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

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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 log10 copies ml−1) and low-positive (3.16 log10 copies ml−1) samples. For precision studies of inter-assay variability, a high-positive (7.0 log10 copies ml−1) and a low-positive (3.0 log10 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 log10 copies ml−1, respectively. The assay was linear from 2.70 to 9.26 log10 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 log10 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 tubulointerstitial nephritis and ureteral stenosis in renal transplant...
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; Marcén, 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). BKVAN leads to renal failure and graft loss, and is characterized by graft dysfunction, and graft loss, and is characterized by graft dysfunction

**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′-CGACGTCCAAAA-GCCAAA-3′; and BKrev, 5′-CTGGGTATAGAAAGCCATTCTA-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/ ZEN/AGTGCCAAAACCTACTATAAAAAG-IBLK1-3′.

All primers and probe reagents were obtained from Integrated DNA Technologies (Coralville; IA, USA). In the initial studies, the probe was compared with a second identical TaqMan probe that did not contain the ZEN quencher. Serial low-level dilutions demonstrated increased sensitivity of the probe containing the ZEN quencher.

**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 2× 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 ($R^2$) 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
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 (>9.0 log₁₀ 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 log₁₀ copies ml⁻¹) and two controls (3.00 and 7.00 log₁₀ copies ml⁻¹) for use in subsequent runs. A minimum of two positive controls (3.00 and 7.00 log₁₀ 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-viral-titre patient urine sample (>9.0 log₁₀ 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 log₁₀ copies ml⁻¹, and low positive, 3.00 log₁₀ copies ml⁻¹) was assessed by 20 runs on 20 different days. Within-run variability for two specimens (high positive, 6.16 log₁₀ copies ml⁻¹, and low positive, 3.16 log₁₀ 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 log₁₀ 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 log₁₀ 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 log₁₀ 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 log₁₀ 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 log₁₀ 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 log₁₀ copies ml⁻¹; a patient HIV-1 plasma to a final concentration of 4.07 log₁₀ copies ml⁻¹; or herpes simplex virus 1 or cytomegalovirus viral DNAs to a final concentration of 5.16 log₁₀ copies ml⁻¹. The pooled plasma used to test the assay for inhibition or interference with the above substances had either 6.00 log₁₀ 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 (Krumholz 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 log₁₀ 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 log₁₀ copies ml⁻¹, compared with the 2.7 and 2.00 log₁₀ copies ml⁻¹ standards. Therefore, the LoQ was defined as 2.70 log₁₀ 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 log₁₀ 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 log₁₀ 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 log₁₀ 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
tested positive in our assay was confirmed positive by a separate laboratory. Qualitative analysis prior to discrepancy testing demonstrated a sensitivity of 89.58% and a specificity of 97.96%. After discrepancy testing, the sensitivity was 89.80% and the specificity was 100.00%.

The two CAP proficiency samples (VLS-07 and VLS-08) had reported mean ± SD values of 2.60 ± 0.99 and 5.38 ± 0.69 log_{10} copies ml^{-1}, respectively. Our assay detected 2.28 and 5.29 log_{10} copies ml^{-1}, respectively.

Fig. 2(a, b) shows linear regression data comparing our BK assay result with positive samples from laboratories A and B in a sample exchange. The results were highly linear with correlation coefficients of 0.876 for laboratory A and 0.809 for laboratory B. Compared with laboratory A, our assay reported a mean copy number measurement that was 0.34 log (twofold) higher (P=0.0413, ratio paired t-test). Laboratory B had a mean reported copy number that was 1.59 log_{10} copies ml^{-1} (39-fold) higher than our BKPyV assay (P<0.0003, ratio paired t-test). Fig. 2(c, d) shows Bland–Altman plots of laboratory A versus the UTMC laboratory and UTMC versus laboratory B.

BKPyV genotyping of plasma and urine samples from our patient population showed that 95.45% were type I (n=21) and 4.55% were type II (one urine sample). Inhibition and interference testing using haemolysed, heparinized, lipaemic, and icteric samples showed normal performance for all samples tested except the lipaemic samples with 1203 mg triglycerides dl^{-1}, which inhibited BKPyV detection. Testing of two patient lipaemic samples at 1157 and 858 mg triglycerides dl^{-1} showed no inhibition of BKPyV detection. Testing with laboratory stocks of C. albicans, Staphylococcus aureus, Streptococcus pneumonia, P. mirabilis, K. pneumoniae, Escherichia coli, HIV, cytomegalovirus and herpes simplex virus 1 showed no cross-reactivity or interference. Four JCPyV-positive, BKPyV-negative plasma samples were negative by the assay.

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 (pBK) 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 pBK, 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 for the same samples, and that the spread of the differences was significant. 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,

![Figure 2](image)

**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) log10 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.
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 analysis (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.

Laboratories includes three FDA-approved assays for differentiation of human polyomavirus DNA in undiluted 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


