An in-house assay for BK polyomavirus quantification using the Abbott m2000 RealTime system

Kenneth L. Muldrew and Jennie L. Lovett

Department of Pathology, University of Toledo Medical Center, Toledo, OH, USA

BK polyomavirus (BKPyV) quantification is useful for monitoring renal transplant patient response to therapy. The Abbott m2000 RealTime System employed by some clinical laboratories to perform US Food and Drug Administration-approved assays can also be used to develop in-house assays such as the one presented here. This study aimed to validate an in-house quantitative real-time PCR assay targeting the BKPyV major capsid VP1 gene for assessment of viral load using the Abbott m2000 RealTime System. BKPyV load was measured in 95 urine and plasma samples previously tested for BKPyV by one of three laboratories (46 BKPyV-positive samples consisting of 35 plasma and 11 urine samples; 49 samples negative for BKPyV consisting of 47 plasma and two urine samples). Two additional plasma specimens from the College of American Pathologists proficiency testing survey were also analysed. Precision studies were performed by diluting a high-viral-titre patient sample into BKPyV-negative pooled plasma to create high-positive (6.16 log_{10} copies ml^{-1}) and low-positive (3.16 log_{10} copies ml^{-1}) samples. For precision studies of inter-assay variability, a high-positive (7.0 log_{10} copies ml^{-1}) and a low-positive (3.0 log_{10} copies ml^{-1}) sample were measured in 20 separate runs. The assay’s limit of quantification and limit of detection were 2.70 and 2.25 log_{10} copies ml^{-1}, respectively. The assay was linear from 2.70 to 9.26 log_{10} copies ml^{-1}. Of the 48 known positives, 43 were detected as positive, with three reported by the reference laboratory as values lower than the limit of detection. Two known positives at 3.27 and 3.80 log_{10} copies ml^{-1} tested negative by the m2000 BKPyV assay. Of the 49 known negative samples, 48 were negative by the m2000 BKPyV load assay, with one sample confirmed positive by a reference laboratory. Qualitative analysis prior to discrepancy testing demonstrated a sensitivity of 89.58 % and a specificity of 97.96 %. Precision studies demonstrated inter-assay coefficients of variation of 0.63 % (high positive) and 4.38 % (low positive). Genotyping was performed on 22 patient samples, of which 21 (95.45 %) were type I and one (4.55 %) was type II. In conclusion, the m2000 BKPyV viral load assay sensitivity, specificity, linear range, precision and cost effectiveness make it an attractive methodology for clinical laboratories using the Abbott m2000 RealTime System.

INTRODUCTION

BK polyomavirus (BKPyV) is a member of the family Polyomaviridae, which includes JC polyomavirus (JCPyV), Merkel cell carcinoma polyoma virus, Trichodysplasia spinulosa polyomavirus and simian virus 40 (Wiseman, 2009). Other human polyomaviruses that currently have not been demonstrated to cause disease include St Louis polyomavirus, WU polyomavirus, Malawi polyomavirus/ human polyomavirus 10 (HPyV10)/MX polyomavirus, KI polyomavirus, HPyV6, HPyV7 and HPyV9 (Korup et al., 2013; Yamaguchi et al., 2013; Yu et al., 2012).

Recently, two new polyomaviruses have been identified: vervet monkey polyomavirus 1 and HPyV12 were found in monkeys and the gastrointestinal tract of humans respectively (Korup et al., 2013; Yamaguchi et al., 2013). There are four major subtypes of BKPyV (I–IV) that play a significant role in disease (Randhawa et al., 2011). These types have sequence variability that may affect the efficiencies of quantitative BKPyV PCR assays, depending on the design of primers and probes (Randhawa et al., 2011). BKPyV and JCPyV share significant sequence similarities, making assay development a challenge in regard to specificity (Luo et al., 2008). BKPyV causes several diseases including tubulo-interstitial nephritis and ureteral stenosis in renal transplant...
BK viral load assay

METHODS

Sample preparation. EDTA anticoagulated blood was centrifuged at 1200 g for 20 min at 25 °C. Urine and plasma samples from renal and bone marrow transplant patients were stored at −80 °C in polypropylene tubes (TS01-5T, TS01Y; Simport), thawed at 25 °C and then centrifuged for 5 min at 2000 g prior to being placed in the m2000sp.

Primers and probe. Previously described BKPyV-specific primers and a probe targeting a conserved sequence within the VP1 locus of the BKPyV genome were used for amplification by TaqMan real-time PCR (Randhawa et al., 2004, 2011). The forward primer and probe sequences were identical in 17 representative BKPyV isolates representing all four subtypes, and the reverse primer binding region had two C→T mismatches located 11 and 17 bp from the 3’ end of the 21 bp sequence (data not shown). The assay is designed to be specific for BKPyV by targeting a region complementary to the forward primer that is not present in JCPyV strains (Luo et al., 2008). The primer sequences were: BKfwd, 5′-GACGCTCCCCAAAAGCCAAA-3′; and BKrev, 5′-CTGGGTTCAGAAGACTTCTA-3′. The previously published probe (Singh et al., 2009) contained 6-carboxyfluorescein (FAM) as the fluorophore and tetramethylrhodamine (TAMRA) as the quencher. We modified the probe by substituting an Iowa Black (IBLK1) quencher in place of TAMRA, and added a ZEN internal quencher: BK-FAM: 5′-ACCCGTGCA/GCCAAA-3′. The m2000sp extraction and PCR set-up. A minimum sample volume of 0.4 ml plasma or urine was placed in the m2000, with 0.2 ml extracted using the m2000sp DNA-Plasma-LL-200-070 laboratory-developed assay software protocol from Abbott Laboratories and Abbott mSample DNA Preparation System reagents (Abbott Molecular). A 50 × TaqMan exogenous internal positive control (29.2 μl; Applied Biosystems) was added to each bottle of lysis buffer sufficient for 24 extractions. The sample elution volume was 70 μl, with 15 μl added to 35 μl PCR master mix, consisting of 25 μl × Universal Master Mix (Applied Biosystems), 5 μl 10 × BKPyV primers/probe at 2.5 μM each, and 5 μl 10 × proprietary exogenous internal positive control primers/VIC probe (Applied Biosystems) by the m2000sp.

m2000rt parameters. Cycling parameters were: 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1.5 min, followed by 2 min at 60 °C and then a 4 °C hold, as described previously (Randhawa et al., 2011). Slow ramp mode, a threshold value of 0.03, an initial sensitivity of 0.005 and a final sensitivity of 2.50 were used according to the manufacturer’s recommendations. Acceptable standard curve slopes were between −3.73 and −3.01. An acceptable correlation coefficient (R²) was defined as >0.990.

Controls and standards. For initial studies, a plasmid DNA standard was used (pBKV strain MM, ATCC 45026) and diluted in

recipients, and haemorrhagic cystitis in bone marrow transplant recipients (Decker et al., 2009; Wiseman, 2009). JCPyV and BKPyV are ubiquitous in the general population, with the majority of children becoming seropositive early in life. Infections are usually subclinical, and the virus establishes latency in kidney tissue (Wiseman, 2009). During periods of immunosuppression, such as solid organ transplant (renal), reactivation can occur (Cimbaluk et al., 2009; Marce´n, 2009). In immunocompetent patients, reactivation can lead to ureteral stricture and haemorrhagic cystitis (Decker et al., 2009; Wiseman, 2009). The use of immunosuppressive drugs during renal transplantation has led to the development of a distinct entity of BKPyV-associated nephropathy (BKVAN) that occurs in approximately 5–10 % of patients (Cimbaluk et al., 2009; Ramos et al., 2009; Wiseman, 2009). BKVAN leads to renal failure and graft loss, and is characterized by graft dysfunction (Cimbaluk et al., 2009; Ramos et al., 2009). Reduction in immunosuppression is an important therapy for treatment of BKVAN, allowing the immune system to suppress virus replication (Cimbaluk et al., 2009; Höcker & Tönshoff, 2009; Marce´n, 2009; Ramos et al., 2009). Cicloforov and lefunomide have been reported to be an option for therapy in select cases (Cimbaluk et al., 2009; Marce´n, 2009; Ramos et al., 2009; Wu & Harris, 2008).

An important tool for monitoring BKPyV disease and response to therapy is PCR assessment of viral load (Bechert et al., 2010; Wiseman, 2009). Following viral load over time enables an adjustment of immunosuppressive therapy to maintain the optimal balance between controlling infection and preventing allograft rejection (Bechert et al., 2010; Limaye et al., 2001; Pang et al., 2007; Randhawa et al., 2004). A value of >4.00 log10 copies BKPyV ml−1 in plasma or >7.00 log10 copies BKPyV ml−1 in urine is a cut-off used in the literature to predict BKVAN, but because different BKPyV assays are currently in use by individual laboratories, serial monitoring using the same assay is more helpful in predicting disease (Bechert et al., 2010; Chung et al., 2012; Limaye et al., 2001; Miller et al., 2012; Randhawa et al., 2004). Multiple laboratory-developed assays have been used to monitor BKPyV viral load (Adeyi et al., 2005; Bechert et al., 2010; Biel et al., 2000; Costa et al., 2009; Funahashi et al., 2010; Iwaki et al., 2010; Kipp et al., 2005; Limaye et al., 2001; Marchetti et al., 2007; Moret et al., 2006; Pang et al., 2007; Randhawa et al., 2004). The Abbott m2000 RealTime System (m2000 hereafter) is a two-instrument extraction, PCR set-up and real-time PCR analysis system comprising the Abbott m2000sp and the m2000rt. The Abbott m2000sp fully automates the extraction of nucleic acid from each specimen and then dispenses the extracted DNA, along with a PCR amplification master mix, into a 96-well optical reaction plate, which is then transferred to the m2000rt for amplification and detection. The m2000 has been used for US Food and Drug Administration (FDA)-approved assays such as human immunodeficiency virus (HIV) viral load and chlamydia/gonorrhoea testing, but no BKPyV load assay using this platform has been reported (Bourlet et al., 2011; Cheng et al., 2011; Møller et al., 2010; Swanson et al., 2006, 2007). The objective of this study was to develop and validate a quantitative PCR assay for assessment of BKPyV viral load using the m2000sp.
pooled BKPyV-negative plasma. An AcroMetrix viral control standard panel containing intact viral particles (OptiQuant BKV Quantification Panel) was used according to manufacturer’s recommendation to generate a standard curve and calculate the copies ml⁻¹ of a high-titre (>9.00 log_{10} copies BKPyV ml⁻¹) patient urine. This high-titre sample was diluted in pooled BKPyV-negative plasma to form four calibrators (3.00, 4.00, 6.00 and 7.00 log_{10} copies ml⁻¹) and two controls (3.00 and 7.00 log_{10} copies ml⁻¹) for use in subsequent runs. A minimum of two positive controls (3.00 and 7.00 log_{10} copies ml⁻¹) and one negative control (BKPyV-negative pooled plasma) were included in every run. The four calibrators were run in duplicate in addition to the three controls above when changes to extraction or PCR reagent lots were made.

**Precision, limit of detection (LoD) and limit of quantification (LoQ).** Precision studies were performed by diluting another high-titre patient urine sample (>9.00 log_{10} copies BKPyV ml⁻¹) into BKPyV-negative pooled plasma to create high-positive and low-positive samples. Between-run variability for two specimens (high positive, 7.00 log_{10} copies ml⁻¹, and low positive, 3.00 log_{10} copies ml⁻¹) was assessed by 20 runs on 20 different days. Within-run variability for two specimens (high positive, 6.16 log_{10} copies ml⁻¹, and low positive, 3.16 log_{10} copies ml⁻¹) was assessed by testing eight replicates of each concentration within a single run. Acceptable precision was defined as coefficient of variation (CV) values of <10 % each for all concentrations analysed.

The LoD was determined by probit analysis and defined as the DNA concentration in log_{10} copies ml⁻¹ with a probit probability of 0.95. This was determined by testing a minimum of 15 replicates of virus standards diluted to the lower range of the assay (3.00, 2.70 or 2.00 log_{10} copies ml⁻¹) and measured in two separate runs. The LoQ (lowest concentration that could be quantified) was determined by analysing the CV and linearity (R²) of at least 13 replicates each of two sources of viral standards diluted to 3.00, 2.70 or 2.00 log_{10} copies ml⁻¹. The two standards used were a diluted positive patient sample and purchased virus standards (OptiQuant BKV Quantification Panel; AcroMetrix). The LoD and LoQ values were defined as acceptable if both were ≤3.00 log_{10} copies ml⁻¹.

**Validation samples.** A total of 97 known positive/negative BKPyV urine and plasma samples were analysed. Ninety-five of the samples were obtained from three separate national academic referral laboratories (laboratory A: 11 positive plasma, 11 positive urine, two negative plasma and one negative urine samples; laboratory B: 20 positive plasma samples; laboratory C: four positive plasma and 46 negative plasma samples). Two were proficiency samples obtained from the College of American Pathologists (CAP).

**Inhibition and interference.** AcroMetrix Inhibition Panel (Life Technologies) plasmas representing haemolysed, heparinized, lipemic and icteric samples were diluted 1:2 with BKPyV-positive plasmas (7.00 log_{10} copies ml⁻¹) and tested. BKPyV-positive or -negative plasma was spiked with laboratory stocks of *Candida albicans*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Proteus mirabilis*, *Klebsiella pneumoniae* and *Escherichia coli* to a final concentration of 6.18 log_{10} copies ml⁻¹; a patient HIV-1 plasma to a final concentration of 4.07 log_{10} copies ml⁻¹; or herpes simplex virus 1 or cytomegalovirus viral DNA to a final concentration of 5.16 log_{10} copies ml⁻¹. The pooled plasma used to test the assay for inhibition or interference with the above substances had either 6.00 log_{10} copies BKPyV ml⁻¹ or tested negative for BKPyV. Cross-reactivity for JCPyV was tested using four plasma samples positive for JCPyV and negative for BKPyV. Plasma triglyceride levels were measured on a Beckman Coulter UniCel DxC 800.

**BKPyV genotyping.** Twenty-two BKPyV-positive samples from our hospital population (12 plasma samples from kidney-transplant patients plus 10 randomly selected patient urine samples) were genotyped using methods described previously to assess our population for uncommon genotypes that may influence quantification (Krumbholz et al., 2006).

**Statistical analysis.** Excel version 14 (Microsoft) and GraphPad Prism version 4.03 were utilized for data analysis. Statistical analyses included linear regression and Pearson correlation (standard curve), an unpaired t-test and a ratio paired t-test (sample exchange comparisons). Probit analysis for the limit of detection was performed utilizing spss version 21.

**RESULTS**

In the initial studies of the dual-quencher ZEN probe, there was a marked decrease in background fluorescence from the traditional non-ZEN TaqMan probe (sevenfold reduction; P<0.0001), with a marginal increased sensitivity. As a result, the ZEN probe was chosen for the assay validation.

Precision studies for within-run variability demonstrated CV percentage values for the high- and low-positive samples were 1.49 and 6.95 %, respectively. For between-run variability, the CV percentage values for the high- and low-positive samples were 0.63 and 4.38 %, respectively. The range of acceptable values for the internal control was established upon analysis of 6 months of weekly patient run data as a cycle threshold (Ct) of between 26.0 and 29.0, correlating to the mean ± 3 SD.

Probit analysis demonstrated an LoD of 2.25 log_{10} copies ml⁻¹. The lower limit of LoQ was the lowest concentration of the analyte that could be quantified. The two standards used were a diluted positive patient sample and purchased virus standards from AcroMetrix. All samples responded linearly, but a higher CV (16.27 %, n=13) was observed in the standard diluted to 2.00 log_{10} copies ml⁻¹, compared with the 2.7 and 2.00 log_{10} copies ml⁻¹ standards. Therefore, the LoQ was defined as 2.70 log_{10} copies ml⁻¹. The assay was linear within this range (R²=0.999).

Serial dilutions of a BKPyV patient sample in pooled plasma demonstrated that the assay was linear (R²=0.997) from 2.70 to 9.26 log viral genome equivalents ml⁻¹ (Fig. 1). The PCR had an efficiency of 98.0 % where efficiency=(10⁻¹ m⁻¹/2)×100 (m is the slope of the line). Use of the BKPyV plasmid showed that the assay was linear up to 12.00 log_{10} copies ml⁻¹.

In our patient sample exchange, 43 of 48 known positive samples were positive and five were negative by the m2000 BKPyV viral load assay. Of the five discrepant samples, three samples (two from laboratory A and one from laboratory B) were reported by the reference laboratory as having values <2.70 log_{10} copies ml⁻¹. The remaining two samples from laboratory A that tested as negative by the m2000 BKPyV viral load assay were reported as having 3.27 and 3.80 log_{10} copies ml⁻¹. Of the 49 known negative samples, 48 tested negative by the m2000 BKPyV viral load assay. One sample reported negative from laboratory A that...
DISCUSSION

In our m2000 BKPyV viral load assay, we evaluated the use of a dual-quencher TaqMan probe design. The probe containing a ZEN internal quencher and 3' black hole quencher IBLK1 showed a marked decrease in background fluorescence compared with a TaqMan probe containing only 3' IBLK1 and therefore may be a better approach for other TaqMan assays. Further studies are required. Our initial studies used a diluted plasmid (pBKV) in pooled plasma to generate standard curves for evaluation of extraction methods, minimum volume requirements and probe comparisons. These results indicated higher levels of within- and between-run variability of plasmid samples compared with a diluted patient virus sample (data not shown), where intact virions are either better protected from degradation or have less variability in extraction. Because of the variability with plasmid pBKV, we used a commercial quantification panel containing intact virus to establish our calibrators and controls as primary reference materials.

As demonstrated in the validation experiments, the m2000 BKPyV viral load assay had an acceptable performance. Linearity ($R^2=0.997$) was shown over a wide range of plasmid and virus concentrations (12.00 and 9.26 log$_{10}$ copies ml$^{-1}$, respectively), which is particularly useful for testing urine samples that can have concentrations up to 5 logs higher than plasma. The LoD and LoQ were below 3.00 log$_{10}$ copies ml$^{-1}$ (2.25 and 2.70 log$_{10}$ copies ml$^{-1}$, respectively), and comparable to other BKPyV viral load assays (Funahashi et al., 2010; Iwaki et al., 2010; Pang et al., 2007). The assay had acceptable precision (CV $<10\%$) for within-run, between-run and between-day variability using virus at the high and low concentrations tested.

The sample exchange demonstrated a significant difference in our BKPyV assay results compared with the results reported by laboratories A and B. These differences are probably due to variability in extraction methods, PCR method, controls and standards as has been described previously (Hoffman et al., 2008; Preiksaitis et al., 2009; Trofe-Clark et al., 2013). Large inter-laboratory variability for BKPyV quantification is common, with results of proficiency testing showing a reported range of more than 3.10 log$_{10}$ copies ml$^{-1}$ for all laboratories (College of American Pathologists, 2012). Our study demonstrated this observation of inter-laboratory variability. The Bland–Altman plot in Fig. 2(c) showed a smaller mean difference when comparing laboratory A with our laboratory (UTMC; Fig. 2d) were more pronounced than the comparison in Fig. 2(c). The differences did not appear to change with increasing concentrations. Differences between laboratory B and our laboratory (UTMC; Fig. 2d) were more pronounced than the comparison in Fig. 2(c). The differences were tighter around the mean difference at higher concentrations, but this was tempered by a smaller number of samples at higher concentrations.
The development of a BKPyV standard is needed to improve the ability to compare assays across institutions; variability across laboratories is a common problem seen with BKPyV and with other assays (Hoffman et al., 2008; Pang et al., 2009; Preiksaitis et al., 2009). Our assay was within 1 SD of the mean with two CAP proficiency samples. The important factor in evaluating BKPyV quantitative viral load testing is to follow patient viral loads over time, measured using the same assay. Therapeutic decisions in renal transplant patients could be adversely affected by using multiple tests for a patient performed at different laboratories, and the interpretation of results must take this into consideration.

Recent data suggest that BKPyV assays may under-quantify genotypes other than type I strains (Luo et al., 2008; Randhawa et al., 2011; Trofe-Clark et al., 2013). Genotyping of a limited number of samples from our patient population (n=22) indicated that strains other than type I may be uncommon in our area. PCR-based studies have suggested that non-type I BKPyV strains infect a minority of adults worldwide, in contrast to the nearly ubiquitous chronic infections seen with type I strains (Pastrana et al., 2012). Estimates of type IV frequencies among populations range from <5 to 54%, but suffer from being based on data using non-universal BKPyV PCR primers (Pastrana et al., 2012). Because the study of non-type I strains on quantification used synthetic oligonucleotides as templates and not whole non-type I viruses, we are cautious in considering expansion of the assay to include genotypes that have not yet been identified in our target population of kidney-transplant patients (Randhawa et al., 2011). Our reporting of BKPyV test results cautions that results must be correlated with each patient’s clinical situation.

Inhibition and interference testing with a variety of substances indicated that only a lipaemic sample from a commercial source inhibited BKPyV detection. PCR inhibition by lipaemic samples from this supplier has been seen by others (Dingle et al., 2013). The inhibition was unable to be repeated using two different lipaemic patient samples, indicating that the commercial plasma may contain an inhibitory compound other than triglycerides. Cross-reactivity was not seen upon testing with a variety of bacteria and viruses (including JCPyV).

We estimated the materials required to be less than US$22 per reaction when batching a minimum of 18 samples. Other real-time BKPyV PCR assays such as Qiagen’s artus BK Virus RG PCR kit and Argene’s BK virus R-gene system are priced at US$37 and US$28 per reaction for materials, but require much greater hands-on time than our automated assay.

Our BKPyV assay is simple and requires little hands-on time for analysis of up to 45 specimens. The m2000sp instrument performs the extraction and PCR plate set-up,

![Fig. 2. Sample exchange studies for 20 positive samples received from laboratories A (a, c) and 17 positive samples received from laboratory B (b, d) respectively. (a) and (b) show reported (reference laboratory measurement) and measured (our BKPyV assay result) log_{10} copies ml^{-1}; (c) and (d) show Bland–Altman plots of laboratory A versus the University of Toledo Medical Center (UTMC) laboratory (c) and UTMC laboratory versus laboratory B (d), with the mean difference shown as a solid line, and bias (mean difference ±1.96 SD of the difference) shown as dashed lines.](image-url)
and utilizes bar codes for entry of specimen information. Once the m2000sp has finished processing, data are uploaded to the m2000rt PCR instrument for seamless transfer of specimen information. After the PCR has been completed, the m2000rt results can be transferred automatically into a laboratory information system using an interface. The hands-on time of 1 h for complete sample processing (sample centrifugation, instrument set-up, extraction, PCR plate set-up, real-time PCR and results review/release) is excellent, along with the overall assay time of 4 h and is comparable to similar assays utilizing automated systems (Amendola et al., 2011). The m2000 by Abbott Laboratories includes three FDA-approved assays for chlamydia/gonorrhoea testing, hepatitis C virus load and HIV load making the BKPyV assay easier to incorporate into a laboratory that has already adopted this testing protocol (Cheng et al., 2011; Möller et al., 2010).

In summary, the m2000 BKPyV viral load assay using the Abbott m2000 RealTime System is sensitive, has a wide linear range, performs well with urine and plasma samples, and is convenient with minimal hands-on time compared with the manual set-up of similar PCR assays.

ACKNOWLEDGEMENTS

We thank the University of North Carolina and ARUP Laboratories for providing the sample exchanges and the UTMC Pathology Laboratory chemistry technologists for providing triglyceride measurements. Biomedical institutional review board ethical approval was obtained for this study in compliance with the Declaration of Helsinki.

REFERENCES


