and later developed gastrointestinal manifestations of PTLD, 15 (88%) of 17 had antibodies to EBV before transplantation [29, 30]. PTLD is a heterogeneous group of lymphoproliferative diseases [31]. In general, PTLD in organ transplant recipients occurs at 18–66 months after transplantation, with the incidence dependent mainly on the transplanted organ (kidney, 1%; heart-lung, 6–10%; lung, ~20%), the immunosuppressive drugs received, the EBV antibody status of the host before transplantation, and the age of the recipient [32].

The technical variables of real-time PCR for detection of CMV DNA similarly apply to quantitative detection of EBV DNA in blood specimens. Even though there is a seemingly endless number of combinations of these variables to eventuate in one assay, the total system can be standardized for use at one institution. A very general approach is to introduce commercial standards of EBV DNA into the assay as described for analysis of CMV DNA [33]. At our institution, 95% of the EBV DNAemias were ≤5.5 × 10⁴ copies/mL and 50% of the reported values were ≤2.0 × 10⁵ EBV DNA/mL.

Comprehensive studies are lacking for evaluation of the most appropriate compartment of blood (e.g., whole blood vs. plasma) to monitor EBV DNA in viremic patients. The most meaningful results are obtained by comparing the detection of EBV DNA from whole blood and plasma from serial or sequential specimens obtained from the same patient. This information should provide guidance relating to the distribution of EBV DNA in cellular (whole) compared with cell-free (plasma) blood during the posttransplantation period. Until definitive, longitudinal comparisons are done, unfractionated whole blood is preferred from these patients, because this specimen matrix combines all blood compartments that may harbor EBV and may most accurately reflect the absolute level of DNAemia in the patient [34].

BKV

BKV is a polyomavirus; each of the 4 subtypes contain ~5000 bp of double-stranded DNA with homology of ~70% with 2 closely related viruses (JCV and SV40). BKV is a recognized etiologic agent of allograft nephropathy in patients who have undergone renal transplantation. BKV infection is common, indicated by the detection of IgG-specific antibodies in 80%–100% of the general population. After primary infection, BKV becomes latent in kidneys and the urinary tract. The virus is reactivated with immunosuppression, and, especially in kidney-transplant recipients, can lead to polyomavirus-associated nephropathy (PVAN) several months after transplantation in 2%–
9% of recipients; PVAN is associated with graft loss in 30%–60% of subjects [35].

A definitive diagnosis of BKV-associated nephropathy requires the demonstration of viral cytopathic changes in tubular epithelium in renal tissue. The presence of decoy cells containing polyomavirus-inclusion bodies in sedimented urine specimens indicates BKV infection; nevertheless, this screening test has a low positive predictive value.

BKV can be detected in both urine and plasma specimens of transplant patients. Urine may have very high BKV DNA loads (10^6–10^8 copies/mL); the virus load may overlap in patients with and without PVAN, so the specificity of BKV DNA as an indicator of PVAN may not be clinically helpful. BKV DNA detected only in urine, and not in plasma, is not associated with PVAN [36]. However, the detection of BKV DNA in urine specimens may provide the first evidence of polyomavirus infection in the patient [37]. Conversely, monitoring for detection of BKV DNA in plasma is more specific for PVAN than detection with urine specimens. In a cohort study of 213 patients who had received a kidney transplant, only 4 patients developed PVAN [38]. In that study, 2.5 x 10^4 BKV DNA copies/mL (urine) and 1.6 x 10^4 BKV DNA copies/mL (plasma) had 100% sensitivity and >90% specificity for PVAN. Interestingly, with use of PVAN as the primary outcome, the presence of decoy cells had a sensitivity of only 25% and a specificity of 84%. Of 190 positive blood specimens tested at the Mayo Clinic over a 2-year period, more than one-half of the reported values were low (<5000 BKV DNA copies/mL; 50th percentile); the 95th percentile was 2.36 x 10^7 BKV DNA copies/mL. Monitoring specimens from these patients by real-time quantitative PCR may provide earlier, more sensitive, and specific information to the clinician for prediction and management of PVAN.

CONCLUSIONS

Development and implementation of quantitative real-time PCR assays for the detection of target nucleic acid of CMV, EBV, and BKV present opportunities for early detection, disease prediction (threshold values), monitoring for significant viral-load values, and providing appropriate medical management of patients posttransplantation. At this time, testing of multiple specimens from each patient (trend analysis) with a single assay will provide reproducible results, because many interlaboratory (nucleic acid–extraction method, gene target, and result reporting) and patient characteristics (age, sex, underlying diseases, immune status, and immunosuppression regimen) vary widely among institutions. Commercial availability and common reagents combined with proficiency challenges are needed to standardize the variability associated with individualized testing protocols.

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References


