Polyomavirus Infection in Renal Transplant Recipients

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List of Abbreviations

AcMNPV	*Autographa californica multiple nucleopolyhedrovirus*
AKI	Acute kidney injury / acute renal failure
AVF	Arteriovenous fistula
BKPyV	Human polyomavirus 1 / polyomavirus BK
BKyVAN	BK polyomavirus-associated nephropathy
BP	Base pair
C	Celsius
CKD	Chronic kidney disease
CMV	Cytomegalovirus
Ct	Cycle threshold
Cq	Cycle quantification
CV%	Coefficient of variation
dsDNA	Double-stranded deoxyribonucleic acid
E score	Efficiency
E. coli	*Escherichia coli*
EBV	Epstein–Barr virus
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assays
ESRD	End-stage renal disease / renal failure
EVGR	Early viral gene region
g	Gravitational force
GFR	Glomerular filtration rate
HBM	Honeybee melittin
HBV	Hepatitis B virus

HCV	Hepatitis C virus
HHV-6	Human herpesvirus 6
HHV-7	Human herpesvirus 7
HHV-8	Human herpesvirus 8
Hi5™	*Trichoplusia ni* insect cells
His-tag	Histidine tag
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPyV12	Human polyomavirus 12
HPyV6	Human polyomavirus 6
HPyV7	Human polyomavirus 7
HPyV9	Human polyomavirus 9
HPyVs	Human polyomaviruses
HRP	Horseradish peroxidase
ICTV	International committee on taxonomy of viruses
IMAC	Immobilized metal affinity chromatography
IU	International unit
JCPyV	Human polyomavirus 2 / polyomavirus JC
Kb	Kilobase
kDa	Kilodaltons
KIPyV	KI polyomavirus
KRT	Kidney replacement therapies
LTag	Large tumour antigen
LVGR	Late viral gene region
m	Slope
MAPK	Mitogen-activated protein kinases
MCPyV	Merkel cell polyomavirus

MCS	Multiple cloning site
miRNA	Micro ribonucleic acid
µL	Microliter
mL	Millilitre
mM	Micromolar
mRNA	Messenger ribonucleic acid
MWPyV / MXPyV	Malawi or Mexico polyomavirus
NCCR	Noncoding control region
ng	Nanogram
NJPyV	New Jersey polyomavirus
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pfu	Plaque forming unit
PML	Progressive multifocal leukoencephalopathy
PMP	Per million population
PP2A	Protein phosphatase 2a
pRb	Retinoblastoma-associated protein
PTLD	Post-transplant lymphoproliferative disease
PyVAN	Polyomavirus-associated nephropathy
qPCR	Quantitative polymerase chain reaction
R^2	Correlation coefficient
rBV	Recombinant baculovirus
RE	Restriction enzyme
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
Sf9	*Spodoptera frugiperda* insect cells

sTag	Small tumour antigen
STLPyV	Saint Louis polyomavirus
SV40	Simian vacuolating virus 40
TSPyV	TS polyomavirus
VL	Viral load
VLP	Virus-like particle
VP1	Viral protein 1
VP2	Viral protein 2
VP3	Viral protein 3
VZV	Varicella zoster virus
WUPyV	WU polyomavirus

Abstract

Polyomavirus-associated nephropathy (PyVAN) occurs in recipients of kidney transplantation. The development of PyVAN can cause a decrease or complete loss of the function of the kidney. Two of the human polyomaviruses BKPyV, JCPyV are known to be involved in the pathogenesis of PyVAN, and a further virus HPyV9 has been found in renal transplant recipients. A multiplex quantitative polymerase chain reaction (qPCR) for BKPyV, JCPyV, and HPyV9 DNA, together with an internal control molecule, was used to investigate polyomavirus infection in the late transplant period. Results for BKPyV, JCPyV were reported in IU/mL. The dynamic range, linearity and reproducibility of the assay was good (intra- and inter-assay variation for each viral target of <1% or less, with a wide range of target concentrations). In a cohort of 466 renal transplant recipients investigated late after transplant, blood samples from 27 were positive for BKPyV with a viral load of between log 2.97 IU/ml to log 8.48 IU/mL; 15 were positive for JCPyV with generally lower viral loads (log 3.55 and 6.56 IU/mL); 4 were HPyV9 positive but with only very low viral load (log 0.42 to log 1.25 copies/mL). The age range, sex, and ethnicity of positive patients were similar to those of the population from which the samples were drawn. BKPyV was detected most frequently (in 27 of the 466 patients; 5.8%), the BKPyV positive samples tended to be collected earlier after transplantation, with a median of 2.33 years (maximal follow up post-transplant was 34 years). Fewer patients were JCPyV positive even though the JCPyV positive blood samples tended to be even earlier after transplant (median collection time of 0.38 years). The low rate of detection and low viral load detected in HPyV9 positive samples does not support the role of HPyV9 in disease causation both early and late after transplant.

The VP1 proteins of BKPyV, JCPyV and HPyV9 were produced in insect cells using a baculovirus protein expression system using pOET8.VE2 transfer vector with flashBAC™ expression system and purified using nickel chelate purification of Histidine tagged proteins. Preliminary analysis of the protein was made in ELISA, but additional optimisation of expression and purification of the antigens will be required for use in the serological investigation of polyomavirus infection.

Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Mohammed K. Nagshabandi

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Copyright Statement

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Only with the grace of God, I could be here. I am forever grateful for his blessings.

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Chapter 1. Introduction

1.1. Background

1.1.1. Kidney function and disease

The renal system is responsible for the production and elimination of urine, which is excreted by the kidneys. The kidneys are the main functioning organs in the renal system. Kidneys serve three major functions: firstly, they act as a filter, clearing the body from toxins and by-products of metabolism, such as urea. Secondly, they act as regulators, controlling the body fluids and electrolytes to maintain a balance between acid and base concentrations. Finally, they perform essential hormonal actions, retaining healthy red blood cells and bones, normal blood pressure, and blood flow control (Boron and Boulpaep, 2016; Koeppen et al., 2009). The loss of kidney functions can be calamitous for patient health and life quality, with various complications that may lead to death. Some of the complications caused by kidney disease are adverse drug toxicity due to inability of clearance; anaemia, as the kidney produces erythropoietin which is fundamental to the production of red blood cells; osteopenia because the kidneys activate vitamin D and regulate calcium; and cardiovascular diseases as a result of defective blood pressure and flow control (Fink et al., 2009). Besides the socio-economic consequences on the individual and society, it is estimated that the National Health Service (NHS) in England spent around 1.5 billion pounds in direct costs on kidney disease treatment in 2010 (Kerr et al., 2012).

There are several terms used to describe kidney diseases, such as acute kidney injury (AKI) or acute renal failure, chronic kidney disease (CKD), and end-stage renal disease (ESRD) or renal failure. These terms describe the kidneys' filtration capacity as an indicator of renal function. Acute kidney injury (AKI) refers to a sudden insult to the kidney or abrupt kidney failure, which is reversible in most cases. Whereas the collective term, CKD comprises a series of heterogeneous disorders altering the kidneys' structure or function for a period of three months or more (KDIGO, 2013; Levey et al., 2005; National Kidney Foundation, 2002). Alteration or damage to the kidneys' structure produces pathologic abnormalities, which are observed by abnormal renal biopsy, abnormal radiological tests, proteinuria or the presence of cell casts in urine. Meanwhile, the reduction of renal function produces a decreased glomerular filtration rate (GFR) and abnormal serum electrolytes levels. Chronic kidney disease varies in severity from a mild reduction of function to ESRD or renal failure, where patients will require kidney replacement therapies (KRT). The different stages of CKD are classified according to the cause of kidney disease, the degree of GFR reduction, and the severity of proteinuria (KDIGO, 2013; Levey et al., 2005). For example, the most common cause of CKD in the UK is diabetic nephropathy accounting for 26.9% in 2014 (Caskey et al., 2016). Estimation of GFR (eGFR) is calculated using the concentration of serum creatinine and/or cystatin C employing multiple differential equations. The categorisation of CKD using eGFR classifies patients into six stages ending with eGFR <15 mL/min per 1.73 $m^{2,}$ which is renal failure and requires KRT. Proteinuria is measured by the presence of albumin in the urine, which is called albuminuria. Albuminuria is used to classify patients into three groups; normal, microalbuminuria, and macroalbuminuria. Albuminuria is calculated using the albumin to creatinine ratio in urine (KDIGO, 2013; Levey et

al., 2005; Levey and Coresh, 2012; National Kidney Foundation, 2002). The purpose of classification and stratification of chronic kidney disease is to allow proper management and monitoring of all patients with deficient kidney functions both before and after the implementation of KRT.

Chronic kidney disease (CKD) usually starts asymptomatically and progresses gradually until clinical manifestations are noticed. Determining the aetiology of CKD affects the management of CKD patients and provides an indication as to whether they will progress to ESRD (Feest, 2007). The most common cause of CKD is diabetes mellitus, where almost half of diabetic patients experience a reduction in their renal function (KDIGO, 2013). A further chronic disorder that causes CKD is hypertension (Feest, 2007). Other causes of CKD are infections such as post-streptococcal infection glomerulonephritis, genetic disorders such as adult polycystic kidney disease, drug-induced kidney injury which can be observed with the use of non-steroidal anti-inflammatory drugs, and mechanical urinary tract conditions such as urinary tract obstruction or vesicoureteral reflux (Evans and Taal, 2011).

The progress of CKD is gradual and silent; many patients first present with CKD complications such as anaemia. Examples of complications related to anaemia are; lethargy, exertional shortness of breath, reduced physical capacity, cold intolerance, and fatigue (MacDougall, 2011). Undiagnosed CKD patients may also present with cardiovascular manifestations such as pericardial effusion and uraemic pericarditis, due to the kidneys' inability to clear excess water as well as metabolic toxins such as urea. Other cardiovascular manifestations that CKD

patients may experience are generalised oedema, pulmonary oedema, shortness of breath, and left ventricular hypertrophy (Lees et al., 2015; Wheeler, 2007).

1.1.2. Renal failure and kidney replacement therapies

Chronic Kidney Disease (CKD) is a challenging worldwide health problem that has been progressively increasing, resulting in a rise in renal failure cases (Fink et al., 2009; Jha et al., 2013; Sharaf El Din et al., 2016; USRDS, 2016a). End-stage renal failure or ESRD is the last stage of kidney disease where the patient does not have a functioning kidney. In 2014 the reported incidence of ESRD in the UK was around 115 per million population (PMP) per year an escalation from 109 PMP per year in 2013, with KRT commencing in 7,411 new patients (Caskey et al., 2016). Similarly, the incidence in the United States was 363 PMP per year in 2013, showing a steady increase over the past thirty years (USRDS, 2016b). Because of the serious adverse consequences of ESRD on the health and the quality of life of patients, initiation of Kidney replacement therapy (KRT) is necessary. Kidney replacement therapies (KRTs) such as haemodialysis or peritoneal dialysis can eliminate toxins and regulate the fluid balance allowing prolongation and improved quality of life, compared to no intervention. On the other hand, renal transplantation can restore most of the native kidney functions such as the hormonal activities. Thus, renal transplantation can produce better long-term outcomes than prolonged dialysis. In 2014, there were 58,968 adult patients in the UK receiving KRTs, showing a 4% increase in comparison to 2013. The prevalence of KRT in 2014 was 913 PMP almost doubling the prevalence in 2000 (Caskey et al., 2016) while displaying a steady increase in survival incidence over the past fourteen years in both short and long-term survival rates.

However, even with the use of KRTs, there is an elevated overall relative risk of death compared to the general population, according to the UK renal registry (Caskey et al., 2016).

Renal dialysis or kidney replacement therapies (KRT) are techniques that artificially mimic the kidneys filtration process, through two main mechanisms: diffusion and convection (solvent drag). KRTs can be either haemodialysis or peritoneal dialysis (Mohammed, 2010). Haemodialysis is carried out by transferring the patient's blood into a dialysis machine, where the filtration process occurs. Haemodialysis was first developed to manage AKI through continuous filtration in emergency cases during the nineteen forties. It was not until the nineteen sixties that haemodialysis became the management of choice for ESRD, due to advances in filtering membranes, blood anticoagulants and the introduction of a dependable access point to the blood circulation in the form of arteriovenous fistulae (AVF) (Wong et al., 2015). The other main dialysis modality is peritoneal dialysis, which is less lifestyle intrusive than haemodialysis and replaces the blood circulation access with a peritoneal cavity catheter. Peritoneal dialysis is the preferred primary dialysis for ESRD patients and around one-fifth of ESRD patients in the United Kingdom start with it. Peritoneal dialysis mechanism leverages the natural filtration capacity of the peritoneal membrane. Filtration occurs by repeatedly introducing an osmotic differential between the patients' circulation and a peritoneal dialysis fluid, forcing toxins to filter through the peritoneal membrane (Ellam and Wilkie, 2007; Mohammed, 2010).

Each KRT modality has some limitation that ultimately favours renal transplantation. The most common complication is depression due to life disruption by frequent and recurring hospital visits (Mohammed, 2010). Another example of haemodialysis complications is the increased risk of fatal systemic infections due to the requirement to have a readily available vascular access (Bray et al., 2012; Davenport, 2015). Different vascular accesses also have specific complications such as the vascular access of choice which is the arteriovenous fistulae (AVF) which can cause thrombosis, haemorrhage, and high shunt failure rate that requires further surgical procedures for shunt correction (Salahi et al., 2006). Meanwhile, peritoneal dialysis increases the risk of death by 16% most commonly due to infectious peritonitis (Cheuk-Chun and Kam-Tao Li, 2010). Other complications of peritoneal dialysis are dialysis catheter blockage, hypoalbuminemia, as well as the eventual progressive alteration of the peritoneal membrane leading to the loss of filtration ability. Another fatal condition called encapsulating peritoneal sclerosis is associated with lengthy dependency on peritoneal dialysis (Ellam and Wilkie, 2007).

1.1.3. Renal transplantation and associated immunotherapy

The favoured treatment for ESRD is kidney transplantation chosen by 53% of patients in the UK in 2014 (Caskey et al., 2016), which has been performed for 3,272 patients in 2017/18 an increase of 7% compared to 2016/17 (NHS Blood and Transplant, 2017). Renal transplantation improves the patients' quality of life and decreases the mortality risk by ten-fold when compared to maintenance renal dialysis (Ojo et al., 1994; Port, 1993; Schnuelle et al., 1998; Suthanthiran and Storm, 1994); as long-term dialysis has a 20% associated annual mortality risk

(Evans and Taal, 2011). In addition, some studies have reported a decrease in the inflammatory status in renal transplant recipients (Cueto-Manzano et al., 2005; Ducloux et al., 2004). Another benefit is the decreased risk of death due to cardiovascular disease compared to dialysis patients (Jardine et al., 2011). Transplantation has better survival rates and quality of life in comparison to peritoneal or haemodialysis (Snyder et al., 2002), due to improvements in pre- and post-transplantation procedures, including patient follow-ups and immunotherapy (Caskey et al., 2016; Matas et al., 2014). Protecting the transplanted kidney is of utmost importance as about seven per cent of patients spend up to seven years on transplantation waiting lists (NHS Blood and Transplant, 2017), and almost ten per cent of patients die waiting for a suitable kidney for transplantation (Rabbat et al., 2000), this requires a delicate balance between graft rejection and control of post-transplant infections such as polyomavirus-associated nephropathy.

Renal transplantation success is measured through graft survival rates. Graft survival rates have been improving over the last five decades. Five-year graft survival has improved using both deceased and living donors' kidneys reaching 86% and 93% respectively. Similarly, ten years of patient survival after receiving a deceased donor kidney has increased and is now at 75% (NHS Blood and Transplant, 2018). These survival improvements can be attributed to advancements in immunosuppression therapies.

Immunosuppression is required post-renal transplantation to prevent rejection of the grafted kidney. However, the overall aggressiveness of the

immunosuppression, whether used in induction, maintenance, or acute allograft rejection has been associated with increased risk of infection. As infections are the most common cause of mortality in the early post-transplantation period and the second most common cause of mortality and graft failure overall, careful monitoring of immunosuppression is recommended (Briggs, 2001; Caskey et al., 2016; Meier-Kriesche et al., 2002; Rubin, 1993).

Despite improvements in the understanding of immunotherapy, there is no consensus on the ideal regimen for immunosuppression, whether for induction or maintenance. The immunosuppression in renal transplantation begins with induction therapy, which is targeted to reduce the incidence of acute rejection in the early post-transplantation period. Induction therapies can be divided into two main regimens where the first one uses high doses of conventional immunosuppressive agents such as calcineurin inhibitors and glucocorticoids, while the second one uses antibody therapies against T-cell (e.g. basiliximab) or B-cell antigens (e.g. rituximab) in addition to lower doses of conventional agents. Meta-analyses have found the second method which utilises antibodies to be more effective in reducing renal allograft rejection (Carpenter, 1990; Szczech et al., 1998, 1997; Webster et al., 2004). Maintenance therapy can consist of double agents calcineurin inhibitors such as tacrolimus and antimetabolite such as mycophenolate mofetil or triple agents with the addition of glucocorticoids; however, minimising immunotherapy is always advocated. Calcineurin inhibitors; cyclosporine, tacrolimus or its newer derivative sirolimus are macrolides that inhibit T-cells activation and proliferation, while antimetabolites such as mycophenolate mofetil, mycophenolic acid, or azathioprine prevent B- and T- cell

proliferation (Galliford and Game, 2009; Halloran, 2004; Jordan et al., 1990; Ponticelli, 2011).

1.1.4. Renal transplantation associated viral infections

Viral infections cause significant morbidity and mortality in renal transplant patients. As mentioned, the continuously required immunosuppression preventing allograft rejection post-renal transplantation is linked to increased risk of developing opportunistic infections, of which viral infections are the most common (Karuthu and Blumberg, 2012; Splendiani et al., 2005). These infections occur at every stage post-transplantation (Fishman, 2017)(Figure 1). They may be acquired through the donated kidney (e.g. cytomegalovirus and Epstein-Barr virus), reactivation of latent infections (e.g. varicella-zoster virus), or by direct exposure (e.g. influenza virus and Respiratory syncytial virus) (Cukuranovic et al., 2012). There are many transplantation-related viral infections, including viruses of the *Herpesviridae* family, hepatitis viruses B (HBV) and C (HCV), human immunodeficiency virus (HIV), and polyomaviruses mainly BK and JC. Viruses of the *Herpesviridae* family causing post-transplantation infections are human herpes simplex virus 1 (HSV1), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (CMV), human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7), and human herpesvirus 8 (HHV-8) (Weikert and Blumberg, 2008).

The most common transplantation-related viral infection is CMV (Cukuranovic et al., 2012). CMV infection has been linked with acute and chronic renal allograft

rejection (Martin-Gandul et al., 2015). Cytomegalovirus (CMV) infection in recent years has seen a decrease in incidence in transplant recipients due to a number of prevention strategies, including improved human leukocyte antigen (HLA) and CMV serological immune status matching between the donors and the recipients, and the availability of antiviral prophylaxis (Fischer and Lu, 2013; Karuthu and Blumberg, 2012). CMV infection has been found to be associated with a higher risk of developing BKPyV infection and polyomavirus associated nephropathy (PyVAN) (Toyoda et al., 2005), as well as EBV, HHV-6, and HHV-7 infections (Kotton, 2005). Established infection with CMV requires immunosuppression modification and antiviral treatment with intravenous ganciclovir or oral valganciclovir (Åsberg et al., 2009).

Post-transplantation lymphoproliferative disease (PTLD) develops as a consequence of EBV infection, which is found to be more aggressive in renal transplant recipients. EBV seronegative renal transplant recipients were noted to be at a higher risk of developing PTLD (Morton et al., 2014). Management of PTLD involves immunosuppression modulation in the form of reduction of immunotherapy and administration of the monoclonal anti-CD20 antibody rituximab (Karuthu and Blumberg, 2012). Furthermore, modulation of the adoptive immunotherapy using EBV specific cytotoxic lymphocytes in addition to rituximab showed promising results (Jiang et al., 2016). Moreover, antiviral chemotherapy has been proposed, however, a specific consensus on antiviral chemotherapy is not yet reached (Dierickx and Vergote, 2019).

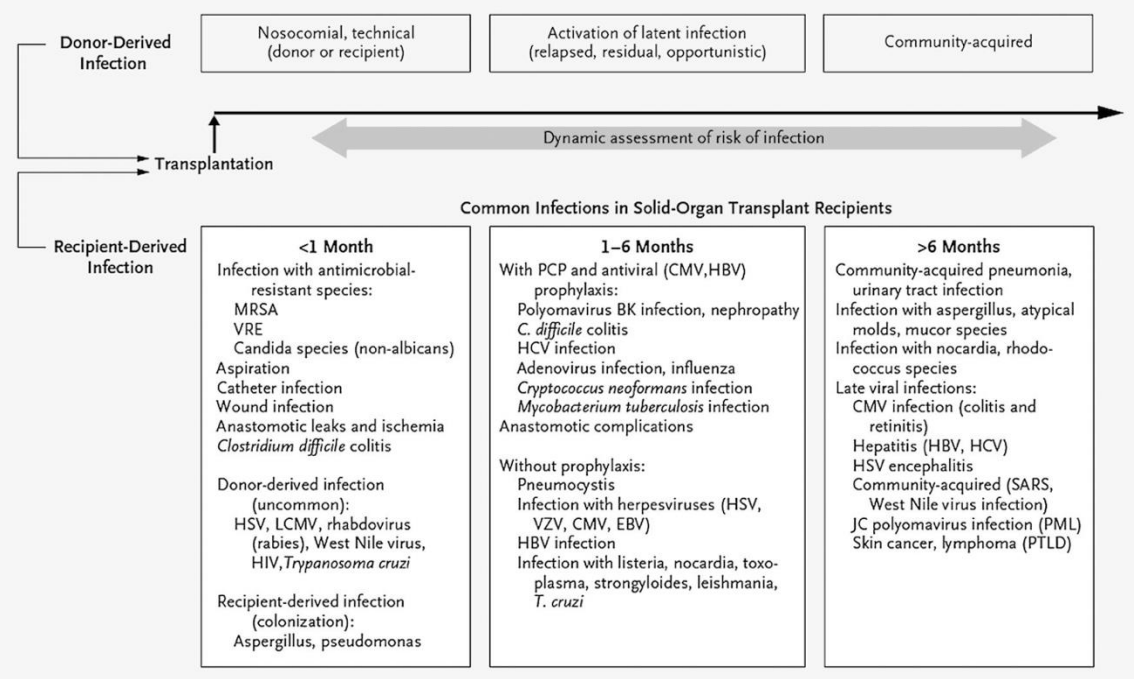
Renal transplantation candidates who are on haemodialysis are at higher risk of contracting viral hepatitis; however, infection with HBV and HCV can be acquired through the donated kidney. Viral hepatitis in renal transplant recipients is associated with higher morbidity and mortality. Therefore, screening before transplantation of both the donor and the recipient is necessary (Unal et al., 2018). Due to increased successfulness of treating HCV infection, pre-transplantation and complicated treatment post-transplantation, it is advised to treat both donor and recipients HCV candidates before transplantation (Karuthu and Blumberg, 2012; Morales et al., 2012; Spearman et al., 2019). Recently HBV infection has been more successfully managed post-transplantation due to the advancement in HBV antiviral treatments thus allowing the consideration of HBV patients for transplantation (Karuthu and Blumberg, 2012; Levitsky and Doucette, 2013).

Patients with HIV infection used to be disqualified from transplantation, however, studies have shown that selected HIV patients, such as patients with well-controlled HIV infection who have no malignancies, have the same survival rate as over 65 years old non-HIV patients (Stock et al., 2010). Nonetheless, the challenge in HIV patients is in controlling the interactions between immunosuppression and antiretroviral treatment (Blumberg and Stock, 2009).

Polyomavirus infections and subsequently (PyVAN), have become more frequently reported with the increasing use of vigorous immune suppression agents (van Doesum et al., 2017).

Some viruses' infections cause high morbidity and mortality in renal transplant recipients such as West Nile virus (WNV) (Cushing et al., 2004) and human T-lymphotropic viruses (HTLV) (Toro et al., 2003); therefore, donors with such viruses are generally avoided.

Figure 1: Solid organ transplantation related infections.



CMV, cytomegalovirus; EBV, Epstein-Barr virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HSV, herpes simplex virus; LCMV, lymphocytic choriomeningitis; MRSA, methicillin-resistant *Staphylococcus aureus*; PCP, *Pneumocystis jiroveci* pneumonia; PML, progressive multifocal leukoencephalopathy; PTLD, post-transplant lymphoproliferative disorder; VRE, vancomycin-resistant enterococci; VZV, varicella-zoster virus. Taken from: (Karuthu and Blumberg, 2012) Original Figure in (Fishman, 2007).

1.2. *Polyomaviridae* family

The *Polyomaviridae* family of viruses are a group of small, non-enveloped, icosahedral viruses. They range in size between 40nm to 45nm and contain a circular double-stranded deoxyribonucleic acid (dsDNA) of around 5000 base pairs (bp) long (Jiang et al., 2009). This family of viruses infects a wide range of hosts, ranging from mammals such as chimpanzees, rodents, and humans, to other vertebrates such as birds and fish (Buck et al., 2016). Ever since the discovery of the first polyomavirus during the fifties in association with multiple murine tumours (where the name *poly-oma* is derived from) the membership of this group of viruses has expanded, now reaching around one hundred different species (Buck et al., 2016; Stewart et al., 1957; Walker et al., 2019). Interest in studying these viruses was triggered by the accidental contamination of the live-attenuated (Sabin) oral polio vaccine used in the fifties and sixties with a monkey polyomavirus called simian vacuolating virus 40 (SV40). This virus is known to be oncogenic in primates. To date, the linkage of SV40 infection as a cause of human disease remains controversial (Dang-Tan et al., 2004; Garcea and Imperiale, 2003). One explanation for the lack of evidence of human disease caused by SV40 is the observed host exclusivity of these viruses, where the group of viruses that infect humans, the human polyomaviruses (HPyVs), are known to only cause human disease.

1.2.1. Taxonomy and nomenclature

Originally, there was only one genus under the *Polyomaviridae* family called *Polyomavirus*, as there were only a small number of viruses. The last decade has

seen an exponential rise in the discovery of polyomaviruses, particularly due to the rapid advancements in novel molecular methods for virus discovery and in next-generation sequencing techniques, which in turn made classification more problematic (Feltkamp et al., 2013). To date, there are 102 different polyomaviruses (Walker et al., 2019). The first classification proposed by the International Committee on Taxonomy of Viruses (ICTV) introduced three genera. Two genera that infected mammalian species (*Orthopolyomavirus* and *Wukipolyomavirus*) and one genus that infected avian species (*Aviopolyomavirus*) (Johne et al., 2011). Recently, the ICTV revised the classification to produce a more robust system utilising primarily host species and genomic sequences of the large tumour antigen protein (LTag) for classification, dividing the family into four genera (*Alpha-, Beta-, Gamma-,* and *Delta-polyomavirus*, and a non-assigned group). *Alphapolyomavirus*, *Betapolyomavirus,* and *Deltapolyomaviurs* are unique to mammalian hosts, whereas *Gammapolyomavirus* are avian specific. This new system also introduced a standardised polyomavirus species naming scheme, where the host species name precedes the word polyomavirus, then the pair is followed by a number that is both consecutive and chronological in order of discovery. For example, human polyomavirus 9 (HPyV9) (Calvignac-Spencer et al., 2016; Walker et al., 2019).

1.2.2. Human polyomaviruses

There are currently fourteen HPyVs (Wang et al., 2019a). The initially discovered HPyVs were BK polyomavirus (BKPyV) and JC polyomavirus (JCPyV), both viruses being named after the initials of the patients from whom they were first

isolated in 1971. BKPyV was discovered in the urine of a renal transplant recipient suffering from ureteral stenosis, while JCPyV was found in the brain tissue of a Hodgkin's lymphoma patient who presented with progressive multifocal leukoencephalopathy (PML) (Gardner et al., 1971; Padgett et al., 1971). BKPyV is known to cause nephropathy and ureteral stenosis in renal transplant recipients, as well as to cause haemorrhagic cystitis in hematopoietic cell transplant recipients. The remaining HPyVs were all discovered in the last decade (Feltkamp et al., 2013; White et al., 2013), these are as follows: KI polyomavirus (KIPyV), WU polyomavirus (WUPyV), and LI polyomavirus (LIPyV), all of which are named after the institutions where they were discovered (Karolinska Institute, Washington University, and Lyon International Agency for Research on Cancer, respectively (Allander et al., 2007; Gaynor et al., 2007; Gheit et al., 2017); Merkel cell polyomavirus (MCPyV) discovered in a patient suffering from Merkel cell carcinoma (Feng et al., 2008); TS polyomavirus (TSPyV) associated with a rare follicular skin condition affecting immunocompromised patients called Trichodysplasia Spinulosa (van der Meijden et al., 2010); Malawi or Mexico polyomavirus (MWPyV, MXPyV); Saint Louis polyomavirus (STLPyV); and New Jersey polyomavirus (NJPyV) all named after the geographical area where they were identified (Ehlers and Wieland, 2013; Mishra et al., 2014). Finally, human polyomavirus 6 (HPyV6), human polyomavirus 7 (HPyV7), human polyomavirus 9 (HPyV9), and human polyomavirus 12 (HPyV12), were all named using the ICTV nomenclature method and have no alternative names (Ehlers and Wieland, 2013; Ho et al., 2015; Schowalter et al., 2010; Scuda et al., 2011). A summary of relevant information about HPyVs and associated illnesses is shown in Table 1.

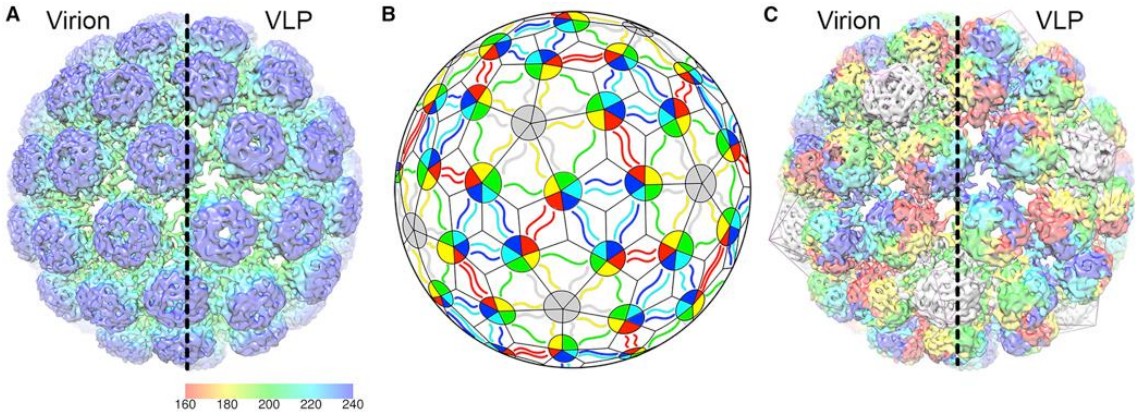
Table 1: Human Polyomaviruses (HPyVs).

Species	Abbreviation	Discovery year	Genome length in (bp)	GenBank accession number	NCBI reference accession number	Associated diseases
Alphpolyomavirus						
Human polyomavirus 5	MCPyV	2008	5387	HM011556	NC_010277	Merkel cell carcinoma
Human polyomavirus 8	TSPyV	2010	5232	GU989205	NC_014361	Trichodysplasia spinulosa
Human polyomavirus 9	HPyV9	2011	5026	HQ696595	NC_015150	PyVAN
Human polyomavirus 12	HPyV12	2013	5033	JX308829	NC_020890	Diarrhoea
Human polyomavirus 13	NJPyV	2013	5108	KF954417	NC_024118	
Beta-polyomavirus						
Human polyomavirus 1	BKPyV	1971	5153	V01108	NC_001538	PyVAN, haemorrhagic cystitis, ureteric stenosis
Human polyomavirus 2	JCPyV	1971	5130	J02226	NC_001699	PML, PyVAN
Human polyomavirus 3	KIPyV	2007	5040	EF127906	NC_009238	Respiratory infections, diarrhoea,
Human polyomavirus 4	WUPyV	2007	5229	EF444549	NC_009539	Respiratory infections, diarrhoea,
Deltapolyomavirus						
Human polyomavirus 6	HPyV6	2010	4926	HMO11560	NC_014406	
Human polyomavirus 7	HPyV7	2010	4952	HMO11560	NC_014407	Pruritic rash, viraemia in lung transplant recipients
Human polyomavirus 10	MWPyV, MXPyV	2012	4927	JQ898291	NC_018102	
Human polyomavirus 11	STLPyV	2013	4776	JX463183	NC_020106	
Unassigned group						
Human polyomavirus 14	LIPyV	2017	5269	KY404016	NC_034253	

1.2.3. Viral structure

The virion structure of this family is conserved, making them indistinguishable morphologically. The virions are small spherical particles ranging between 40 to 45 nm, composed of 88% protein while the remainder is made up of DNA. They are resistant to heat, preserving their infectious properties even after submersion in water at 55° C for 1 hour. These viruses are non-enveloped with a skewed icosahedral protein capsid in a T=7 Dextro quasi-symmetry. The protein capsid is composed of three structural proteins encoded by the virus; viral protein 1, 2, and 3 (VP1, VP2, VP3), where VP1 makes the majority of the viral capsid accounting for 80%, while the remaining one-fifth is made up by both VP2 and VP3. An additional structural viral protein (VP4) has not been observed in human polyomaviruses, despite the finding of its' ORF in BKPyV and JCPyV genome (Daniels et al., 2007). There are 360 copies of VP1 making 72 VP1 beta-barrel shaped pentamer or capsomers in six rotational axes conformation, linked together by C-terminal segment found in each VP1 monomer, as well as holding different pentamers together, creating the major capsid (Figure 2) (Hurdiss et al., 2016). VP1 protein capsomers are the facilitator of viral attachment and cellular uptake. Inside each VP1 pentamer shell, reside minor capsid proteins VP2 and VP3 in a ratio of 1:1:5 in relation to VP1, bound to the inner surface, creating the minor capsid. These minor capsid proteins aid in viral packaging and may have a role in the viral attachment (Belnap et al., 1996; Boothpur and Brennan, 2010; Hirsch et al., 2013; Hurdiss et al., 2016; Shah, 2001). However, recombinant VP1 major capsid alone has the ability to self-assemble into a virus-like particle (VLP), giving it the capacity to be used to package dsDNA molecules as a delivery system, as well as a method to elicit an immune response against HPyV infections (Gillock et al., 1997; Li et al., 2003).

Figure 2: BKyV structure schematics derived from Cryo-EM images of the VLP and Virion.



(A) External view of the virion and VLP. (B) An architectural representation of the outer virion structure. (C) Cryo-EM image of the virion and VLP, highlighting structural units. (Adapted from (Hurdiss et al., 2016).

1.2.4. Genomic construction and organization

The genome is a circular dsDNA coiled around four host cell-derived chromatin histones H2a, H2b, H3, and H4, creating a super-helical mini-chromosome, which is around 5k BP in size. Each viral capsid carries a single circular dsDNA. The genome is divided into three major regions; the early viral gene region (EVGR), the late viral gene region (LVGR), and the noncoding control region (NCCR). The transcription of the EVGR and LVGR occurs in opposite directions away from the NCCR (Figure 3).

The NCCR is a nucleosome-free region about 400 bp in size and resides in between the EVGR and LVGR. The viral DNA replication origin sequences (ori, TATA-, and TATA- like) of both early and late viral gene transcription as well as

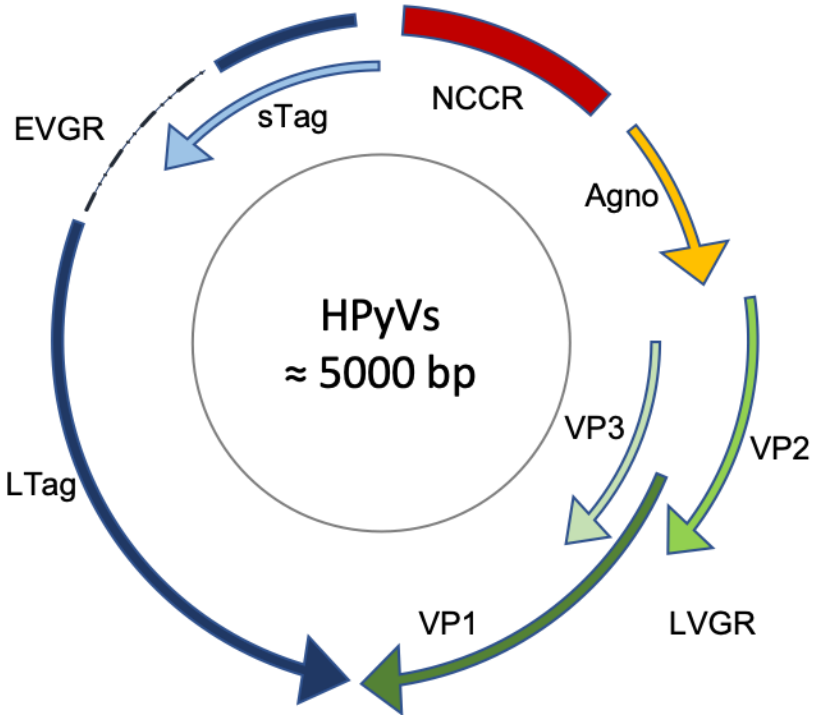
the bidirectional promoter/enhancer sequences are found within the NCCR. The NCCR is also referred to as the transcription control or regulatory region of the genome, as it coordinates the expression of viral genes according to the host cellular state. It also harbours the binding sites of the viral large tumour antigen (LTag) (Hirsch et al., 2013; Knipe and Howley, 2013; Krumbholz et al., 2009; Rinaldo et al., 2013; Shah, 2001). The configuration of the NCCR has been shown to be susceptible to mutations in the form of partial or complete duplication, or insertion or deletion of certain blocks. These mutated NCCRs are called rearranged NCCR (rr-NCCR). Rearranged NCCRs (rr-NCCR) has been found in patients suffering from polyomavirus infections such as BKyVAN in a renal transplant recipient and are associated with increased plasma viral load and disease severity. Also, progressive multifocal leukoencephalopathy (PML) patients have higher severity when associated with rr-NCCR JCPyV infection (Gosert et al., 2010, 2008).

The EVGR encodes the viral non-structural proteins the LTag and the small tumour antigen (sTag), which interact with the target host cells. These open reading frames (ORFs) are transcribed by alternating splicing of a single messenger ribonucleic acid (mRNA) prior to viral replication. Furthermore, LTag and sTag have identical N-terminal amino acids as they utilise the same start codon. There are other unique non-structural proteins produced in some HPyVs through different mRNA splicing (e.g. BKPyV, JCPyV, and MCPyV) (Hirsch et al., 2013; Knipe and Howley, 2013; Krumbholz et al., 2009; Rinaldo et al., 2013; Van Ghelue et al., 2012; White et al., 2013).

The LVGR contains genes encoding the viral capsid proteins (VP1, VP2, and VP3) which assemble to form the nucleocapsid. These structural proteins are transcribed in the same manner as the non-structural proteins, through alternating splicing of a common mRNA, in the opposite direction and strand of the EVGR. In addition to the structural proteins encoded by LVGR, both BKPyV and JCPyV also encode agnoprotein a small non-structural cytoplasmic protein that possesses regulatory abilities (Myhre et al., 2010; Unterstab et al., 2010; Van Ghelue et al., 2012; White et al., 2013).

Multiple HPyVs such as BKPyV and JCPyV produce microRNA (miRNA) which has been found to control both host and viral gene expression. HPyVs miRNA may play an important role in immune evasion mechanism (Bauman and Mandelboim, 2011; Broekema and Imperiale, 2013; Seo et al., 2008).

Figure 3: HPyVs genome.



HPyVs have circular dsDNA. Early viral gene region (EVGR) encodes large tumour antigen (LTag) and small tumour antigen (sTag). Late viral gene region (LVGR) encodes structural viral proteins; VP1, VP2, VP3, and agnoprotein (Agno). The noncoding control region (NCCR) located between EVGR and LVGR contains TCR and Ori. Adapted from (Java et al., 2012).

1.2.5. Viral proteins

The HPyVs produce two main groups of proteins, structural and non-structural proteins. The non-structural proteins LTag and sTag are responsible for controlling viral replication through interaction with host cells. They are produced early after the infection of the host cell and have been suggested to play a role in oncogenesis through binding to the host tumour suppressor retinoblastoma-associated protein (pRb) and related proteins (Trang et al., 2017). Additionally, TSPyV produces other tumour antigens such as; middle tumour antigen (mTag),

and alternative tumour antigen (ALTO) (van der Meijden et al., 2015). The other group of proteins produced by HPyVs are the capsid structural proteins VP1, VP2, and VP3. Other than providing the structure of the virion, they play a significant role in the attachment and entry of the virus to the host cell (DeCaprio and Garcea, 2013).

1.2.6. Genotypes and subtypes

Several HPyVs have been further classified into serotypes according to the immune response to their antigens as well as genotypes by differences in the genomic sequences. BKPyV has been further classified into four (I-IV) main genotypes according to variation in the major capsid protein VP1 gene sequence. Each BKPyV genotype presents a different serotype (Pastrana et al., 2013). BKPyV genotype I, which, is the predominant group, is further sub-divided into four sub-types (Ia, Ib-1, Ib-2, and Ic). The second most common BKPyV genotype is IV and has the following sub-types (IVa1, IVa2, IVb1, IVb2, IVc1, and IVc2) (Ikegaya et al., 2006; Zheng et al., 2007). On the other hand, JCPyV only one dominant serotype with several genotypes, classified either based on the complete genome or a sub-section of the genome (Shah, 2001). Using a sub-section of the genome called the IG region, which is 601bp long, JCPyV is found to have 12 different genotypes, although the complete genomic sequence of JCPyV suggests there are three different super-clusters (A, B, and C) in addition to several minor clusters (Khalili and Stoner, 2004).

HPyVs have distinct geographical distributions. BKPyV type I is most common and has a global geographical distribution. This distribution may be explained by the work of (Nukuzuma et al., 2006) who showed this type is more successful at replication in human renal epithelial cells. While type IV has been mainly detected in Europe and Asia, type III is more frequently found in Africa. JCPyV also shows recognizable geographical distribution, for example, IG genotypes; type 1 is found in the United States, type 2 in Asia and type 3 in Africa (Boukoum et al., 2011; Cubitt, 2006; Gard et al., 2015; Knowles, 2005; Luo et al., 2009; Nukuzuma et al., 2006; Sugimoto et al., 2002; Takasaka et al., 2004; Tremolada et al., 2010; Trofe-Clark et al., 2013).

1.2.7. Epidemiology

Seroprevalence studies of HPyVs infections revealed that this group of viruses is ubiquitous among humans with seroprevalence increasing with age (Kamminga et al., 2018). The most extensively studied viruses, BKPyV and JCPyV, are known to establish lifelong persistent infections explaining the increase in seroprevalence with increasing age (Chesters et al., 1983). Currently, favoured serological tests are enzyme-linked immunosorbent assays (ELISA) that detect antibodies to the major capsid protein VP1 (Li et al., 2015, 2003). In adults, BKPyV seroprevalence ranges between 40 and 94%, while JCPyV seroprevalence ranges between 32 and 85%. Seroconversion to BKPyV peaks in late childhood, while JCPyV shows a steady and gradual increase until adulthood (Egli et al., 2009; Kean et al., 2009; Knowles, 2005; Viscidi et al., 2011). Despite the high percentage of serologically positive individuals, HPyVs are not associated with causing significant diseases in immunocompetent

individuals. The detection of the viral dsDNA in the blood using molecular techniques such as polymerase chain reaction (PCR) termed viraemia is uncommon. However, viral detection in urine termed viruria is more common, affecting almost one-third of the adult population. The percentage of viruria is even higher in pregnant women and patients with altered immune status such as those receiving immune-modulatory therapies, where it may reach 50% (Drachenberg et al., 2007; Egli et al., 2009; Hirsch et al., 2002; Knowles, 2005; Polo et al., 2004).

1.2.8. Transmission

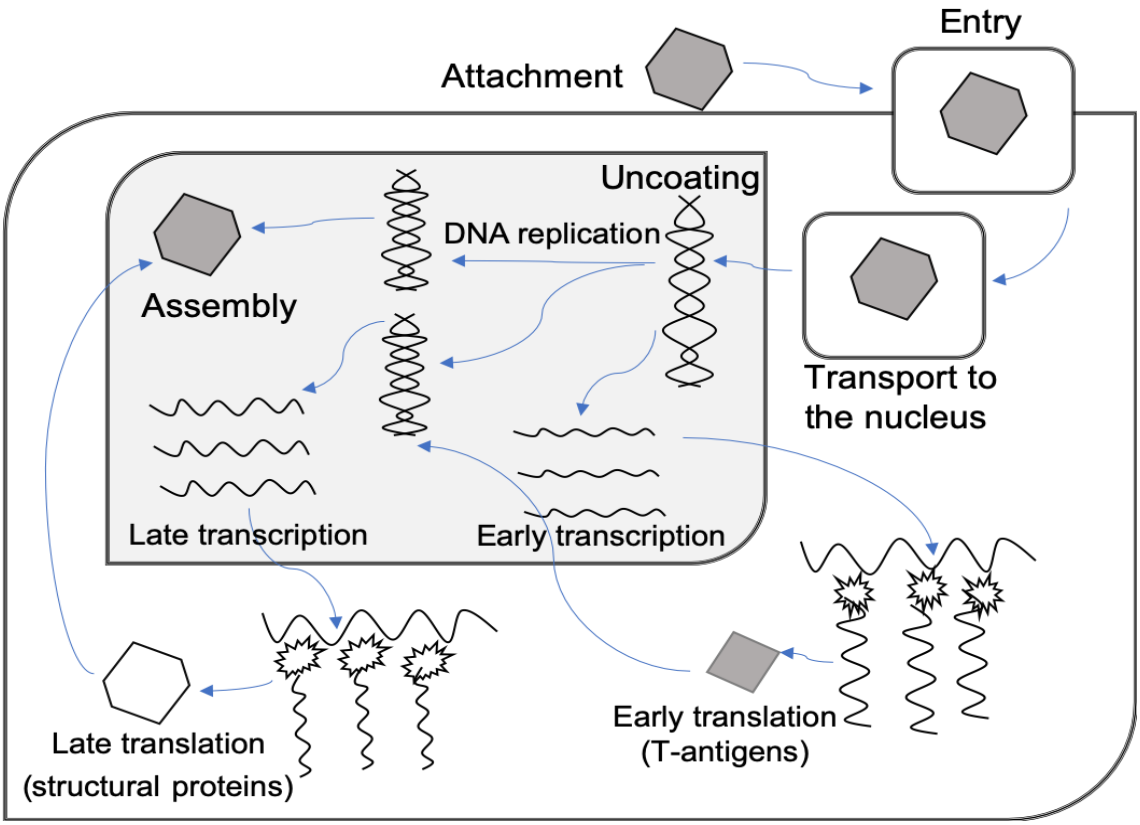
The exact transmission routes of HPyVs have been challenging to identify despite extensive study of these viruses, because of lifelong persistent infection with recurrent asymptomatic viral shedding (Chesters et al., 1983). The most plausible routes of infection transmission are the oral and respiratory routes, especially, for BKPyV, JCPyV, KIPyV and WUPyV (Babakir-Mina et al., 2009). This is supported by the detection of these viruses plus MCPyV in tonsillar samples (Babakir-Mina et al., 2009; Goudsmit et al., 1982; Kantola et al., 2009; Monaco et al., 1998). Another suggested route is the direct contact route which is thought to be the route of transmission for MCPyV, TSPyV, HPyV6 and HPyV7. There are also other routes suggested such as faecal-oral due to the detection of viral DNA in stool samples of hospitalised children as well as healthy adults, and urban sewage material (Bofill-Mas et al., 2010, 2001, 2000; Vanchiere et al., 2009, 2005; Wong et al., 2009). Vertical transmission of the viruses is also possible, because of the evidence of viral shedding during pregnancy and the detection of IgM antibodies in newborns, as well as the identification of identical viruses in

parent and child (Gibson et al., 1981; Kitamura et al., 1994; Kling et al., 2012; Kunitake et al., 1995; McClure et al., 2012).

1.2.9. Viral life cycle

The life cycle of HPyVs starts with the attachment of virions to their specific cell tissue target. The cycle can then be divided into two main phases an early and late phase (Figure 4). The replication capacity is influenced by the archetype of NCCR with some rearranged NCCRs showing altered viral replication speeds (Olsen et al., 2009).

Figure 4: Polyomavirus life cycle.



Virions enter the cell by endocytosis and are transported to the nucleus by cytoskeletal transportation. The viral genome uncoats inside the nucleus. Early transcription and translation produce tumour antigens (T-antigens). Late transcription and translation produce structural proteins. Assembly and release then occur. Adapted from (Java et al., 2012).

1.2.9.1. Attachment and receptors

Polyomaviruses use multiple specific receptors for their initial attachment to cells; this produces organ tropism and restriction of host range. These receptors are located on the cellular surfaces of target tissues, for example, BKPyV VP1 capsid protein attaches to an N-linked glycoprotein consisting of an alpha 2-3-linked sialic acid, GT1b, GD1b as receptors (Dugan et al., 2006; Low et al., 2006) and JCPyV VP1 attaches to lactoseries tetrasaccharide c (LTSc) a sialic

pentasaccharide and 5HT2A serotonin receptors that are found on the surfaces of kidney, lung, oligodendrocytes, and glial cells (Elphick et al., 2004; Neu et al., 2010). While, HPyV9 attaches to sialyllactosamine compounds terminating in 5-N-glycolyl neuraminic acid (Neu5Gc) (Khan et al., 2014).

1.2.9.2. Viral entry, intracellular transportation, and uncoating

Polyomaviruses have a non-enveloped virion and obtain cellular entry by endocytosis (Aktories et al., 2010). In the case of BKPyV, the virion utilises invaginations of the host cellular membrane in the form of caveolae, while JCPyV employs clathrin-coated pits acquired from the cell membrane (Dugan et al., 2006; Eash et al., 2004; Neu et al., 2009).

After internalisation by endocytosis, polyomavirus virions reaches the early endosome. Transport using the cytoskeleton of the host cell carries the virion to the endoplasmic reticulum (ER), where an acidic late endosome allows uncoating. The virion nucleic acid enters the nucleus where transcription begins (Dugan et al., 2006; Moriyama and Sorokin, 2008).

1.2.9.3. Transcription and replication (early phase)

After gaining entry to the nucleus, the EVGR of the genome starts to express the early common precursor mRNA, using a cellular RNA polymerase. Transcription of this mRNA produces the tumour antigens LTag and sTag by alternative splicing. LTag is a large viral protein that interacts with host cell proteins such as

the suppressor genes P53 and Rb and is essential for virus replication. The interaction with these host cell proteins triggers the host cell to enter the S-phase of cell cycle progression, which may help explain the oncogenic capacity of these viruses. LTag binds to the origin of replication (Ori) to unwind the genome dsDNA and begin the replication process. LTag promotes the bidirectional DNA replication through controlling the host DNA polymerase and other replication factors, as polyomaviruses do not encode their own DNA polymerase (Cuesta et al., 2010; Harris et al., 1996; White and Khalili, 2006). The production of LTag has a negative feedback on EVGR activation while activating LVGR in the genome; the role of sTag remains elusive.

1.2.9.4. Assembly and release (late phase)

The LVGR also expresses a common mRNA that encodes the structural proteins VP1, VP2, VP3 by alternative splicing. In this stage, agnoproteins which are specific to certain HPyVs are also produced. Viral particles are assembled in the nucleus after required proteins are transported back from the cytoplasm. VP1 showed the ability to assemble a VLP and package foreign DNA using recombinant expression techniques (Knipe and Howley, 2013; Shah, 2001). The exact mechanism of viral assembly and packaging is still obscure.

After the production of progeny virus, the virus is released from the cell by either cell lysis (as in renal tubular epithelial cells infection) or cellular detachment (as in human urothelial cells infection) (Li et al., 2013; Seemayer et al., 2008).

1.2.10. Pathophysiology

HPyVs causes subclinical asymptomatic infections in an immunocompetent host. However, immunocompromised individuals may develop severe infections. After the initial infection, HPyVs establish lifelong persistent infection with multiple recurrences of viral shedding.

1.2.10.1. Primary infection

After the virion successfully enters the host cell and start expressing the viral antigens and produce viral proteins. The virus takes over the host cell production machinery and starts to actively replicate, leading to cytopathic changes in the host cell, increasing the size of the nucleolus and the entire cell. These altered cells eventually swell and lyse to release the virus to infect neighbouring cells or to haematologically spread the infection (Knipe and Howley, 2013; Shah, 2001). The replication of HPyV depends primarily on the immune response of the host as the cycle takes about two to three days until it is controlled by the healthy immune system (Funk et al., 2008). It is suggested that the first replication cycles are carried out in primary infection sites such as the urinary tract, tonsils, or the respiratory system, then by haematological spread, the virus reaches its primary site of replication, for example, BKPyV in the reno-urinary tract (Chesters et al., 1983; Hirsch and Steiger, 2003; Monaco et al., 1998).

1.2.10.2. Persistent and Latent infection

The high seropositivity to HPyV along with the evidence of active viral replication post-infection leads to the conclusion that after the primary infection HPyV establishes lifelong persistence or latency (Knipe and Howley, 2013; Shah, 2001). For example, BKPyV infection of the kidney leads to periodic detection of the virus in urine samples without causing any disease (Khalili and Stoner, 2004; McClure et al., 2012). JCPyV is found in different sites such as the brain, bone marrow and the kidneys (Ferenczy et al., 2012; White and Khalili, 2011) and KIPyV and WUPyV are present in the mucous membranes of infants (Allander et al., 2007; Gaynor et al., 2007). Rather than persistent infection, viral latency could provide an explanation for the effective immune evasion exhibited by HPyVs to defy elimination by the immune system (White et al., 2013). Recently, studies have shown circulating BKPyV miRNA in both BKPyV DNA positive as well as negative individuals (Martelli and Giannecchini, 2017). The miRNAs were carried in exosomes, which protect them from degradation by the immune system RNAase (Pegtel et al., 2010).

1.2.11. Polyomavirus immune response

A clear understanding of the interplay between polyomaviruses infection and the immune system is not entirely understood. However, several studies have explored different aspects of the immune system and their relation to polyomaviruses infections, especially BKPyV infection. The innate immune system gives general initial defence against all viral infections (Babel et al., 2011). The availability of dendritic cells, which are responsible for presenting antigens,

has been linked with the development of BKPyV viremia post-transplantation (Womer et al., 2010). Furthermore, the renal transplantation recipients' human lymphocyte antigen (HLA) class, specifically HLA-C7, or the mismatching of the HLA class between recipient and donor has been associated with increased risk of developing PyVAN (Awadalla et al., 2004; Bohl et al., 2005). Meanwhile, other HLA classes such as; HLA-A2, HLA-B44 and HLA-DR15 were associated with decreased risk of developing PyVAN (Masutani et al., 2013). Cellular immune responses are observed in the form of specific T-cells against BKPyV proteins (Comoli et al., 2008). BKPyV antigens targeted by T-cells can be the structural proteins VP1, VP2, and VP3, or the tumour proteins LTag and sTag (Mueller et al., 2011). Further, the degree of viral clearance is directly correlated with the level of BKPyV specific T-cell activation (Stervbo et al., 2019). Sero-epidemiological studies highlight the existence of developed humoral immunity towards polyomaviruses infections (Gossai et al., 2016). Further, specific immunoglobulin G (IgG) has been found in adult renal transplant recovering from BKPyV infection (Hariharan et al., 2005). Additionally, specific BKPyV antibodies (IgG, IgM, and IgA) titers are higher in patients with BKPyV infection (Randhawa et al., 2008). Meanwhile, a study in paediatric renal transplant recipients showed a correlation between the lack of BKPyV antibodies and the increase in BKPyV infection (Ginevri et al., 2007). However, the degree of infection protection can be inadequate, explaining the occurrences of re-infections and reactivation (Randhawa et al., 2008).

1.2.12. Relation to oncogenesis

Because some of the expressed proteins by polyomaviruses have been suggested to play a role in human oncogenesis, specifically, LTag and sTag, it has been suggested that HPyVs may play a role in human cancer development. LTag has been associated with the transformation of host cells due to its ability to inhibit retinoblastoma (Rb) proteins p53, as well as p107, and p130, this result in cells that may proliferate uncontrollably (Harris et al., 1996), further, prostate cancer samples have been found to contain p53-LTag complexes (Das et al., 2008). sTag also contributes to uncontrollable host cell proliferation by stimulating mitogen-activated protein kinases (MAPK) pathway through inactivating protein phosphatase 2A (PP2A; Sontag et al., 1993). These effects of polyomavirus proteins and the association of polyomavirus infection with the development of carcinomas in animal models led to the suspicion of a similar association in human tumorigenesis (Takemoto and Martin, 1976). However, a causal association with human carcinomas is yet to be proven, except for MCPyV. MCPyV is linked with a locally aggressive skin tumour called Merkel cell carcinoma, where, MCPyV DNA is found in 95% of these tumours (Grundhoff and Fischer, 2015).

1.3. Laboratory diagnosis

1.3.1. Multiplex quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) assays allow not only the detection of infection but also the monitoring of the progress of a viral infection (Watzinger et al., 2006). Real-time qPCR assays determine the amount of viral

DNA by continuous measuring of the emissions produced by specific fluorescent hydrolysis probes (Heid et al., 1996). Accurate quantification of the amount of viral DNA present in a sample is possible and this, in turn, can be used to determine the amount virus present in a fluid sample (the so-called 'viral load' of a sample) by correlating the amount of template DNA in a series of standard samples with known amounts of DNA with the cycle quantification (Cq) value of the sample in the test, i.e. the amplification cycle where the fluorescence exceeds a chosen threshold (Raymaekers et al., 2009). In order to, standardise DNA measurement these control samples are calibrated with known quantities of external positive controls particularly, where available, a WHO international standard, to allow reporting in international units to achieve a robust method comparable between different laboratories and different test methodologies to achieve assays with low intra-, inter-assay variation (Madej et al., 2010). To enhance confidence in negative results and discern qPCR inhibitors in samples, an internal control (IC) should be examined in every reaction (McHugh et al., 2015). Utilising multiple hydrolysis probes with different fluorescent dyes, multiple viruses, as well as the IC, can be tested in a single qPCR reaction forming a multiplex qPCR. Multiplex qPCR can reduce sample and reagents consumption, thereby lowering cost and decreasing test turn-around time (Kurkela and Brown, 2009).

1.3.2. Recombinant protein production

Polyomavirus capsid is composed of three structural proteins; viral protein 1, 2, and 3 (VP1, VP2, VP3). The major capsid protein VP1 makes the outer portion with an 80% majority of the total viral capsid and facilitate viral attachment and

cellular uptake. Meanwhile, VP2 and VP3 form 20% of the capsid split equally (Daniels et al., 2007; Hurdiss et al., 2016). The major capsid protein VP1 can be produced by several recombinant protein expression systems; most commonly used is the insect baculovirus expression system.

Baculoviruses are a wide group of insect-specific viruses, mainly the larvae of the order *Lepidoptera*. These viruses enveloped with a rod-shaped nucleocapsid containing circular double-stranded dsDNA. The most prominent baculovirus used in recombinant protein expression is *Autographa californica nucleopolyhedrovirus* (AcMNPV) (Jarvis, 2009; Possee and King, 2016) These viruses can incorporate large foreign genes for recombinant expression (Smith et al., 1983). The foreign gene expression utilises the polyhedron promoter, as the polyhedron is a non-essential for baculovirus replication in insect cell cultures (Kost et al., 2005). Insect cells considered safe and easy to maintain, as they do not require CO^2 supplementation and can be grown as suspension or monolayer at 28ºC (Kelly et al., 2007). The most commonly used insect cell lines are *Spodoptera frugiperda* Sf9 or Sf21 cells which are used for co-transfection and recombinant rBV baculovirus amplification, and *Trichoplusia ni* High Five™ which are used for recombinant protein production (Kost et al., 2005). Recombinant baculoviruses are generated by homologous recombination when insect cells are co-transfected with a cloned transfer vector containing the gene of interest and modified AcMNPV virus. Homologues recombination exchanges DNA region between two flanking homologous regions resulting in a recombinant virus with the gene of interest at the polyhedron locus. Recent modifications in AcMNPV such as *flashBAC* Ultra improved protein expression. Included modifications are; truncation of the essential open reading frame (ORF) 1629 stopping no-

recombinant viruses from replication, the insertion of a bacterial artificial chromosome (BAC) at the polyhedron locus allows propagation in bacterial cells, and the deletion of the non-essential gene chitinase (*chiA*) enhancing protein production (Rohrmann, 2019).

Furthermore, recombinantly produced VP1 major capsid alone retain its immunogenicity and has the ability to self-assemble into a virus-like particle (VLP), eliciting the immune response against HPyV infections allowing its utilisation to assess seroreactivity of polyomavirus infections (Gillock et al., 1997; Li et al., 2003; Zhou et al., 2019).

1.4. Polyomavirus associated nephropathy

HPyVs cause significant diseases among particular groups of patients, mainly immunocompromised individuals. In the case of renal transplant recipients where immunosuppressive therapy is used to prevent host-allograft rejection, HPyVs are the primary cause of viral nephropathy and are the main cause of tubulointerstitial nephritis of the grafted kidney which is called polyomavirus-associated nephropathy (PyVAN). BKPyV infection accounts for around 95% of PyVAN cases. Another common condition caused by BKPyV in renal transplant recipients is ureteral stenosis. The remaining 5% of PyVAN cases are found to be caused by JCPyV which is thought to cause a milder and slower clinical course (Delbue et al., 2013; Drachenberg et al., 2007). In addition, the newly discovered HPyV9 first described by (Scuda et al., 2011) in renal transplant patients. Furthermore, (van der Meijden et al., 2014) found HPyV9 prevalence in renal

transplant recipients to be as high as 20%, while (Wang et al., 2019b) detected HPyV9 in a patient with high BKPyV viral load and evidence of PyVAN. Therefore, the emergence of HPyV9 is of interest in PyVAN development, whether as a primary cause of PyVAN or as co-infection with BKPyV.

PyVAN is a serious complication of renal transplantation with a prevalence of up to 10%. In severe cases, it may lead to a reduction of renal function or complete loss of function of the transplanted kidney (15% of cases). Most PyVAN cases present in the first year post-transplantation, followed by a small proportion of cases that are diagnosed in the second and third year (Bressollette-Bodin et al., 2005; Hirsch et al., 2005; Hirsch and Randhawa, 2013).

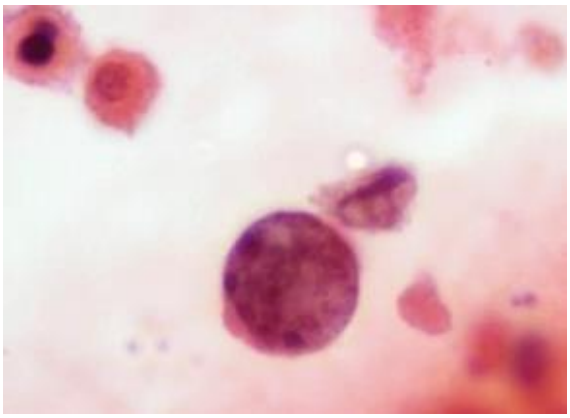
1.4.1. Risk factors

It has been suggested that the type and aggressiveness of immune therapy is the leading risk factor for developing PyVAN. Immune therapies associated with PyVAN development include tacrolimus alone, or in combination with mycophenolate mofetil, or anti-lymphocyte globulin; however, a definitive association is still lacking. The addition of steroid maintenance therapy increases the chances of PyVAN development. Other risk factors are; old age, male gender, chronic diseases such as diabetes mellitus, concurrent cytomegalovirus (CMV) infection (Toyoda et al., 2005), evidence of ureteral trauma, human leukocyte antigen (HLA) or ABO blood group mismatch, serologically naïve recipient (Wunderink et al., 2017), graft hypoxemia (Signorini et al., 2016), delayed graft function, or acute graft rejections (Pham et al., 2014; Wu et al., 2006).

1.4.2. Clinical presentation

The majority of PyVAN cases present as an asymptomatic gradual reduction of the engrafted kidney functions (e.g. decreased GFR). Some patients can be diagnosed early by the detection of polyomavirus viruria and viremia. Other cases may present with symptoms such as haematuria or ureteral obstruction. Urine analysis may detect associated pyuria or decoy cells. Decoy cells are enlarged urothelial cells with a swollen nucleus and intranuclear inclusion bodies (Figure 5) (Hirsch et al., 2002).

Figure 5: Decoy cell microscopy.



Decoy cells visualised by cytosine staining in a urine sample. Adapted from (Geetha et al., 2012).

1.4.3. Screening and diagnosis

As PyVAN can potentially cause loss of the engrafted kidney and there is a lack of effective antiviral therapy once the disease is established, rigorous routine screening is necessary to allow earlier intervention to reduce the risk of graft

failure (Hirsch et al., 2005). The stepwise progression of PyVAN aids implementation of simple screening regimens as PyVAN begins with viruria which in 50% of the cases precedes viraemia that begins about eight weeks prior to the detection of PyVAN findings in the kidneys (Ramos et al., 2009). Routine quantitative PCR (qPCR) screening for HPyVs, especially BKPyV in urine and blood samples of renal transplant recipients at least every three months for the first two years is advocated (Hirsch and Randhawa, 2013). However, more frequent monthly screening has been suggested to help in early intervention in the first six months (Alméras et al., 2011a; Brennan et al., 2005; Knight et al., 2013; Randhawa and Brennan, 2006). Although viruria has a high negative predictive value in ruling out PyVAN (Hirsch et al., 2002), this must be interpreted with caution, due to the lack of a universal standardised qPCR technique (Hirsch et al., 2005). A presumptive diagnosis of PyVAN can be made by obtaining a positive polyomavirus viruria result with positive polyomavirus viraemia, and evidence of renal dysfunction such as high urea and creatinine, has a high predictive value in diagnosing PyVAN (Gosert et al., 2008). These positive results are confirmed by renal biopsy (Dadhania et al., 2010; Drachenberg et al., 2004).

To reach a definitive diagnosis of PyVAN, a positive tissue analysis must be obtained. There are difficulties in obtaining a tissue diagnosis as it is required to have large biopsies that include the medullary tubules and there is a general lack of pathognomonic cytopathic effects to distinguish PyVAN from other viral infections (Drachenberg et al., 2004). Nonetheless, it is important to differentiate between PyVAN and graft rejection as the management of the two conditions entirely different. Such differentiation can be achieved by correlating clinical information, molecular results, and histopathological studies (Hirsch et al., 2014).

Serological testing of polyomavirus infection in renal transplant patients is not done routinely in the clinical setting (Hirsch et al., 2014). This is due to previous seroprevalence studies reporting HPyVs infection ubiquity in humans (Kamminga et al., 2018) and periodic reactivation of latent/persistent infections questioning the usefulness of serological tests in the diagnosis of PyVAN (Gardner et al., 1984). However, evaluation of the serological status for polyomavirus infection in renal transplant patients has shown a correlation between BKPyV seroreactivity and infection (Bohl et al., 2008). Assessing the kidney donor serostatus can also be beneficial, as it is speculated that the development of polyomavirus infection may originate from the allograft kidney (Schmitt et al., 2014). Moreover, the difference in serostatus between donor and recipient such as serologically positive donor and the serologically negative recipient has been suggested as a risk factor for developing PyVAN (Wunderink et al., 2017).

Other methods used for polyomavirus identification are urine cytology and virus culture. Urine cytology is used to screen for decoy cells and has high sensitivity but suffers from a low positive predictive value, which can be as low as 20% (Hariharan, 2006; Summers et al., 2005). While virus culture may be used in research, it is not utilised in clinical settings as it is a slow, expensive, insensitive, and laborious (Arthur and Shah, 1989).

1.4.4. Management

The primary therapy is the reduction of immunosuppression because there is a lack of effective antiviral treatment for established PyVAN (Saad et al., 2008; Vasudev et al., 2005), although there is no consensus on how to precisely reduce the immunosuppression. Most commonly, a 25 to 50% reduction of immunotherapy along with the cessation of mycophenolate or azathioprine treatment is used (Johnston et al., 2010). Unfortunately, with reduced immunotherapy, acute graft rejection can be observed in up to a quarter of patients. Steroid therapy can successfully treat these episodes of acute rejection without recurrence of PyVAN (Hirsch et al., 2005, 2002), but the response to treatment has to be monitored periodically (Hirsch et al., 2005).

Antiviral therapy in the form of cidofovir has been attempted in addition to immunosuppression reduction, and showed the ability to briefly maintain kidney function after PyVAN (Raval et al., 2011). Furthermore, brincidofovir which changes intracellularly into cidofovir and is not associated with cidofovir nephrotoxicity (Marty et al., 2013; Rinaldo et al., 2010), has been successful in controlling BKPyVAN in a single case report (Papanicolaou et al., 2015). However, nephrotoxicity, or lack of a large controlled trial study hinders specific antiviral therapy use (Costa, 2012; Dylewski and Al-azragi, 1999; Hirsch et al., 2014; Smith et al., 2004). Immunoglobulins against BKPyV are available, but due to scant information about their effectiveness they are still not widely used, and further study of their use is required (Randhawa et al., 2015; Sener et al., 2006; Vu et al., 2015). Leflunomide an antimetabolite with both antiviral and immunosuppressive effects has been used and demonstrated activity against

BKPyVAN (Nesselhauf et al., 2015), but it has a significant liver and haematological toxicity profile (Chong et al., 2006; Faguer et al., 2007; Josephson et al., 2006). Quinolone antibiotics like ciprofloxacin and levofloxacin have also been suggested due to *in vitro* activity against BKPyV. However, two randomised control trials have shown little evidence to support using quinolones, and they have known side effects such as nephrotoxicity and tendinitis (Knoll et al., 2014; Lee et al., 2014).

1.5. Aims and Objectives

HPyVs are a rapidly expanding group of viruses, which are found to be ubiquitous among the human population. At least 2 of the known HPyVs (BK and JC) are known to develop distinct infections among immunosuppressed renal transplant recipients in the form of PyVAN (Hirsch and Steiger, 2003; Querido et al., 2015; van der Meijden et al., 2014; White et al., 2013). However, in the past few years, many new HPyVs has been identified, such as HPyV9 which has been detected in the blood and urine of some renal transplant recipients (Scuda et al., 2011; van der Meijden et al., 2014). Currently, there is a lack of any standardised screening and diagnostic approach for PyVAN and lack of knowledge about the exact role of the newly discovered HPyV9 in the development of PyVAN.

The aim of this project is to investigate the incidence and role of HPyVs infections in renal transplant recipients to establish an understanding of the dynamics of PyVAN among this group of patients and to explore and propose an optimal screening and diagnostic approach for HPyV infections in renal transplant patients that is predictive of the development of PyVAN.

In order to reach this aim, this project will:

- Develop an internally controlled multiplex qPCR to detect BKPyV, JCPyV and HPyV9 viral DNA.
- Develop a VP1 specific ELISA to detect antibody to BKPyV, JCPyV and HPyV9.

- Utilise these assays to test stored samples from renal transplant recipients to ascertain the incidence of current and previous infection with each polyomavirus.

Chapter 2. Materials and Methods

2.1. Patient samples

Samples of blood (in EDTA) were collected as a part of a study of Post-transplant Lymphoproliferative Disease; UK National Research Ethics Board (GM West NW Rec 9 ref: II/NW/0001). On completion of that study, residual samples were transferred to the Manchester Institute for Nephrology and Transplantation (MINT) Biobank. Both original samples and nucleic extracts of these samples were retained. Permission for reuse of samples was granted under the Biobank ethics (UK National Research Ethics Board approval; 16/NW/0119), and the project was further approved by the University of Manchester Research Ethics Committee (UREC reference number: 2018-4298-6502, Polyomavirus Infection in Renal Transplant Patients).

Samples of blood (200µl) collected in EDTA were extracted in the Clinical Virology Laboratory, Manchester Royal Infirmary (MRI) using the semi-automated Qiagen BioRobot® MDx (Hombrechtikon, Switzerland) and the QIAamp DNA Blood BioRobot MDx Kit (Qiagen, UK) according to the manufacturer's instructions and stored at - 80° C upon transfer to MINT; then at - 20° C upon transfer to the Virology and Microbiology Department, University of Manchester). All samples were anonymized, and limited clinical information linked to the samples via a study code (to ensure full anonymization of samples) was made available by Dr Muir Morton, Renal Unit, Manchester Royal Infirmary. A total of 1156 extracted DNA samples from 466 patients were analysed.

2.2. Multiplex qPCR design

2.2.1. Retrieval of viral genomic sequences

Three genomic sequences (BKPyV, JCPyV, and HPyV9) were retrieved from the National Center of Biotechnology Information (NCBI) GenBank® (www.ncbi.nlm.nih.gov/genbank/; Clark et al., 2016). Using the following search lines: First BKPyV - (((((((Human Polyomavirus BK[Organism]) AND Genome) AND complete) NOT partial) NOT fragment)) NOT coding) NOT sv40), second JCPyV - (((((JC Polyomavirus[Organism]) AND Genome) AND Complete)) NOT partial) NOT fragment), third HPyV9 - (Human Polyomavirus 9[Organism]). Last checked at 31/1/2020.

2.2.2. Sequence alignment

The retrieved genomic sequences were analysed using Clustal Omega version CLUSTAL-O-1.2.4 from EMBL-EBI, (McWilliam et al., 2013) available at (http://www.ebi.ac.uk/Tools/msa/clustalo/). Areas of consensus inside each viral genome were assessed, then discrimination between the three polyomaviruses was checked.

2.2.3. Selection of target sequences

Real time PCR techniques were researched and primers within conserved regions within the large tumour antigen (BKPyV, JCPyV) and the VP1 region (HPyV9) were analysed to confirm primers reacted with all newly published sequences. Mismatched areas between the three viruses were further analysed

within each virus. The regions providing the greatest mismatch between the three viruses and the most conserved in each individual virus were selected as PCR targets.

2.3. Multiplex qPCR Primers and probes

2.3.1. Primers and Probes design

The target regions were analysed using Primer3Plus (Untergasser et al., 2007) to select oligonucleotide primers and probes (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). In order to ensure multiplexing compatibility each oligonucleotide was examined using the Oligo Calc online tool (Kibbe, 2007; http://biotools.nubic.northwestern.edu/OligoCalc.html) and the Thermo Fisher Scientific, Multiple Primer Analyser online tools (https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html). Several parameters were assessed including melting temperature, ideal length, GC content percentage, self-complementarity, primer-dimer estimation, and hairpin formation.

Specificity was confirmed using the NCBI database Nucleotide Basic Local Alignment (BLAST) search tool BLAST N (Altschul et al., 1990; https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The results obtained BLAST N were evaluated by query coverage, identity percentage, and expectation value (E-value). Each

primer or probe was searched to ensure it only amplified the desired target. Amplicons were analysed individually to verify amplification of only the desired targets.

2.3.2. Primers and probes sequences and chemistry

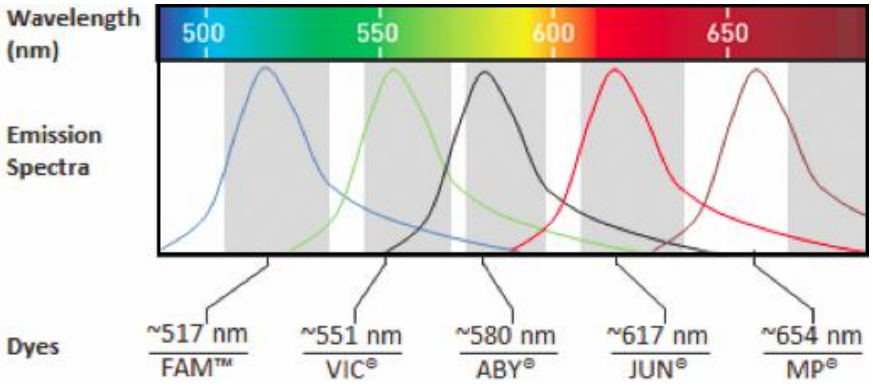
The primers and probes sequences are detailed in Table 2. All primers and probes were supplied by Life Technologies Ltd., (UK). Dual labelled TaqMan® hydrolysis probes with Applied Biosystems™ QSY™ quenchers were synthesised, and the emission spectra of the hydrolysis probes are shown in Figure 6.

Table 2: List of oligonucleotide primers, probe sequences and target genes

Target	Gene name	Oligonucleotide name	Sequence (5'–3')
BKPyV	LTag	Forward primer	CAGAAGGAAAGTCTTTAGGGTC
		Reverse primer	GATGAAGATTTATTTTGCCATGA
		TaqMan® hydrolysis probe	[FAM]GTGTTGAGAATCTGCTGTTGCTTC[QSY]
JCPyV	LTag	Forward primer	TTTAGGTGGGGTAGAGTGTTG
		Reverse Primer	CCAACCTATGGAACAGATGAA
		TaqMan® hydrolysis probe	[VIC]GTGGAATACATTTAATGAGAAGTGGGAT[QSY]
HPyV9	VP1	Forward primer	GCTCTCTCCTTAATAGCTTGTTC
		Reverse primer	CCTGATAAATTCTGACTTCTTCCA
		TaqMan® hydrolysis probe	[JUN]TATGCCTCAAATTCAGGGACAAC[QSY]
Internal control (Pumpkin)	N/A	Forward primer	GATGAGGTGCTTCGAGAG
		Reverse primer	ATCACGGGTCCCCTACTAC
		TaqMan® hydrolysis probe	[ABY]AGCCATGAAGAAGGATGCAATTC[QSY]

Dyes attached to the 5' end of the hydrolysis probe are: FAM™ (6-carboxyfluorescein), and Applied Biosystems™ proprietary dyes (VIC™, JUN™, and ABY™).

Figure 6: Fluorescence spectra of selected hydrolysis probes.



The four qPCR Applied Biosystems™ QSY™ proprietary dual labelled probes, and the passive reference (MP®) fluorescence wavelengths are shown, which have no interferences. Image supplied by Life Technologies Ltd., UK.

2.4. Multiplex qPCR Calibration controls

2.4.1. positive control and quantitation standard

A DNA sequence containing the qPCR amplification targets for BKPyV, JCPyV and HPyV9 (Table 3) was designed using SnapGene software version 3.2.1 (GSL Biotech; USA. available at Snapgene.com). A 692 bp long synthetic dsDNA standard was synthesised by Eurofins Genomics (Epsberg, Germany). The sequence represented the amplicons of BKPyV, JCPyV, and HPyV9 specific PCRs together with other viruses (herpes simplex virus type 1, herpes simplex virus type 2 and cytomegalovirus). The synthesized DNA was cloned into plasmid pEX-K4 (2507 bp) by (Eurofins Genomics), illustrated in Figure 7. The plasmid was propagated in *Escherichia coli,* then purified and sequenced using bidirectional sequencing by (Eurofins Genomics). The purified plasmid was provided lyophilised. The freeze-dried plasmid DNA was reconstituted in Tris/EDTA (TE) buffer at pH 8.0 at a concentration of 1443 ng/μL and stored in

aliquots at - 80° C. A 25 μL aliquot was serially diluted (10^{-1} to 10^{-11}) in sterile nuclease-free molecular grade water to be used in standard curve generation. To calculate the copy number of the DNA insert, the following formula was used:

$$\text{Number of copies (molecules)} = \frac{\text{X ng} * 6.0221 \times 10^{23} \text{ molecules/mole}}{(\text{N} * 650 \text{ g/mole}) * \times 10^{9} \text{ ng/g}}$$

Where X= amount of amplicon (ng), N= length of dsDNA amplicon and 650 g/mole is the average mass of 1 bp of dsDNA. The DNA length, in this case, (3199 bp) includes the size of the vector backbone (2507 bp) and the insert size (692 bp). This calculation was calculated using the online tool copy number calculator for real-time PCR found on https://scienceprimer.com/copy-number-calculator-for-realtime-pcr.

Table 3: qPCR amplicons sequences and size for BKPyV, JCPyV, and HPyV9.

Virus	Amplicon Length	Amplicon sequence
BKPyV	122bp	CAGAAGGAAAGTCTTTAGGGTCTTCTACCTTTCTT TTTTTCTTGGGTGGTGTTGAGTGTTGAGAATCTG CTGTTGCTTCTTCATCACTGGCAAACATATCTTCA TGGCAAAATAAATCTTCATC
JCPyV	138bp	TTTAGGTGGGGTAGAGTGTTGGGATCCTGTGTTT TCATCATCACTGGCAAACATTTCTTCATGGCAAAA CAGGTCTTCATCCCACTTCTCATTAAATGTATTCC ACCAGGATTCCCATTCATCTGTTCCATAGGTTGG
HPyV9	102bp	GCTCTCTCCTTAATAGCTTGTTCTCTGGTCTTATG CCTCAAATTCAGGGACAACCCATGGAAGGAACTT CAGGGCAAGTGGAAGAAGTCAGAATTTATCAGG

Figure 7: Positive control sequence.

Illustration of the 676 bp sequence of the positive control. Shown in purple are the binding sites and direction for all primers and probes. qPCR amplicons colour coded as follows; BKPyV in green, JCPyV in blue, and HPyV9 in red.

2.4.2. Internal control

As a dsDNA internal control, a synthetic plasmid was also ordered from (Eurofins Genomics, UK) containing a fragment (492 bp in length) of pumpkin (*Cucurbita cv. Kurokawa Amakuri*) DNA including part of the hydroxypyruvate reductase gene (NCBI GenBank® Accession D49433) this gene was previously used as an internal control (McHugh et al., 2015). This synthetic standard was cloned into plasmid pEX-A2 (2450 bp; Figure 8) by (Eurofins Genomics, UK) and was provided at a concentration of 1340 ng/µL. The number of copies of the internal control molecule was determined as described in section 2.5.1. and for use 4150 copies of the internal control was added to the primers and probes mixture

(section 2.6.1; Table 4). The final concentration of IC per each reaction was 166 copies/mL.

The primer pair detecting the internal control amplified the 140 bp sequence: GATGAGGTGCTTCGAGAGGCTGATGTGATAAGTCTTCATCCAGTACTGGAT AAAACCACCTTCCATCTGGTGAACAAAGAAAGTCTTAAAGCCATGAAGAAG GATGCAATTCTCATTAACTGTAGTAGGGGACCCGTGAT.

Figure 8: Internal control molecule target sequence.

Illustration of the 492 bp sequence of the internal control molecule. Shown in purple are the binding sites and direction for both forward and reverse primers, and probe. Internal control molecule qPCR 140bp amplicon highlighted in purple.

2.4.3. International standards

The first WHO International Standards for BKPyV (Govind et al., 2015; NIBSC 14/212) and JCPyV (Govind et al., 2015b; NIBSC 14/114) were purchased from the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK). These were used to re-calibrate the positive control in international units, IU/mL.

Freeze dried International Standards at a concentration of 7.2 log_{10} IU/mL (15,848,932 IU/mL) for BKPyV and 7.0 log_{10} IU/mL (10,000,000 IU/mL) for JCPyV, respectively were reconstituted in 1 mL sterile nuclease-free molecular grade water and stored in aliquots at - 80° C.

200 μL of each standard was serially diluted (from 10^{-1} to 10^{-7}) in human plasma (NGS Blood and Transplant, Manchester, UK). The human plasma was negative for HSV1, HSV2, EBV, CMV, HBV, BKPyV, JCPyV, and HPyV9 DNA and, HCV and HIV RNA (Brown, 2020). 200 μL of each dilution was extracted using the Qiagen QIAamp MinElute Virus Spin Kit (Qiagen, UK) according to the manufacturer's instructions. In brief, 200 μL of each sample were combined into a 1.5 mL microcentrifuge tube, with 25 μL QIAGEN Protease reconstituted in Buffer AVE, and 200 μL of Buffer AL containing carrier RNA reconstituted in Buffer AVE. The mixture was then incubated at 56° C for 15 min in a heating block. Then 250 μL of absolute ethanol was added to each sample and left to incubate for 5 min at room temperature. The mixture was transferred to a QIAmp MinElute column, centrifuged at 8000 x *g* for 1 min and the flow-through was

discarded. The columns were washed three times, 500 μL of each in order; Buffer AW1, Buffer AW2, and absolute ethanol, each wash was centrifuged at 8000 x *g* for 1 min and discard of the flow-through. The columns were centrifuged at 18000 x *g* for 3 min and incubated at 56° C for 3 min, to dry the collection membrane. Columns were then placed in a clean 1.5 mL microcentrifuge tube, 100 μL of sterile molecular grade water was loaded to the membrane, left to incubate at room temperature for 1 min, and centrifuged at 20000 x *g* for 1 min to elute the DNA. The extracts were then stored at - 20° C.

2.5. Multiplex qPCR protocol

2.5.1. Primers and probes mixture

A mixture of BKPyV, JCPyV, HPyV9, IC primers and probes as well as the internal control molecule was prepared and stored in aliquots of 100 reactions (750 μL) at – 20° C. The primers and probes mixture concentrations was as follows; BKPyV, JCPyV, and HPyV9 forward and reverse primers was 1 μM/μL, BKPyV, JCPyV, and HPyV9 probes was 0.67 μM/μL, and internal control molecule forward and reverse primers and probe were 0.34 μM/μL. Plus the internal control molecule was added at a concentration of 553 copy/mL. The primers and probes mixture was diluted in PCR grade sterile molecular water and 7.5 μL of the mixture was added to each qPCR reaction. The final qPCR reaction concentrations are in Table 4.

Table 4: Primers and probes final concentrations per reaction.

Reagents	Final concentration per reaction
BKPyV, JCPyV, and HPyV9 forward and reverse primers	0.3 μM/μL
BKPyV, JCPyV, and HPyV9 probes	0.2 μM/μL
IC forward and reverse primers and probe	0.1 μM/μL
Internal control molecule	166 copies/mL

2.5.2. PCR reaction mixture and parameters

The 25 μL reaction mixture was made of 12.5 μL of TaqMan™ Multiplex Master Mix 2X (Applied Biosystems, UK) which contains AmpliTaq® Fast DNA Polymerase, Uracil-N Glycosylase (UNG), dNTPs with dUTP, MUSTANG PURPLE® dye (passive reference) plus 7.5 μL of primers and probes mix (section 2.6.1) and 5 μL of a sample (genomic extract, positive control, or WHO International standards).

Thermocycling was performed on the Applied Biosystems™ StepOnePlus™ Real-Time PCR system according to the cycling parameters found in Table 5 using MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL and MicroAmp™ Optical Adhesive Film (Applied Biosystems, UK).

Table 5: Thermocycling parameters.

Step	Temperature (C)	Time (minutes:seconds)	Cycles
Initial hold	95°	00:40	X1
Denature	95°	00:04	X45
Anneal/extend	60°	00:40	

Each assay run included a negative control (sterile distilled water), five dilutions of the positive control were run to determine assay reproducibility. Extraction negative control (extraction of sterile distilled water) done with the WHO international standards genomic extraction were tested during qPCR calibration.

2.6. Multiplex qPCR quality control

2.6.1. Reproducibility of the standard curve

10-fold serial dilutions of the positive control were run in triplicate on different days and on different PCR machines. Then the mean Cq for each dilution was calculated and used to develop the standard curve. Each dilution was evaluated for inter-assay, and intra-assay standard deviation (SD) and coefficient of variance (CV).

Linearity was assessed using the correlation coefficient (r^2), while the slope (*m*) was utilised to interpolate the international standard curves and positive standard result.

The qPCR efficiency (E) score. The efficiency was calculated as: E= $10^{-1/slope}$ using the QPCR Standard Curve Slope to Efficiency Calculator online tool (https://www.chem.agilent.com/store/biocalculators/calcSlopeEfficiency.jsp?_requestid=252705).

2.7. VP1 transfer vector cloning

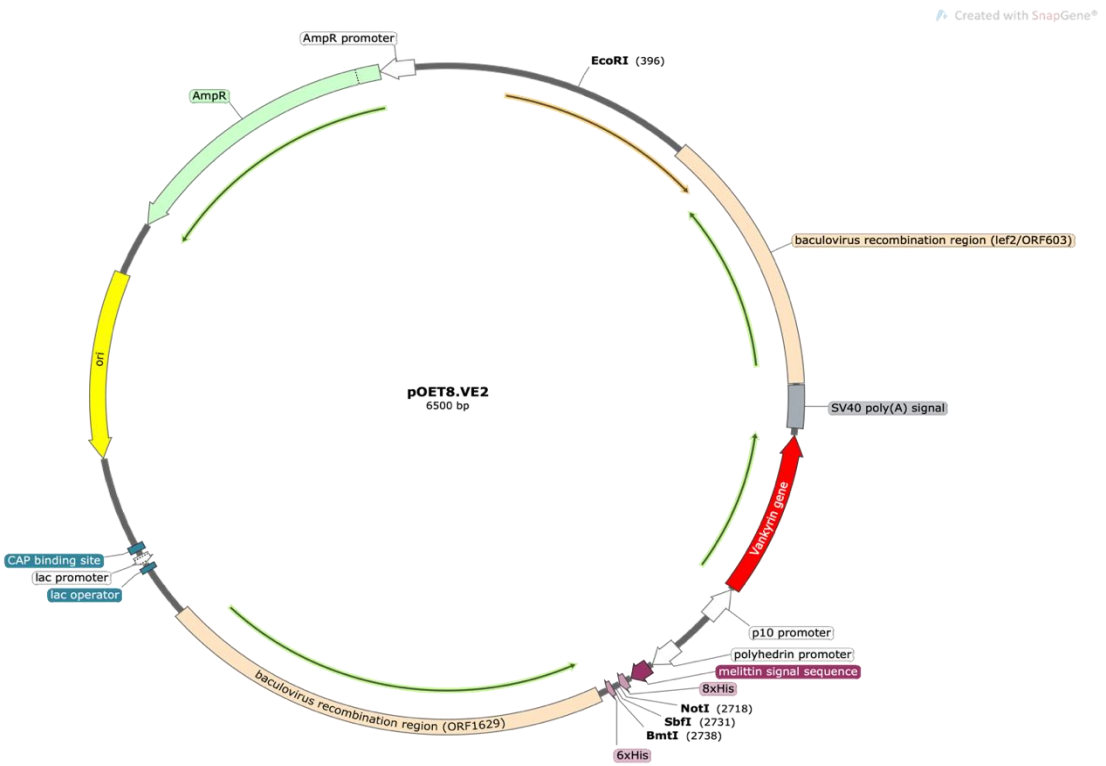
2.7.1. Transfer vector design

The baculovirus transfer vector used was pOET8.VE2 plasmid (Figure 9) purchased from Oxford Expression Technologies (Oxford, UK). The VP1 gene sequence data were obtained from the NCBI GenBank database as follows; BKPyV (NCBI GenBank® AB301093), JCPyV (NCBI GenBank® AF281625), and HPyV9 (NCBI GenBank® HQ696595).

The source used to amplify the VP1 gene for BKPyV and HPyV9 were two separate synthetic constructs which contain the sequence of the VP1 gene data of the GenBank® references mentioned earlier. These two synthetic constructs were manufactured by (Eurofins Genomics, UK). While JCPyV VP1 gene was amplified using JCPyV type 8 full-length clone plasmid (ATCC® VRMC-24) purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The amplification process detailed in (section 2.7.2).

The genetic information of both the transfer vector and the amplified products were analysed in-silico to simulate cloning with restriction enzyme digestion and ligation using SnapGene software. The VP1 gene insertion site and orientation were assessed according to the VP1 amplification with the flanking restriction enzymes sequences. Also, the complete incorporation into the polyhedrin promoter translation frame along with the Honey Bee Melittin (HMB) signal sequence and the N-terminal 8X histidine fusion tag (8xHis) was analysed.

Figure 9: The pOET8.VE2 baculovirus transfer vector map.



pOET8.VE2 map showing the recombinant expression promoter used the polyhedrin promoter, the recombinant protein secretion signal the melittin signal sequence, the N-terminal purification 8X histidine fusion tag (8xHis), the insect cells anti-apoptotic protein the Vankryin gene, the *E. coli* selection marker the ampicillin resistance gene (AmpR), and the restriction digestion enzymes used; *Bmt*I, *Eco*RI, *Not*I, and *Sbf*I which are inside the multiple cloning site region (2717-2739 bp). As well as, the baculovirus recombination regions lef2/ORF603 and ORF1629 utilised for homologues recombination in the co-transfection step.

2.7.2. Amplification of VP1 genes

Amplification primers were selected using SnapGene software version 3.2.1, and the desired restriction enzyme sequences were added as flanking tails to the selected primers. Further, the primers were checked for melting temperature, ideal length, GC content percentage, self-complementarity, primer-dimer

estimation, and hairpin formation using OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html).

The sequence of the VP1 specific primers are detailed in Table 6, reaction components for the VP1 PCR are shown in Table 7 with thermocycling parameters as detailed in Table 8.

Table 6: VP1 insert amplification primers.

virus	oligo name	sequence	RE
BKPyV	pOET1N-For-NotI	TACGTAGCGGCCGCTGAAGCTT CTAGAGTCGACATGGC	*Not*I-HF®
	pOET1N-Rev-BmtI	TGCAATGCTAGCGCCGCGGTAC CTCGAGAGCTC	*Bmt*I-HF®
JCPyV	JC ATCC-for-NotI	TACGTAGCGGCCGCTGAGATGG CCCCAACAAAAAGAA	*Not*I-HF®
	JC ATCC-Rev-BmtI	ACACTGAGCTAGCCATTACAGCA TCTTTGTCTGCAA	*Bmt*I-HF®
HPyV9	pOET1N-For-NotI	TACGTAGCGGCCGCTGAAGCTT CTAGAGTCGACATGGC	*Not*I-HF®
	pOET1N-Rev-SbfI	TGCAATCCTGCAGGGCCGCGGT ACCTCGAGAGCTC	*Sbf*I-HF®

Table 7: VP1 amplification reaction mixture components and concentrations.

Component	50-µL rxn	Final Concentration
Molecular grade water	to 50 µL	---
AmpliTaq Gold® 360 Master Mix	25 µL	1X
10 µM forward primer	1 µL	0.2 µM
10 µM reverse primer	1 µL	0.2 µM
Template DNA	10 µL	< 1 µg

Table 8: VP1 amplification thermocycling parameters.

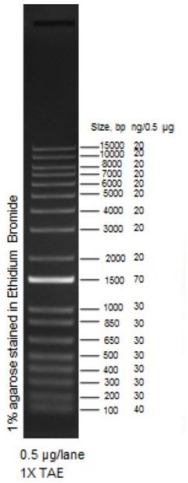
Step	Temperature (°C)	Time	Cycles
Initial denature	95° C	10 min	X1
Denature	95° C	30 sec	X40
Anneal	61° C	30 sec	
Extend	72° C	90 sec	
Final extension	72° C	7 min	X1
Hold	4° C	indefinitely	X1

2.7.3. Confirmation of VP1 PCR amplification

PCR amplification products were analysed in a 1.2% E-Gel™ single comb agarose gel with SYBR™ Safe DNA gel stain, using the E-Gel™ Precast Agarose Electrophoresis System (Thermo Fisher Scientific, UK); 4 µL of the PCR product was mixed with 16 µL of sterile molecular grade water; 20 µL was loaded on the gel. The product sizes were estimated using Invitrogen™ 1 Kb Plus DNA Ladder (Figure 10; Thermo Fisher Scientific, UK), 2 µL was mixed with 18 µL of sterile

molecular grade water and 20 µL applied to a well. After running the gel for 30 minutes using E-Gel® PowerBase™ V.4 (Thermo Fisher Scientific, UK), the molecular bands were visualised using the FastGene Blue/Green Transilluminator (Geneflow Limited, U.K.) and documented.

Figure 10: Invitrogen™ 1 Kb Plus DNA Ladder



2.7.4. PCR amplification products purification

PCR product purification was performed using the QIAquick PCR Purification Kit (Qiagen, UK) according to the manufacturer protocol. In brief, 250µl of Buffer PB was added to the PCR product then transferred to QIAquick spin column and centrifuged at 18000 x *g* (MiniSpin® Plus, Eppendorf, Germany) for 1 min. Followed by washing with 750 µl buffer PE then centrifugation at 18000 x *g* for 1 min and discard the flow-through. The QIAquick spin column was placed in a sterile 1.5 ml Eppendorf tube, and 35 µl of sterile molecular grade water was added into the centre of the column and left for 1 min in room temperature then centrifuged at 20000 x *g* for 1 min. The purified DNA material concentrations were determined using the ND1000 NanoDrop Spectrophotometer (Labtech, UK).

2.7.5. Restriction enzyme digestion

In a total volume of 50 µl: 1 µg of insert DNA, 5 µl of 10x CutSmart® Buffer (New England Biolabs, UK), 1µl (10 units) of restriction enzymes according to the restriction enzymes combinations in Table 9. The volume was adjusted to 50 µl with molecular grade water. This mixture was incubated at 37° C for 45 min then heat-inactivated at 65° C for 15 min in the MiniAmp Plus Thermal Cycler (Thermo Fisher Scientific, UK). All restriction enzymes were purchased from (New England Biolabs, UK). Both the PCR product and the matching plasmid were cut with restriction enzymes in separate 0.2 mL PCR tubes.

Table 9: Restriction enzymes combinations.

Virus	5' enzyme	3' enzyme
BKPyV	*Not*I-HF®	*Bmt*I-HF®
JCPyV	*Not*I-HF®	*Bmt*I-HF®
HPyV9	*Not*I-HF®	*Sbf*I-HF®

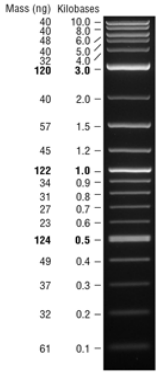
Restriction enzymes reactions were set up on ice. After 30 minutes, shrimp alkaline phosphatase (SAP) was added to the plasmid only, to dephosphorylate the cut ends and reduce the possibility of the plasmid self-annealing. 1 µL (1 unit/µL) of recombinant Shrimp Alkaline Phosphatase (rSAP) (#M0371; New England Biolabs, UK) was added to each pmol of DNA (about 0.5 µg of a 6 kb plasmid).

2.7.6. Gel extraction

After restriction digestion, the digested products were analysed and resolved according to molecular weight by gel electrophoresis.

A 1% agarose gel was prepared using UltraPure agarose (Thermo Fisher Scientific, UK). The DNA fragment sizes were determined by approximation to Quick-Load® 2-Log DNA Ladder (Figure 11; New England Biolabs, UK). 50 µL of restriction digestion product was mixed with 10 µL of 6x Gel Loading Dye (New England Biolabs, UK), 60 µL was added per well. The DNA fragments were resolved by electrophoresis at 120 Volts for 1 hour. Gels were stained with Invitrogen™ SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, UK) by adding 1 µL of stain to 10 mL of gel.

Figure 11: New England Biolabs Quick-Load® 2-Log DNA Ladder



Extraction of DNA fragments was performed by excision of the band of interest using a disposable scalpel (Swann-Morton, UK). The bands were visualised over the FastGene Blue/Green Transilluminator. The DNA from the excised gel slice was dissolved and DNA purified using QIAquick® Gel Extraction Kit (Qiagen, UK)

according to the manufacturer protocol. In brief, the excised fragment was weighed in a 1.5 ml Eppendorf tube; then 3 times the volume weight of Buffer QG was added. The gel with Buffer QG was incubated at 50° C for 10 min in a heating block with frequent vertexing. After the complete dissolving of the gel, 1 gel volume of pure isopropanol alcohol was added, and the mixture transferred to a QIAquick spin column and centrifuged at 18000 x *g* (MiniSpin® Plus, Eppendorf, Germany) for 1 min. This was followed by two washes with 500 µl Buffer QG and 750 µl buffer PE respectively with centrifugation at 18000 x *g* for 1 min and discard of the flow-through after each wash. Then the QIAquick spin column was placed in a sterile 1.5 ml Eppendorf tube, and 35 µl of sterile molecular grade water was added into the centre of the column and left for 1 min in room temperature then centrifuged at 20000 x *g* for 1 min. The purified DNA concentrations were measured using the ND1000 NanoDrop Spectrophotometer (Labtech, UK).

2.7.7. Transfer vector ligation

The purified RE cut PCR product, and plasmid were ligated using Instant sticky end ligase master mix (New England Biosciences). A ligation calculator tool (http://nebiocalculator.neb.com/#!/ligation) was used to calculate a 3:1 insert to vector ratio, based on the DNA sequence length (in Kb) of both the insert DNA and vector DNA.

All reagents were kept on ice during the ligation procedure. Purified vector DNA of purified DNA (section 2.7.6) was gently mixed and 20–100 ng of the pOET8.VE2 vector was combined to ensure a 3 to 5 fold molar excess of insert: vector. The volume was adjusted to 5 µl with molecular grade nuclease-free

water, and 5 µl of Instant Sticky-end Ligase Master Mix was added to the ligation mix and mixed gently. The resulting plasmids were used immediately in *E. coli* transformation (section 2.7.8).

2.7.8. Transformation of *E. coli*

2.5-5 µl of ligated plasmid mix (50-100ng DNA; section 2.8.7) was mixed with 50 µl of *E. coli* stellar™ (Takara Bio Inc., USA) competent cells and incubated on ice for 30 min. The mixture was then heat shocked at 42°C for 2 min and chilled on ice for 2 min. Then, 950 µl of super optimal broth (SOC) transformation medium (Thermo Fisher Scientific, UK) was added into the mixture and incubated with shaking at 300 rpm at 37° C for 1 hour (Eppendorf ThermoMixer C, Eppendorf, Germany). The bacterial suspension (250µl) was spread on selective media (Lennox broth (LB) agar with ampicillin [100 mg/ml]; Sigma-Aldrich Life Science, UK) and incubated at 37° C overnight. Controls were prepared by mixing the competent cells with water instead of plasmid and plated on two LB agar plates; the first included ampicillin while the second was without ampicillin.

2.7.9. Cloned plasmid propagation and extraction

After the successful transformation of *E. coli*, a single isolated colony was picked off and cultured in 3 ml of LB with ampicillin [100mg/ml] broth (Sigma-Aldrich Life Science, UK). The bacteria were incubated overnight at 37° C with shaking at 300 rpm (New Brunswick Scientific, Cambridge, UK).

The following day plasmid extraction and purification were done using QIAprep® Spin Miniprep Kit (Qiagen, UK) following the manufacturer's instructions. Briefly, 3 ml of the bacterial overnight culture was centrifuged at 6000 x *g* for 10 min at room temperature. Then, the resulting bacterial pellet was resuspended in 250 µl buffer P1 and transferred into a sterile 1.5 ml Eppendorf tube. After that, 250 µl buffer P2 was added and mixed thoroughly. Then 350 µl buffer N3 was added and mixed. The mixture was centrifuged at 18000 x *g* (MiniSpin® Plus, Eppendorf, Germany) for 10 min and the supernatant was transferred into QIAprep spin collection column before centrifugation at 18000 x *g* for 1 min. This was followed by two washes with 500 µl buffer PB and 750 µl buffer PE respectively with centrifugation at 18000 x *g* rpm for 1 min and discard of the flow-through after each wash. To elute the plasmid, the QIAprep spin column was placed in a sterile 1.5 ml Eppendorf tube, and 35 µl of sterile molecular grade water was added into the centre of the column and left for 1 min in room temperature then centrifuged at 20000 x *g* for 1 min. Purified plasmids concentrations were measured by NanoPhotometer® (Geneflow Ltd., UK).

2.7.10. Cloned plasmids confirmation

2.7.10.1. Restriction digestion confirmation

Primary confirmation was carried out using restriction enzyme digestion technique. A restriction enzyme (RE) was chosen to produce two distinctive fragments for each cloned plasmid (BKPyV, JCVPyV, and HPyV9) visualised by agarose gel electrophoresis. Restriction enzyme digestion method is described in details in (section 2.7.5).

2.7.10.2. Sequencing

Positive candidates after restriction enzyme digestion with *Eco*RI confirmation were further confirmed by Sanger nucleotide sequencing technique. Plasmid sequencing used gene-specific primers (Table 10) obtained from (Eurofins Genomics). Big Dye Terminator v1.1 cycle sequencing reagent (Applied Biosystems, UK) was used for the sequencing reactions using the MiniAmp Plus Thermal Cycler (Thermo Fisher Scientific, UK) according to the parameters detailed in Table 11. Then the products were sent out to be sequenced on the ABI 3130 genetic analyser (Applied Biosystems, UK).

Table 10: Sequencing primers.

Primer name	Sequence 5'-3'	Direction
pOET8.VE2 polyhedrin promoter	ATAGCACACAAGACTCCAACG	Forward
pOET8.VE2 MCS for	ACCACCATCACCACCATCATCA	Forward
pOET8.VE2 MCS Rev	TAACTAGTGATGGTGATGGTGATGT	Reverse
BK-VP1-Seq-F1	TTCCCCTCCCCAATTTAAATGAG	Forward
BK-VP1-Seq-F2	AGCCCAGTCCCAAGTAATGAA	Forward
BK-VP1-Seq-R1	TTTACAAAGAGGCCCCACACC	Reverse
JC-VP1-Seq-F1	ATGTGCACTCTAATGGGCAAG	Forward
JC-VP1-Seq-F2	ACAGAGCACAAGGCGTACCT	Forward
JC-VP1-Seq-R1	ATGCCACAGACATCAACAGCT	Reverse
HP9-VP1-Seq-F1	CTAATGTGGGAGGCTGTGTC	Forward
HP9-VP1-Seq-F2	GTTTTCAGTGGGTGGAGAGC	Forward
HP9-VP1-Seq-R1	AGGTAGGCCTCTCCAGTTTTG	Reverse

Table 11: Sequencing thermocycling parameters.

Temperature	Time	cycles
95° C	2 min	X1
95° C	10 sec	X25
50° C	5 sec	
60° C	4 min	

2.8. Culture of Sf9 and Hi5 insect cells

Sf9 insect cells (IPLB Sf21-AE) were obtained from Novagen® (Merck, New Jersey USA), Hi5 cells (BTI-TN-5B1-4) were obtained from (Thermo Fisher

Scientific, UK). All cell culture work was performed in a Class II Bio Safety Cabinet (ESCO Hatbaro, USA).

2.8.1. Recovery of cells

A cell ampoule was removed from liquid nitrogen and placed in a beaker of warm water for rapid thawing. When completely thawed, the ampoule was removed from the water and dried, before wiping down with 70% isopropanol (Sigma-Aldrich Life Science, UK) to reduce the chance of bacterial contamination. Using a plastic pipette (STARLAB Ltd., UK), the thawed cells were split between two 25 cm^3 Nunc culture flasks (Thermo Fisher Scientific, UK) each containing 5 mL of pre-warmed media. The flasks were placed in an incubator (Heraeus, Frankfurt Germany) set at 28° C for 1 hour to allow the cells to settle. After an hour, the media was removed and replaced with 5 mL of fresh media. The flask was placed in a 28° C incubator and left until cells reached 90-95% confluency.

2.8.2. Passaging of adherent cell cultures

Both Sf9 and Hi5 insect cells were passaged when they reached 85-95% confluence. Cell media was pre-warmed to 28° C. To dislodge adherent cells flasks were tapped 1-2 times until the majority of cells were re-suspended in the media. The cells were counted, and cell viability determined using trypan blue solution, 0.4% (Gibco™, Thermo Fisher Scientific, UK). Cells were diluted to a final concentration of 5 x 10^5 cell/mL, in a total volume of 20 mL. For cell counting, 0.1 mL of re-suspended cells was added to 0.9 mL of media 1:10 dilution. From

this dilution at 1:1 dilution, 100 µL to 100 µL, was performed in trypan blue solution, 0.4% (Gibco™, Thermo Fisher Scientific, UK) and mixed, then 50 µL was added to a cell of a FastRead counting chamber (Immune systems, Devon UK).

Cells were counted from the 4 large corner squares (each large corner square contained 9 small squares), and the following calculation performed:

Total No. of cells counted in 4 large squares:

85

No. of cells per large square:

$\frac{85}{4} = 21.25$

No. of cells per cm^3 of diluted cell suspension:

$\frac{85}{4} x\ 10^5 = 2.125\ x\ 10^6$

No. of cells per cm3 in original suspension allowing for dilution with trypan blue:

$\frac{85}{4} x\ 10^5 x\ \frac{3}{2} = 3.1875\ x\ 10^6$ cell/cm^3

To create a cell suspension at 5 x 10^5 cell/cm^3 dilution:

$$\frac{0.5}{3.1875}\ x\ 20 = 3.14\ mL\ \ of\ cell\ suspension + 16.86\ mL\ of\ cell\ medium$$

To determine cell viability, the cell counting was repeated, including the dyed non-viable cells, and the percentage of viable to overall cell numbers calculated. Only cultures with cell viability above 90% were passaged, cultures dropping below this level were discarded, and a new culture set up. Dilutions of cells were

prepared in sterile vented capped Nunc 75 cm^3 culture flasks (Thermo Fisher Scientific, UK). Caps were added tightly, and the culture flasks placed in a 28º C incubator to grow until 90-95% confluency.

Cell cultures were generally passaged around 30 times before discarding.

2.8.3. Passage of Suspension Cell Cultures

5 x 10^5 cell/cm^3 of Sf9 cells in 50 mL of media, in 250 mL Erlenmeyer flasks with vented caps (Corning, Tewksbury USA). Hi5 cultures used 3 x 10^5 cell/cm^3 in 50 ml of media. Cultures were placed in a shaking incubator set at 28° C and 150rpm (New Brunswick Scientific, Cambridge, UK). These were sub-cultured when they had reached a level of 3-6 x 10^6 cell/ml. Cell viability was determined during cell counting, and only cells above 90% viability were passaged. Cell cultures were generally passaged around 30 times before discarding. Sf9 infected cells were cultured using serum-free and protein-free Gibco™ Sf-900™ II SFM culture media (Thermo Fisher Scientific, UK). Hi5™ infected cells were maintained in Gibco™ Express Five™ serum free medium (Thermo Fisher Scientific, UK).

2.9. Recombinant baculovirus generation and amplification

One hundred microliters of SF-900™ insect cell culture media, 2.5µl of *flash*BAC ULTRA expression system, and 250 ng of the cloned transfer vector plasmid, were mixed gently. A second tube contained 100 µl of SF-900™ insect cell culture media combined with 6 µl of Escort IV™ transfection reagent (Sigma-Aldrich Life

Science, UK). The two tubes were combined and mixed gently, then left to incubate for 45 min at room temperature. 2 ml of 5 X 10^5 cells/ml (1 X 10^6 cells total) of Sf9 cells were seeded into wells of 6-well culture plate (Thermo Fisher Scientific, UK) and left to settle for one hour at 28° C in an incubator (Heraeus, Frankfurt Germany). After leaving the Sf9 cells to settle for one hour, the culture media was removed from the 6-well plate. Then the combined co-transfection mixture was added to the wells in a dropwise manner, followed by 800 µl of SF-900™ culture media. The plates were incubated for 24 hours at 28° C. The following day, 1 ml of SF-900™ culture media was added. Then the plates were left to incubate for six days at 28° C in an incubator (Heraeus, Frankfurt Germany). The supernatant containing the recombinant baculovirus (rBV) was carefully harvested to be used for amplification of the rBV.

2.9.1. Recombinant baculovirus amplification

To achieve high titer stock of rBV, the harvested supernatant from the previous step for each virus were added to a 50 ml cell suspension of Sf9 cells at 2 X 10^6 cells per mL in 250 mL Erlenmeyer flasks with vented caps (Corning, Tewksbury USA). This was left to incubate at 28° C orbital shaken at 150 rpm for a week in a shaking incubator (New Brunswick Scientific, Cambridge, UK). After one week, the entire 50 ml cell suspension was collected in a 50 ml light safe centrifuge tube (Greiner Bio-One, UK), and stored in the fridge at 4° C.

2.9.2. Baculovirus DNA extraction

In order to, perform a qPCR titration of the rBV, DNA extraction from the Sf9 baculovirus amplification suspension was performed using the Invitrogen™ PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, UK) according to the manufacture recommendations. In brief, 200 µl of the sample was placed in a sterile 1.5 Eppendorf tube combined with 20 µl Proteinase K, 20 µl RNase A, and 200 µl of PureLink™ Genomic Lysis/Binding Buffer. The mixture was vortexed then incubated at 55° C for 10 min in a heating block. Following incubation 200 µl of 100% ethanol was added. Then the entire mixture was transferred to a PureLink™ Spin Column and centrifuged at 18000 x *g* (MiniSpin® Plus, Eppendorf, Germany) for 1 min. This was followed by two washes with 500 µl of Wash Buffer 1 and Wash Buffer 2 respectively with centrifugation at 18000 x *g* for 1 min and discard of the flow-through after each wash. To elute the purified rBV DNA, the PureLink™ spin column was placed in a sterile 1.5 ml Eppendorf tube, and 35 µl of sterile molecular grade water was added into the centre of the column and centrifuged at 20000 x *g* for 1 min. The purified rBV DNA was collected and stored at - 20° C to be used for rBV particles quantitation using qPCR.

2.9.3. Recombinant baculovirus titration

The recombinant viruses were quantitated using a real-time qPCR assay (Brown, 2020). This qPCR assay was calibrated to quantify rBV in relative pfu/ml. Primers and probes were obtained from Eurofins (Eurofins Genomics, UK; Table 12). The reaction mixture comprised of TaqMan™ Fast Universal PCR Master Mix (2X)

without AmpErase™ UNG (Thermo Fisher Scientific, UK) as detailed in Table 13 with cycling parameters as detailed in Table 14 on an Applied biosystems StepOne™ real-time PCR system.

Table 12: rBV titration qPCR primers and probe.

Oligonucleotide	Sequence (5'-3')
Bac-Forward	CGGCGTGAGTATGATTCTCAAA
Bac-Reverse	ATGAGCAGACACGCAGCTTTT
Bac-Probe	[FAM]AAAAGTCTACGTTCACCACGCGCCAAA[BHQ]

Table 13: rBV titration qPCR cycling reaction mixture.

Reagent	Volume in µl	Final concentrations
TaqMan™ Fast Master Mix	12.5	1X
Forward primer at 3µM	2.5	0.3 µM
Reverse primer at 3µM	2.5	0.3 µM
Probe at 1µM	2.5	0.1 µM
Sample	5	

Table 14: rBV titration qPCR cycling parameters.

Temp	Time	Cycles
95° C	02:00	X1
95° C	00:03	X35
60° C	00:20	

2.10. Recombinant baculovirus protein expression

2.10.1. Hi5™ small scale protein expression screening

Hi5™ cells were seeded into wells of 6-well culture plate (Thermo Fisher Scientific, UK), 2 ml of 5 X 10^5 cells/ml (1 X 10^6 cells total) were left to settle for one hour at 28° C in an incubator (Heraeus, Frankfurt Germany). After one hour, the culture media was carefully removed, and 200 µl of Express Five™ media/rBV was added dropwise in different concentrations (Table 15). The plates were incubated for 1 hour at 28° C with occasional tipping. After one-hour 2 ml of Express Five™ media was added to the wells of the plates and were left to incubate at 28° C for 5 days (Heraeus, Frankfurt Germany).

Table 15: Express Five™ SFM/rBV ratios

well number	1	2	3	4	5	6
Media volume in µl	200	175	150	100	50	0
rBV volume in µl	0	25	50	100	150	200

2.10.2. Hi5™ large scale protein expression

Hi5™ cell 100 ml of 3 x 10^5 cell/cm^3 suspension cultures in 250 mL Erlenmeyer flask with vented caps (Corning, Tewksbury USA) were infected with a multiplicity of infection (MOI) between 3-5 (ratio of qPCR titration of rBV pfu/ml to cells/cm^3) of the amplified rBV harvested from Sf9 cultures and incubated at 28° C for 5 days orbital shaken at 150 rpm in a shaking incubator (New Brunswick Scientific, Cambridge, UK) were used to produce the desired proteins.

2.11. Recombinant protein analyses

2.11.1. Cell lysis and protein extraction

After Hi5™ protein expression, suspension cultures were transferred into 50 ml Falcon centrifuge tubes (Thermo Fisher Scientific, UK) and centrifuged at 700 x *g* for 5 minutes at 4° C (HARRIER 18/80 refrigerated centrifuge, MSE, UK), while monolayer cell cultures were harvested using a 25 cm cell scraper and transferred into 2 mL Eppendorf tube and centrifuged at 700 x *g* for 5 minutes at 4° C (Sigma-Aldrich Life Science, UK). The supernatant was collected, and cells pellets were flash-frozen using liquid nitrogen and stored at - 80° C.

Cell lysis buffer, 150 mM NaCl (Thermo Fisher Scientific, UK), 25 mM Tris-HCl (Formedium, UK), 1% Triton-X-100 (Sigma-Aldrich Life Science, UK), and 5 mM Imidazole (Sigma-Aldrich Life Science, UK) was prepared and adjusted to pH 8.2 then chilled to 4° C. Before use, 10 ml of lysis buffer was combined with 100 µl Halt™ Protease and Phosphatase Inhibitor Cocktail (100X) 1:100 (Thermo Fisher Scientific, UK) and 10 µl Benzonase 1:10000 (Novagen, UK).

The frozen monolayer scraped cell pellets were thawed on ice then re-suspended in 500 µl of lysis buffer, while the suspension pellets were resuspended in lysis buffer at a ratio of 1:10 of lysis buffer to original suspension culture volume. Then the resuspended cell lysate was incubated in a cold room (4° C) on a tube the AG6 ORMA tilted tube roller (Eurotek, Italy) for 1 hour with occasional pipetting up and down to break down the cells.

2.11.2. Recombinant protein electrophoresis sample preparation

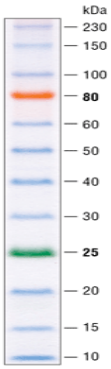
To screen the protein expression total fraction (TF) containing both soluble and insoluble proteins, 30 µl of the sample was collected and mixed with 60 µl of SDS-PAGE Sample Buffer, Laemmli 2× Concentrate (Sigma-Aldrich Life Science, UK) in a 0.2 ml PCR tube (STARLAB, UK). The remaining cell lysate was centrifuged at 15000 x g for 30 minutes at 4° C (Sigma-Aldrich Life Science, UK). The clear supernatant containing the soluble fraction (SF) of the protein was carefully transferred into a fresh 2 mL Eppendorf tube, and 30 µl of the sample was collected and mixed with 60 µl of SDS-PAGE Sample Buffer, Laemmli 2× Concentrate (Sigma-Aldrich Life Science, UK) in a 0.2 ml PCR tube. Both the soluble fraction clear lysate and the remaining pellet were snap-frozen and stored at -80° C. The SF and TF samples mixed with SDS-PAGE Sample Buffer, Laemmli 2× Concentrate (Sigma-Aldrich Life Science, UK) were placed in a the MiniAmp Plus Thermal Cycler (Thermo Fisher Scientific, UK) and incubated for 7 minutes at 99° C, to denature and linearise the protein for further analysis. This was applied on all rBV expressions of VP1 (BKPyV, JCPyV, and HPyV9), positive control (*Trichoplusia ni* insect Hi5™ cells infected with empty pOET8.VE2 transfer vector), and negative control (un-infected *Trichoplusia ni* insect Hi5™ cells).

2.11.3. Protein electrophoresis

Fifteen microliters of each denatured protein (section 2.11.2) in Laemmli sample buffer were loaded into each well of a NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.0 mm (Thermo Fisher Scientific, UK). Five microliters of protein ladder

(ColorPlus™ Prestained Protein Ladder, Broad Range 10-230 kDa; Figure 12; New England Biolabs, UK) was loaded in a separate well in an XCell SureLock Mini-Cell Electrophoresis System (Thermo Fisher Scientific, UK). The SDS-PAGE gel cast was placed in XCell SureLock Mini-Cell tank filled with chilled 1 x of Invitrogen™ NuPAGE® MES SDS-PAGE running buffer (Thermo Fischer Scientific, UK) prepared by mixing 50 ml 20x NuPAGE MES SDS-PAGE running buffer with 950 ml of deionised water. The gel was run at 200 V and 150 W for 45 minutes. After completion, the gel cast was removed, and the gel was extracted. The gel was placed in a square petri dish (Scientific Laboratories Supply, UK) then washed with deionised water twice. The gel was covered with 20 ml of InstantBlue™ Coomassie Blue Protein Stain (Expedeon, U.K.) and shaken at 150 rpm on a platform orbital mixer (Denley, UK) for 30 minutes until the staining colour developed. Finally, the gel was washed with deionised water twice then placed on FastGene White Light Plate (Geneflow Limited, Lichfield, U.K.) for Protein Gel visualisation and documentation. To predict the size of the recombinant proteins in relation to a molecular weight standard, the proteins sequence were analysed using SnapGene software.

Figure 12: ColorPlus™ Prestained Protein Ladder.

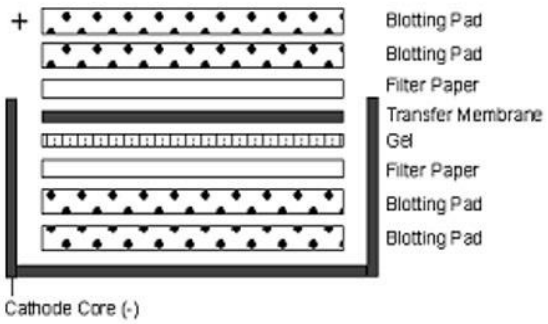


2.11.4. Protein Western blotting

In brief, 15 µl of each denatured protein samples and 10 µl of the protein ladder was loaded into separate wells of NuPAGE™ 4-12% Bis-Tris Protein Gels. The gels were first run at 200 V and 150 W for 45 minutes in a chilled 1 x of Invitrogen™ NuPAGE® MES SDS-PAGE running buffer. An Invitrogen™ Nitrocellulose membrane/Filter Paper Sandwich, 0.45 µm, 8.3 x 7.3 cm (Life Technologies Ltd., U.K.) and blotting pads were soaked in the 1X NuPAGE® Transfer Buffer for 20 minutes. The transfer buffer was made of 50 ml of 20X NuPAGE® Transfer Buffer (Life Technologies Ltd., U.K.) mixed with 100 ml of 100% methanol (Thermo Fisher Scientific, UK) and 850 ml of deionised water. After completion of the initial protein gel run, the extracted NuPAGE® gel, Nitrocellulose membrane/Filters, and the blotting pads were assembled in layers (Figure 13) and placed into the XCell II™ Blot (Life Technologies Ltd., U.K.) cassette. Then the cassette was placed into XCell SureLock Mini-Cell Electrophoresis System and filled with chilled 1X NuPAGE® Transfer Buffer, while the outer chamber was filled with deionised water. The transfer run took 60 minutes at 60 Volts. Meanwhile, 5% blocking buffer was prepared by mixing 2.5 g of skimmed milk (Marvel, Lincolnshire, UK), 50 ml of Phosphate buffer saline (PBS) and 50 µl of Tween 20 (Sigma-Aldrich Life Science, UK) and chilled to 4° C. After completion of the protein transfer run, the Nitrocellulose membrane was removed and blocked with 20 ml of blocking buffer for 1 hour at room temperature in a square petri dish (Scientific Laboratories Supply, UK) shaken at 50 rpm on a platform orbital mixer (Denley, UK). After 1 hour the membrane was rolled into a 50 ml Falcon tube (Thermo Fisher Scientific, UK) combined with 6 ml of the primary antibody (Anti-6X His tag® antibody [HIS.H8] ab18184 (Abcam, UK) diluted 1:1000 in blocking buffer) and left to incubate overnight in a cooled room

at 4° C on the AG6 ORMA tilted tube roller (Eurotek, Italy). The following day, the membrane was washed three times with 0.2% PBS/Tween 20 each time for 5 minutes inside the Falcon tube and on the tube roller. Then 6 ml of the secondary antibody (Rabbit Anti-Mouse IgG H&L (HRP) ab6728 (Abcam, UK) diluted 1:3000 in blocking buffer) was added to the Falcon tube and left to incubate for 1 hour in room temperature on a tube roller. The membrane was washed again three times with 0.2% PBS/Tween 20 each time for 5 minutes and one time with PBS for 5 minutes. Then the membrane was placed in a square petri dish, covered with 1-Step™ Ultra TMB-Blotting Solution (Thermo Fisher Scientific, UK) and left to develop. Finally, the membrane was washed with deionised water then dried and documented on FastGene White Light Plate (Geneflow Limited, Lichfield, U.K.).

Figure 13: Western blotting cassette.



Western blot protein transfer layers arrangement. Showing the NuPAGE® gel, Nitrocellulose membrane/Filters, and the blotting pads in the transfer cassette.

2.12. Recombinant protein purification

Several buffers were prepared for purification at pH 8.2 with different concentrations of Imidazole (Sigma-Aldrich Life Science, UK) solubilised in PBS;

equilibration buffer: 10 mM of Imidazole, wash buffers: 20 or 30 mM of Imidazole, and elution buffer: 250 mM of Imidazole.

Before purification, 200 µl resin beads HisPur™ Ni-NTA Resin beads (Thermo Fisher Scientific, UK) were centrifuged at 700 x *g* for 2 minutes (Sigma-Aldrich Life Science, UK), and the supernatant discarded. After this, the beads were washed with 400 µl equilibration buffer, centrifuged at 700 x *g* for 2 minutes, and the supernatant discarded.

Then the frozen, lysed, soluble fraction of the recombinant proteins was thawed on ice, and 200 µl mixed with 200 µl of equilibration buffer and the 400 µl of lysed protein/buffer was mixed with 200 µl of prepared HisPur™ Ni-NTA resin beads. The mixture was and incubated in a cold room (4° C) on the AG6 ORMA tilted tube roller (Eurotek, Italy) for 2 hours, and then spun at 700 x *g* for 2 minutes and the supernatant collected. Beads were washed five times with 1 mL equilibration buffer (10 mM Imidazole in PBS at pH 8.2). Each wash was left to incubate in a cold room (4° C) on a tube roller for 5 min then spun at 700 x *g* for 2 minutes and the supernatant collected. After two further washes with wash buffers containing 20 mM and 30 mM Imidazole in PBS at pH 8.2, elution was performed using 250 mM Imidazole in PBS at pH 8.2 with incubation in a cold room (4° C) on a tube roller for 5 min. The supernatant collected after centrifugation at 700 x *g* for 2 minutes, the elution step was repeated 3 times. All collected samples were then snap-frozen and stored at - 80° C.

2.13. ELISA

2.13.1. ELISA preparation

Purified antigens were quantified using Invitrogen™ Qubit™ Protein Assay Kit running on Qubit Fluorometer (Thermo Fisher Scientific, UK). A working protein solution was prepared by mixing Qubit protein reagent with Qubit buffer, in a 1:200 ratio. Enough working solution was prepared to test eluates in duplicate. Protein eluates were added in 1 µL volumes (or controls 10 µL) to 199 µL of protein working solution, and vortexed for 2-3 seconds. The samples and controls were left to incubate for 15 minutes at room temperature. Tubes were then placed in the Qubit Fluorometer for reading. The 3 controls were used to generate a standard curve. The mean average was calculated from the duplicate values for each protein eluate. Antigens were diluted in 0.05 M Carbonate-Bicarbonate at pH 9.6 coating buffer to a concentration of 5 µg/ml.

2.13.2. ELISA protocol

8 well ELISA strips were coated by 50 µL of antigens diluted to 5 µg/ml in 0.05 M Carbonate-Bicarbonate coating buffer pH 9.6 by overnight incubation at +4° C. The following day the liquid was aspirated, and the wells were washed with washing buffer (0.05% Tween20/PBS) for 2 min 5 times using an automated plate washer (BioTek™ ELx50™ Microplate Strip Washer, Agilent Technologies, UK). Then 300 µL of blocking buffer (1% bovine serum albumin (BSA)/PBS) was added to each well and incubated at 37° C for 1 hour. The blocking buffer was aspirated, and the wells washed with washing buffer for 2 min 5 times. Then 100

µL patient serum samples diluted 1:20 in PBS containing 1% BSA and 0.05% Tween20 were added to the wells and incubated at 37° C for 1 hour. Wells were washed with washing buffer for 2 min 5 times. Following, 100 µL secondary antibody (mouse mAb Anti-Human IgG HRP diluted 1:20000 in PBS containing 1% BSA and 0.05% Tween20; Thermo Fisher Scientific) was added to the wells and incubated at 37° C for 1 hour. Next 100 µL of TMB substrate solution (1-Step™ Ultra TMB-ELISA Substrate Solution; Thermo Fisher Scientific, UK) was added to each well and left to incubate at 37° C for 1 hour. Finally, 100 µL of 1N sulphuric acid stopping solution was added to stop the reaction, and the plate was read at 450 nm using ELx808™ Absorbance Microplate Reader (Agilent Technologies, UK).

2.14. Statistical data presentation and analysis

Continuous data were expressed by mean, median, standard deviation, and range. While categorical data were presented as frequencies and percentages. Comparative analysis between positive samples and negative samples time of collection after transplantation, as well as, comparison between patients with positive samples and patients with negative samples age was done using the independent t-test in Excel Software (Microsoft, USA).

Chapter 3. Results

3.1. qPCR design

3.1.1. Viral genomic sequences

An NCBI GenBank® search for complete genome sequences (last checked 31/1/2020) yielded 501 entries for BKPyV, 608 entries for JCPyV, and five entries for HPyV. Sequences of primers within existing PCR procedures targeting conserved regions of the viruses were compared with these sequences, and selected primer sets were further analysed to ensure specificity of the resultant PCR techniques. Clustal Omega alignment results guided the selection of qPCR target regions. Primers and probes within each of the conserved regions were designed with the aid of Primer3Plus, Oligo Calc and Multiple primer analyser software and the specificity of each primer and probe confirmed by Blast N search against the entire GenBank sequence database.

3.1.2. Amplification targets

The primers (Table 2) for each of the selected targets amplified conserved regions of each virus (LTag gene of BKPyV; LTag gene of JCPyV; VP1 gene of HPyV9). The size of each amplicon was; 122bp BKPyV, 138bp JCPyV, and 102bp HPyV9 (Table 3). The hydrolysis probe binding region in each of the sequences amplified is highlighted in grey below:

BKPyV:CAGAAGGAAAGTCTTTAGGGTCTTCTACCTTTCTTTTTTTCTGGGT
GGTGTTGAGTGTTGAGAATCTGCTGTTGCTTCTTCATCACTGGCAAACATA
TCTTCATGGCAAAATAAATCTTCATC,

JCPyV:TTTAGGTGGGGTAGAGTGTTGGGATCCTGTGTTTTCATCATCACTG
GCAAACATTTCTTCATGGCAAAACAGGTCTTCATCCCACTTCTCATTAAATG
TATTCCACCAGGATTCCCATTCATCTGTTCCATAGGTTGG,

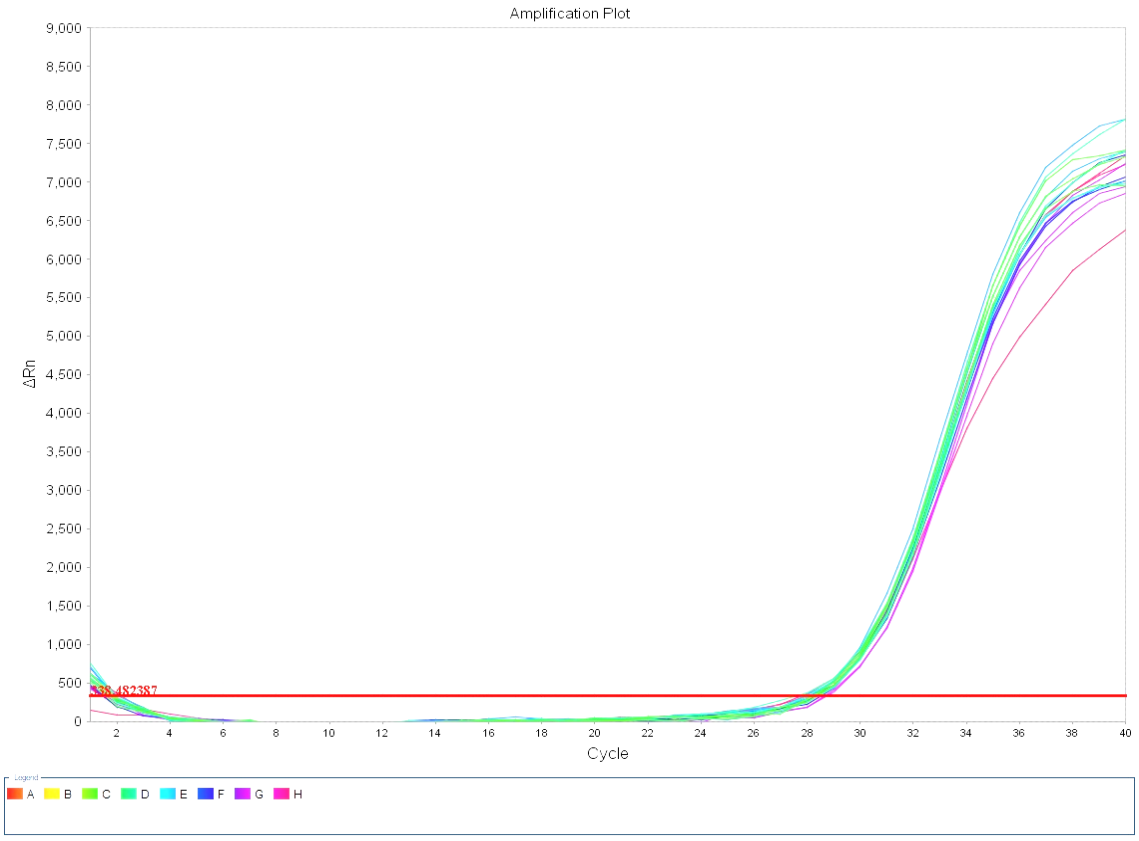
HPyV9:GCTCTCTCCTTAATAGCTTGTTCTCTGGTCTTATGCCTCAAATTCAG
GGACAACCCATGGAAGGAACTTCAGGGCAAGTGGAAGAAGTCAGAATTTA
TCAGG.

The internal control molecule, designed as a control to be added to each sample to monitor for potential inhibition of the PCR amplification in each sample, was a 140 bp DNA sequence of pumpkin DNA. This sequence is found in hydroxypyruvate reductase gene of pumpkin (*Cucurbita cv. Kurokawa Amakuri*), and the gene was previously developed as an internal control molecule for DNA amplification in PCR by (McHugh et al., 2015). The hydrolysis probe binding region in the internal control sequence is highlighted in grey below: GATGAGGTGCTTCGAGAGGCTGATGTGATAAGTCTTCATCCAGTACTGGAT
AAAACCACCTTCCATCTGGTGAACAAAGAAAGTCTTAAAGCCATGAAGAAG
GATGCAATTCTCATTAACTGTAGTAGGGGACCCGTGAT.

The sequence for the internal control was made synthetically and cloned into plasmid pEX-A2. Purified plasmid DNA containing the insert was used, and the number of copies of the internal control molecule was calculated as described in section 2.5.1. The internal control was serially diluted, and an optimum

concentration of the internal control molecule was selected on the basis that it could be reliably detected and did not interfere with the amplification of the target molecules. The final concentration of IC in each reaction was chosen to be 166 copies/mL. The control was consistently positive (Figure 14), giving an average Cq value of 29.34 ±1.41 Cq (SD) (1728 data points).

Figure 14: Internal control molecule amplification.



The fluorescent signal for ABY was read using the TAMRA filter on the StepOne instrument as this instrument lacks a specific filter for ABY. The fluorescent signal is lower than observed with the dye signals used for the other targets.

3.1.3. PCR Development

The performance of the selected primer and probe combinations were evaluated as duplex assays with each of the three viral targets individually, together with the internal control molecule PCR and then combined with all 3 viral targets plus the internal control molecule in one multiplex PCR.

The positive control was calculated to contain 4.2×10^{11} copies per microliter of each of the BKPyV, JCPyV, and HPyV9 targets using the calculation at https://scienceprimer.com/copy-number-calculator-for-realtime-pcr, as described in section 2.5.1. Dilutions of this positive control material were made from 10^{-5} to 10^{-11} equivalent to 4.2 X 10^{6} copies/μL to 4.2 copies/μL. 5 μL of this concentrate was added per reaction; thus the positive control dilution series equated to 4.116 X 10^{5} per reaction tube to 0.4116 copies per reaction tube. Each dilution was tested in triplicate, and the Cq value measured was plotted against copy number.

A similar dilution series was prepared using each of the WHO international standards for BKPyV and JCPyV DNA. Dilutions of the standards were prepared from 10^{-1} to 10^{-7} equivalent to 1,584,893 IU/mL to 1.58 IU/mL for BKPyV and 1,000,000 IU/mL to 1 IU/mL for JCPyV. Aliquots of 200 μL of each dilution was extracted as described in section 2.4.3 and resuspended in 100 μL, Final concentration in each reaction tube would equate to 1/10th of each of these concentrations.

3.1.4.1. BKPyV

The results of the titration of the positive control are shown in Table 16.

Table 16: BKPyV positive control titration.

Dilution factor	Mean Cq. value	Copies per reaction	Copies/mL
10^{-5}	16.26	4.2×10^{5}	4.2×10^{6}
10^{-6}	20.24	4.2×10^{4}	4.2×10^{5}
10^{-7}	23.97	4.2×10^{3}	4.2×10^{4}
10^{-8}	27.33	4.2×10^{2}	4.2×10^{3}
10^{-9}	31.41	4.2×10	4.2×10^{2}
10^{-10}	34.86	4.2	4.2X 10
10^{-11}	37.46	0.42	4.2

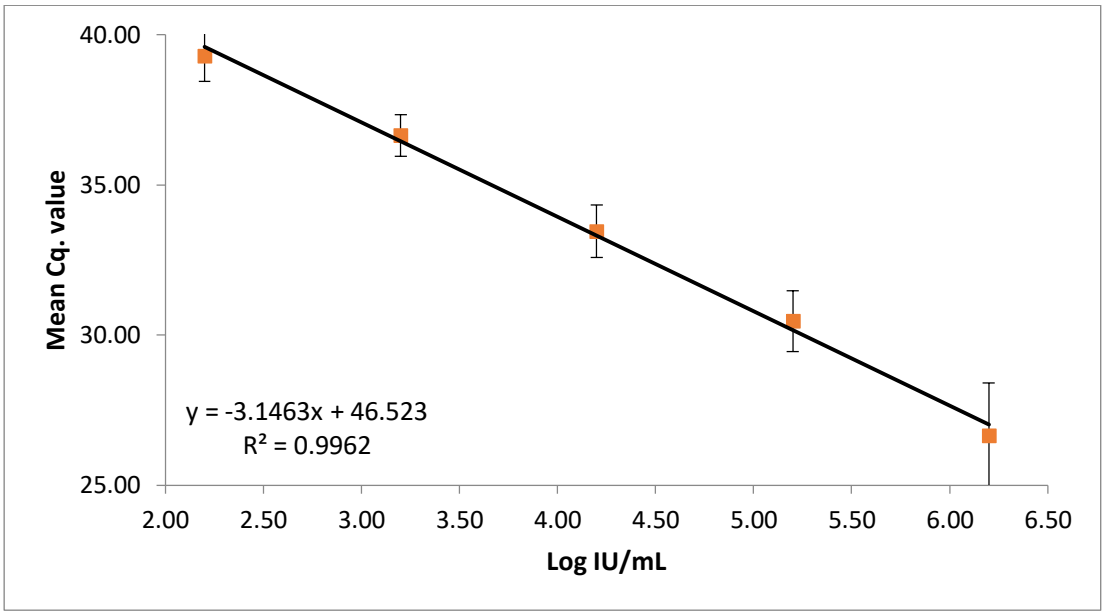
And the results of the titration of the WHO BKPyV standard in the same assay are shown in Table 17.

Table 17: BKPyV WHO international standard.

Dilution factor	Mean Cq. value	Mean Log IU/mL
10^{-1}	26.66	6.20
10^{-2}	30.47	5.20
10^{-3}	33.46	4.20
10^{-4}	36.65	3.20
10^{-5}	39.30	2.20

To determine the amplification efficiency of the PCR, the slope of the log linear portion of the calibration curve of each of the controls was determined. Where PCR efficiency = $10^{-1/slope}$ -1 (Bustin et al., 2009). The calibration curve for the BKPyV WHO international standard is shown in Figure 15.

Figure 15: BKPyV WHO international standard calibration curve.



The y-axis shows the concentration $\log_{10}$ International Units/ml, and the x-axis shows the Cq value at each point. The equation of a straight line with gradient m and intercept c on the y-axis is y = mx + c, thus the slope = -3.146.

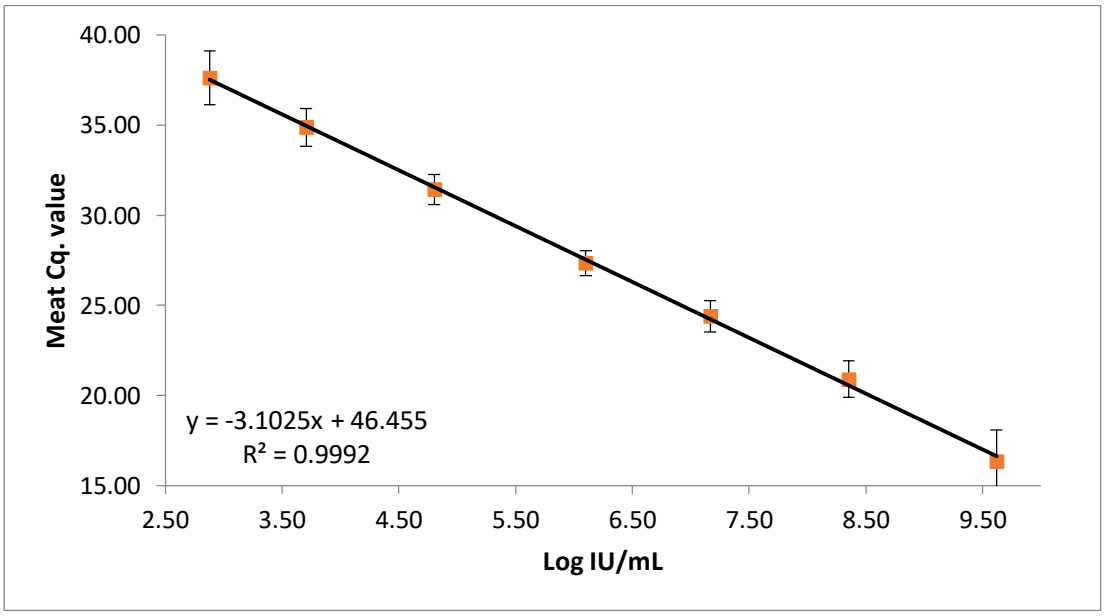
The slope of the graph shown in Figure 15 was -3.1463 (correlation coefficient (r^2) = 0.9962) thus the PCR efficiency was calculated as $10^{-1/-3.1463} - 1 = 107.90\%$. The apparent detection of the assay was less than 158 IU/mL. The calibration curve for the positive control material was analysed in the same way (correlation coefficient r^2 = 0.9975) and slope -3.5062, suggesting a PCR efficiency of 92.85%. Using the data in Figure 15 and Table 18 and the Cq values generated for the positive control the positive control was re-calibrated in IU/ml rather than

in copies/ml creating a secondary standard from the WHO reference standard. The positive control calibration curve was replotted in IU/ml in Figure 16.

Table 18: BKPyV positive control

Dilution factor	Mean Cq. value	Mean Log IU/mL
10^{-5}	16.26	9.62
10^{-6}	20.24	8.35
10^{-7}	23.97	7.17
10^{-8}	27.33	6.10
10^{-9}	31.41	4.80
10^{-10}	34.86	3.71
10^{-11}	37.46	2.88

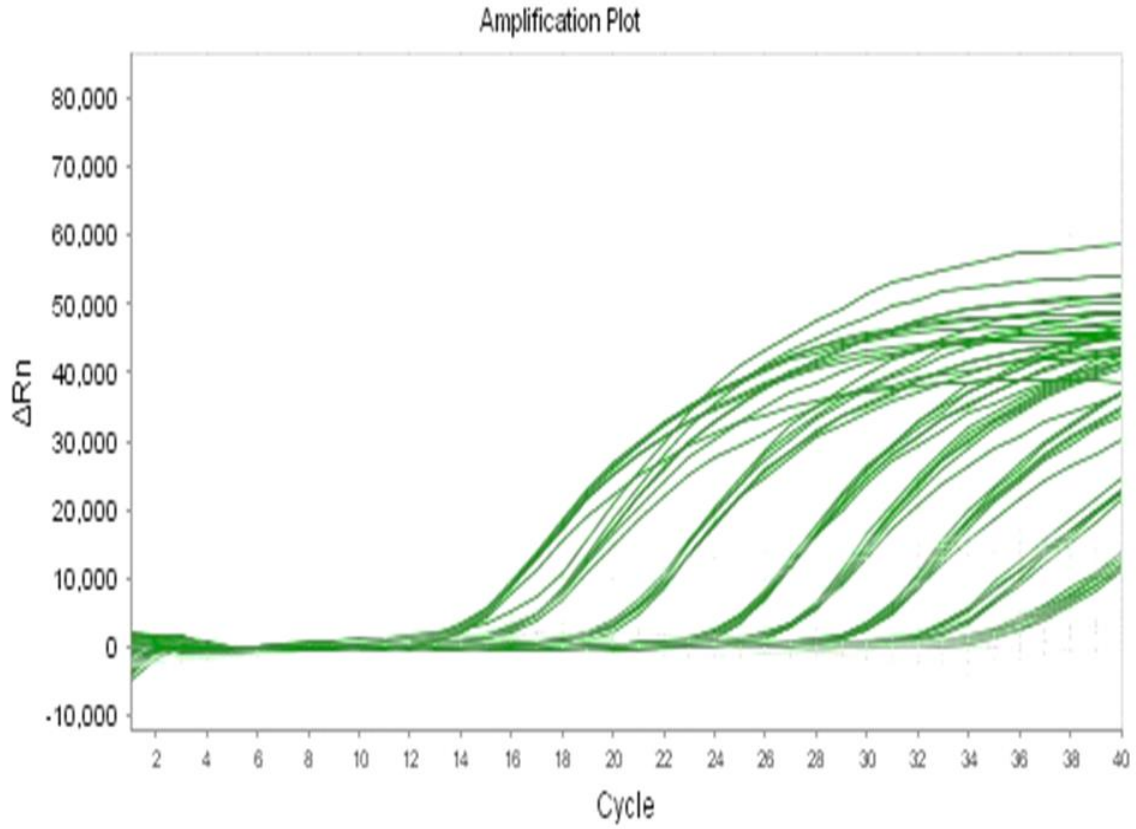
Figure 16: BKPyV positive control (secondary WHO standard) calibration curve.



Calibration curve of BKPyV positive control. Error bars represent the Co-efficient of Variation (CV%) of each data point.

The reaction was linear across the dynamic range represented by the positive control extending over a 7-log concentration range (Figure 17). The limit of detection was not formally assessed in this experiment but was ≤log 2.75 IU/mL.

Figure 17: Amplification plot for BKPyV positive control (primary WHO standard and positive control (secondary standard).



Amplification Curves of BKPyV qPCR WHO standard and secondary standard (positive control) where the fluorescence intensity of the probe reporter dye (FAM) shown in green traces.

3.1.4.2. JCPyV

The JCPyV WHO international standard was titrated as described for BKPyV (section 3.1.4.1) using log_{10} serial dilutions from 10^5 IU/mL to 10^2 IU/mL (Table 19; Figure 18) PCR efficiency was 92.46%, r^2 = 0.9996. As for JCPyV, the synthetic positive control was also titrated, and copies/mL in this control converted into IU/mL as described for BKPyV. (PCR Efficiency 92.46%, r^2 1) (Table 20; Figure 19) The PCR was linear across the dynamic range represented by the positive control extending over a 6-log concentration range (Figure 20). The limit of detection was not formally assessed in this experiment but was ≤log 2.92 IU/mL.

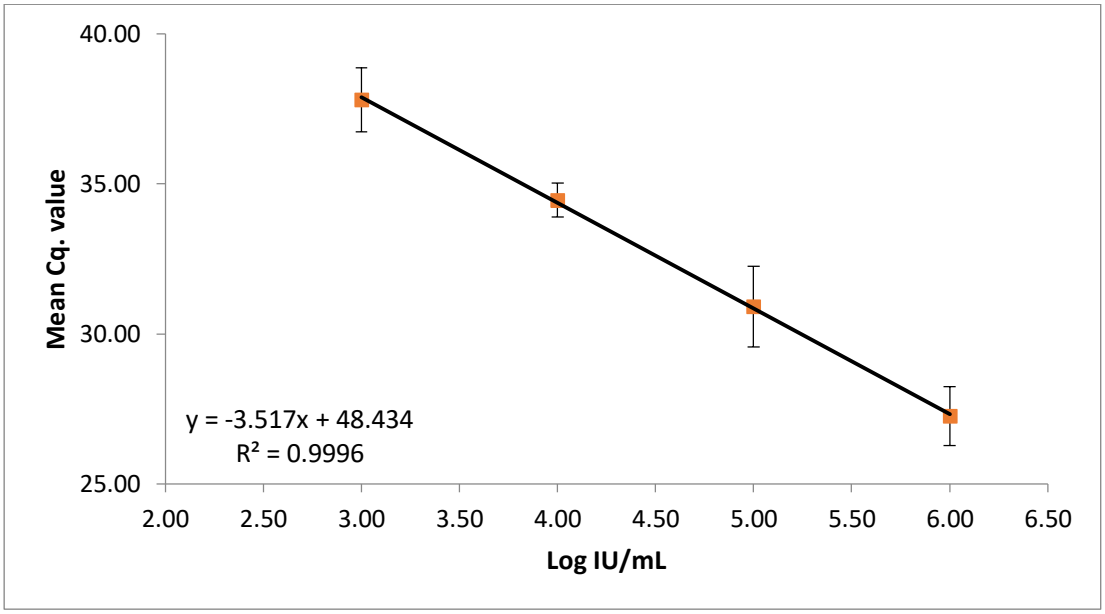
Table 19: JCPyV WHO international.

Dilution factor	Mean Cq value	Log IU/mL
10^{-1}	27.26	6.00
10^{-2}	30.91	5.00
10^{-3}	34.46	4.00
10^{-4}	37.80	3.00

Table 20: JCPyV positive control.

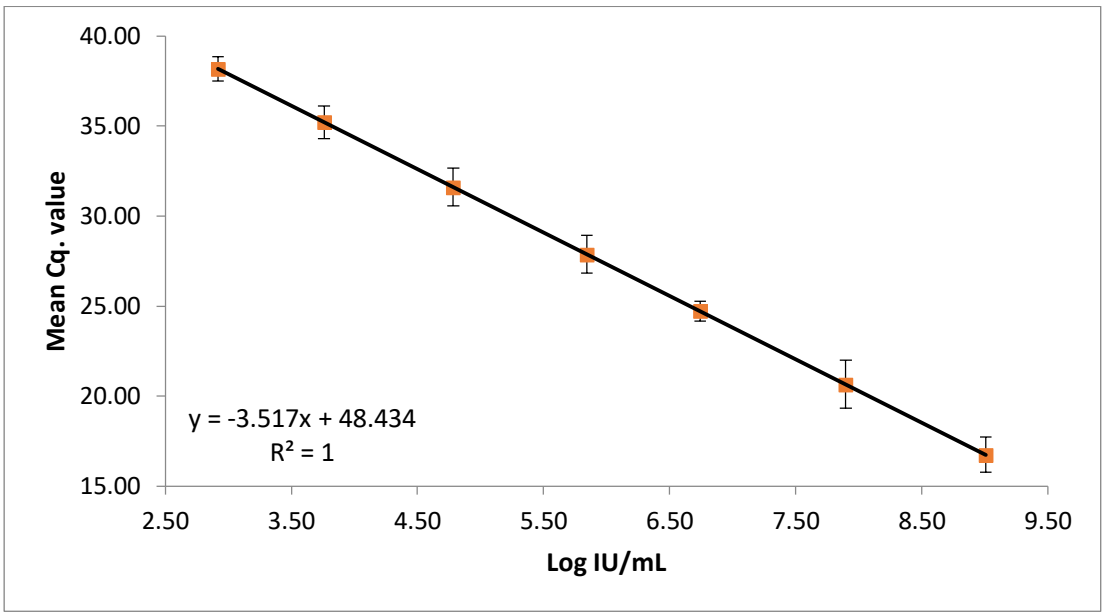
Dilution factor	Mean Cq value	Mean Log IU/mL
10^{-5}	16.75	9.01
10^{-6}	20.66	7.90
10^{-7}	24.72	6.74
10^{-8}	27.88	5.84
10^{-9}	31.61	4.78
10^{-10}	35.21	3.76
10^{-11}	38.17	2.92

Figure 18: JCPyV WHO international standard calibration curve.



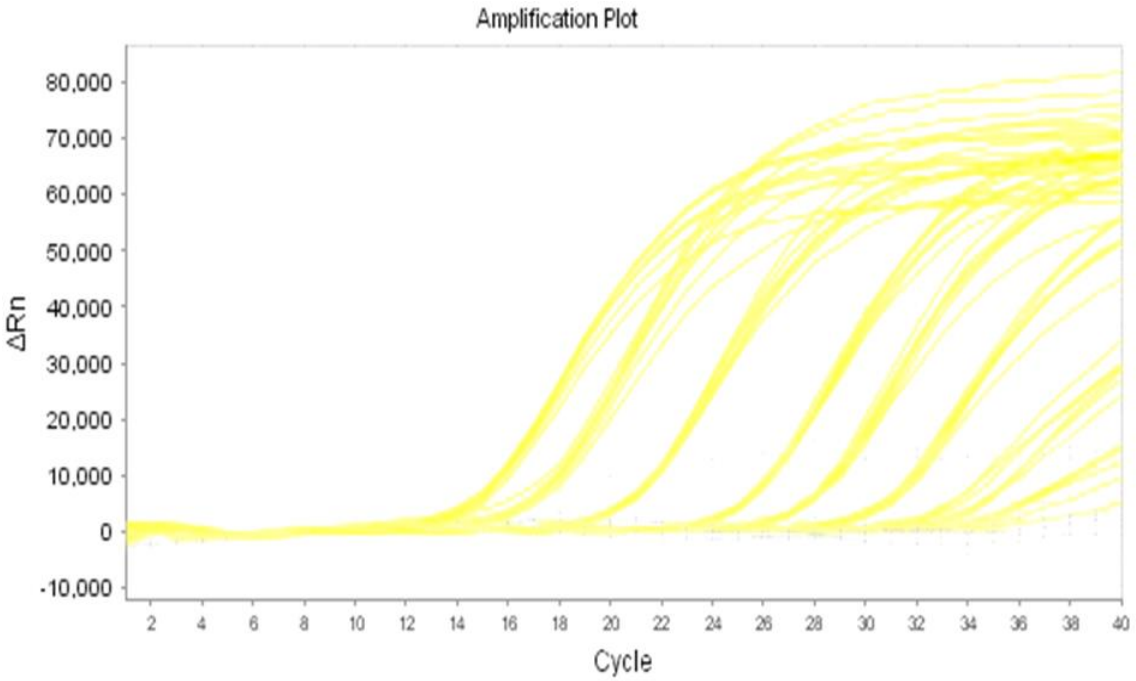
Calibration curve of JCPyV international standard. Error bars represent the Coefficient of Variation (CV%) of each data point.

Figure 19: JCPyV positive control (secondary WHO standard) calibration curve.



Calibration curve of JCPyV positive control. Error bars represent the Coefficient of Variation (CV%) of each data point.

Figure 20: Amplification plot for JCPyV positive control (primary WHO standard and positive control (secondary standard).



Amplification Curves of JCPyV qPCR WHO standard and secondary standard (positive control) where the fluorescence intensity of the probe reporter dye (VIC) shown in yellow traces.

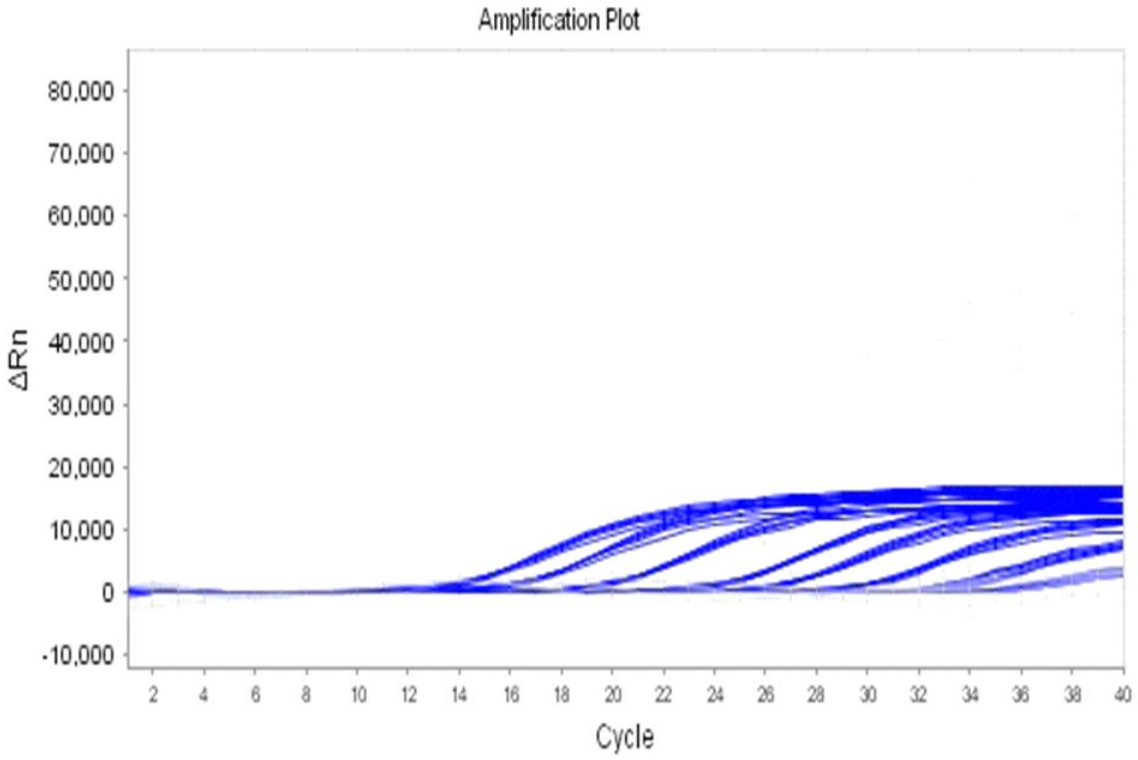
3.1.4.3. HPyV9

The positive control material was also used to assess the linearity and dynamic range of the HPyV9 PCR. The PCR efficiency was 91.35%, with $r^2 = 0.9964$ (Table 21; Figure 21). The PCR was linear across the dynamic range represented by the positive control extending over a 6-log concentration range. The limit of detection was not formally assessed in this experiment due to the lack of an international standard but was calculated using a synthetic plasmid. The calculated limit of detection was ≤ 4.116×10^3 copies/mL.

Table 21: HPyV9 positive control.

Dilution factor	Mean Cq. value	Mean Log copy/mL	Copies/mL
10^{-5}	15.15	6.60	4.2×10^9
10^{-6}	19.10	5.60	4.2×10^8
10^{-7}	22.78	4.60	4.2×10^7
10^{-8}	26.24	3.50	4.2×10^6
10^{-9}	30.65	2.50	4.2×10^5
10^{-10}	34.02	1.50	$4.2X\ 10^4$
10^{-11}	36.40	0.50	$4.2X\ 10^3$

Figure 21: Amplification plot for HPyV9 positive control.



Amplification Curves of HPyV9 positive control where the fluorescence intensity of the probe reporter dye (JUN) shown in blue traces.

3.2. Development and Validation TaqMan® multiplex real-time qPCR

These individual PCRs were then combined in a multiplex polymerase chain reaction (qPCR) to detect BKPyV, JCPyV, HPyV9, and an internal control (IC) molecule. The assay was recalibrated for each target using the positive control materials. The linearity of the PCR and PCR efficiency was re-evaluated in a multiplex format. The efficiency and correlation coefficient for BKPyV was 110% r^2 0.9992; for JCPyV 93.73%, r^2 0.9996; and for HPyV9 90.90%, r^2 0.9961, showing equivalent efficiency and linearity across the dynamic range of detection of each of the assays.

To determine whether the addition of the internal control molecule led to competition for amplification with the BKPyV, JCPyV or HPyV9 targets, differences in Cq values of each dilution of the control tested in the single target assay and in a multiplex assay were compared for each target.

Also the effect of combining all three viral targets; BKPyV, JCPyV, and HpyV9, and the internal control molecule for simultaneous detection in the final multiplex assay was analysed (Table 22).

Table 22: Singleplex versus multiplex positive control performance.

	concentration	Singleplex Mean Cq	Multiplex Mean Cq	difference
BKPyV (log IU/mL)	9.62	16.26	16.34	0.07
	8.35	20.24	20.89	0.65
	7.17	23.97	24.40	0.43
	6.1	27.33	27.35	0.02
	4.8	31.41	31.43	0.02
	3.71	34.86	34.89	0.03
	2.88	37.46	37.62	0.16
JCPyV (log IU/mL)	9.01	16.75	16.96	0.21
	7.90	20.66	21.13	0.47
	6.74	24.72	24.81	0.09
	5.84	27.88	27.87	0.02
	4.78	31.61	31.91	0.29
	3.76	35.21	35.36	0.15
	2.92	38.17	38.19	0.02
HPyV9 (log copies/ mL)	6.60	15.15	15.17	0.02
	5.60	19.10	19.68	0.58
	4.60	22.78	23.26	0.48
	3.50	26.24	26.25	0.01
	2.50	30.65	30.70	0.05
	1.50	34.02	34.27	0.25
	0.50	36.40	36.92	0.52

The developed multiplex qPCR assay proved a robust assay with good concordance between singleplex and multiplex assays with an only minor difference in observed Cq.

3.3. Precision and reproducibility assessment

The inter-assay and intra-assay variability analyzed using 6 replicates of the positive control. The standard deviation (SD) and cumulative percentage variance were calculated for each concentration of the control (Table 23).

Table 23: positive control multiplex reproducibility.

	Mean Cq. value	Cq. value SD	Cq. value CV%	Inter-assay SD	Intra-assay CV%	SD	CV%
BKPyV (log IU/mL)	16.34	0.29	1.75	0.29	1.78	0.09	0.56
	20.89	0.21	1.02	0.21	1.01	0.01	0.07
	24.4	0.21	0.88	0.18	0.72	0.10	0.40
	27.35	0.19	0.69	0.17	0.62	0.14	0.52
	31.43	0.27	0.85	0.28	0.88	0.13	0.41
	34.89	0.37	1.05	0.39	1.11	0.04	0.10
	37.62	0.56	1.49	0.39	1.03	0.27	0.72
JCPyV (log IU/mL)	16.96	0.17	0.98	0.18	1.04	0.04	0.21
	21.13	0.28	1.34	0.30	1.40	0.07	0.35
	24.81	0.14	0.56	0.14	0.55	0.05	0.21
	27.87	0.30	1.06	0.30	1.08	0.16	0.57
	31.91	0.33	1.05	0.31	0.98	0.24	0.75
	35.36	0.32	0.92	0.29	0.82	0.08	0.23
	38.19	0.26	0.68	0.19	0.51	0.16	0.42
HPyV9 (log copies/mL)	15.17	0.18	1.20	0.14	0.89	0.00	0.02
	19.68	0.18	0.92	0.20	1.00	0.10	0.49
	23.26	0.27	1.16	0.25	1.09	0.15	0.65
	26.25	0.19	0.73	0.17	0.63	0.14	0.55
	30.70	0.47	1.52	0.41	1.33	0.38	1.23
	34.27	0.38	1.10	0.40	1.16	0.16	0.46
	36.92	0.51	1.38	0.51	1.38	0.51	1.38

The multiplex BKPyV assay had Cq SD < 0.56 with an average of 0.30 and CV% < 1.75% with an average of 1.10%. Inter-assay variation showed SD < 0.39 with average of 0.27 and CV% < 1.78% with an average of 1.02%. Intra-assay variation SD < 0.27 with an average of 0.11 and CV% < 0.72% with an average of 0.40%.

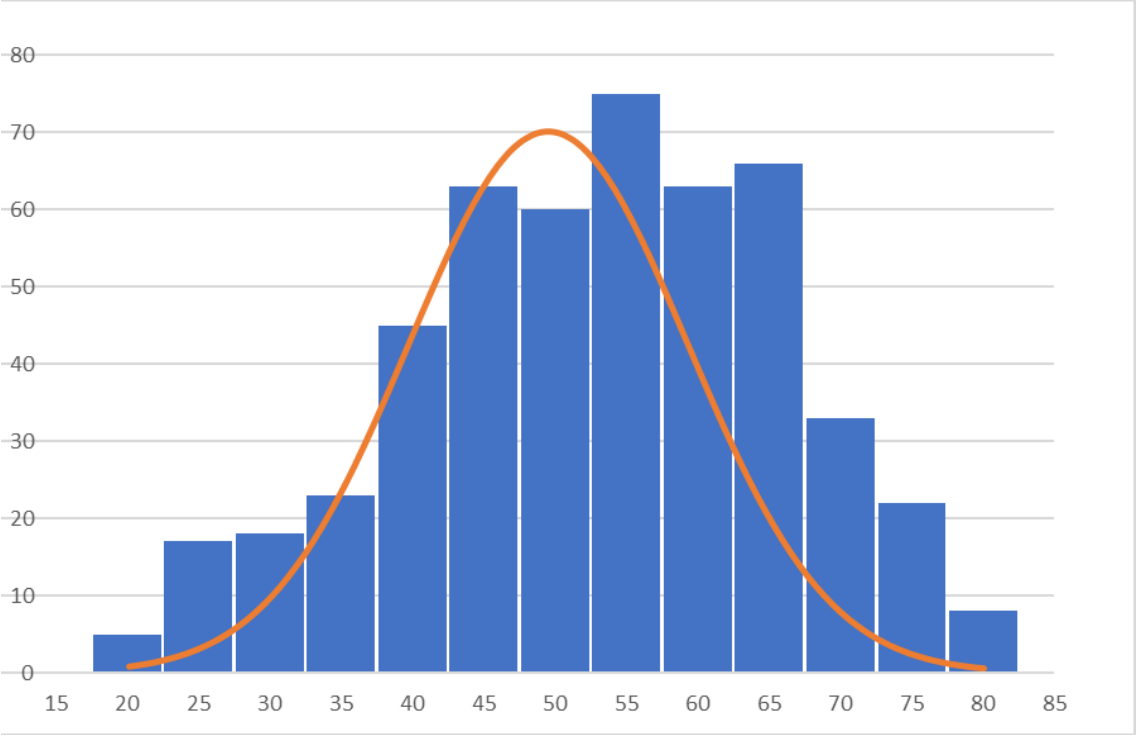
The multiplex JCPyV assay had Cq values SD < 0.33 with average of 0.26 and CV% < 1.34% with average of 0.94%. Inter-assay variation showed SD < 0.31 with average of 0.24 and CV% < 1.40% with average of 0.91%. Intra-assay variation SD < 0.24 with average of 0.11 and CV% < 0.75% with average of 0.39%.

The multiplex assay for HPyV9 showed similar reproducibility with Cq values SD < 0.51 with average of 0.31 and CV% < 1.52% with average of 1.14%. Inter-assay variation showed SD < 0.51 with average of 0.30 and CV% < 1.38% with average of 1.07%. Intra-assay variation SD < 0.51 with average of 0.21 and CV% < 1.38% with average of 0.68%.

3.4. Renal Transplant recipients

The study population was drawn from patients attending the renal medicine Department at Manchester Royal Infirmary. Informed consent for participation in the study was obtained from 466 Patients including 193 female, and 273 males their age range is illustrated in Figure 22 (range 19-80 years (mean 50.8 ±13.02 years; median 52 years; mode 62 years).

Figure 22: Age range of study population.



Frequency distribution of age (years) of study population the expected normal distribution is shown by the orange line. The data is negatively skewed (-0.25) showing a higher proportion of elderly patients

Samples of blood were collected at intervals from each of the study patients, and nucleic acid was extracted from whole blood and stored as described in Section 2.1. In contrast to most studies which investigate infection in the immediate transplant period, the average time of collection of these samples after transplant was 9.22yrs ±10.66yrs; Median 7.01yrs. For 44 samples, no transplant date was given. Most patients had been on haemodialysis prior to transplant, approximately 10% of the patient population had been on peritoneal dialysis.

3.5. PCR testing

The DNA extracts (1156) of samples were tested (Table 24) using 96-well PCR plates; 90 wells of each were used for clinical samples, and the remaining six wells were used for controls, five wells contained dilutions of the positive control, and the final well contained the negative control. The IC molecule was consistently positive in all sample runs. All positive samples were repeated to confirm results.

Seventy two samples from 44 patients were found to be polyomavirus positive (6.2% of samples).

Table 24: Polyomavirus PCR test results.

Patent ID	Sample Number	BKPyV (Cq; log IU/mL)	JCPyV (Cq; log IU/mL)	HPyV9 (Cq; log IU/mL)
MMEBV-0001	10.9538	-ve	-ve	-ve
MMEBV-0001	10.92396	-ve	-ve	-ve
MMEBV-0001	10.90886	-ve	-ve	-ve
MMEBV-0002	10.92819	-ve	-ve	-ve
MMEBV-0002	10.90884	-ve	-ve	-ve
MMEBV-0003	10.95261	-ve	-ve	-ve
MMEBV-0003	10.92709	-ve	-ve	-ve
MMEBV-0003	10.90884	-ve	-ve	-ve
MMEBV-0003	11.91613	-ve	-ve	-ve
MMEBV-0004	10.9526	-ve	-ve	-ve
MMEBV-0004	10.92396	-ve	-ve	-ve
MMEBV-0004	10.90886	-ve	-ve	-ve
MMEBV-0004	11.91347	-ve	-ve	-ve
MMEBV-0005	10.90914	-ve	-ve	-ve
MMEBV-0005	10.93667	-ve	-ve	-ve
MMEBV-0005	10.92661	-ve	-ve	-ve
MMEBV-0006	10.90914	-ve	-ve	-ve
MMEBV-0006	11.90803	-ve	-ve	-ve
MMEBV-0007	10.93448	19.1; 8.34	-ve	-ve
MMEBV-0007	10.93667	30.7; 5.04	-ve	-ve
MMEBV-0007	10.90914	-ve	-ve	-ve
MMEBV-0007	10.92866	31.6; 4.79	-ve	-ve
MMEBV-0008	10.93367	-ve	-ve	-ve
MMEBV-0008	10.90914	-ve	-ve	-ve
MMEBV-0008	11.90739	-ve	-ve	-ve
MMEBV-0009	11.90105	-ve	-ve	-ve
MMEBV-0009	10.90914	-ve	-ve	-ve
MMEBV-0009	11.92072	-ve	-ve	-ve
MMEBV-0010	10.90968	-ve	-ve	-ve
MMEBV-0010	11.90855	-ve	-ve	-ve

MMEBV-0011	10.95488	-ve	-ve	-ve
MMEBV-0011	10.90968	-ve	-ve	-ve
MMEBV-0011	11.91294	-ve	-ve	-ve
MMEBV-0012	10.90968	-ve	-ve	-ve
MMEBV-0012	11.92426	-ve	-ve	-ve
MMEBV-0013	10.90968	-ve	-ve	-ve
MMEBV-0013	11.91287	-ve	-ve	-ve
MMEBV-0014	10.95328	-ve	-ve	-ve
MMEBV-0014	10.90986	-ve	-ve	-ve
MMEBV-0014	10.92866	-ve	-ve	-ve
MMEBV-0014	11.90883	-ve	-ve	-ve
MMEBV-0014	11.92687	-ve	-ve	-ve
MMEBV-0015	10.93646	-ve	-ve	-ve
MMEBV-0015	10.92398	-ve	-ve	-ve
MMEBV-0015	10.90986	-ve	-ve	-ve
MMEBV-0015	11.90984	-ve	-ve	-ve
MMEBV-0016	10.91259	-ve	-ve	-ve
MMEBV-0016	10.90986	-ve	-ve	-ve
MMEBV-0017	10.95379	-ve	-ve	-ve
MMEBV-0017	10.90986	-ve	-ve	-ve
MMEBV-0017	11.92627	-ve	28.3; 5.72	-ve
MMEBV-0018	10.93059	-ve	-ve	-ve
MMEBV-0018	10.91949	-ve	-ve	-ve
MMEBV-0018	11.91509	-ve	-ve	-ve
MMEBV-0019	10.93165	-ve	-ve	-ve
MMEBV-0019	10.91025	-ve	33.2; 4.33	-ve
MMEBV-0019	11.90927	-ve	-ve	-ve
MMEBV-0020	10.93031	-ve	-ve	-ve
MMEBV-0020	10.93489	-ve	-ve	-ve
MMEBV-0020	10.91025	-ve	-ve	-ve
MMEBV-0022	10.93168	-ve	-ve	-ve
MMEBV-0022	10.93616	-ve	-ve	-ve
MMEBV-0022	10.91602	-ve	-ve	-ve
MMEBV-0022	10.92613	-ve	-ve	-ve
MMEBV-0022	11.91612	-ve	-ve	-ve
MMEBV-0023	10.93551	-ve	-ve	-ve
MMEBV-0023	10.91025	-ve	-ve	-ve
MMEBV-0023	10.92734	-ve	-ve	-ve
MMEBV-0024	10.93706	-ve	-ve	-ve
MMEBV-0024	10.91025	-ve	-ve	-ve
MMEBV-0024	11.90739	-ve	-ve	-ve
MMEBV-0025	11.90634	-ve	-ve	-ve
MMEBV-0025	10.91025	-ve	-ve	-ve
MMEBV-0025	10.9273	-ve	-ve	-ve
MMEBV-0025	11.91613	-ve	-ve	-ve
MMEBV-0026	11.90634	-ve	-ve	-ve
MMEBV-0026	10.93401	-ve	-ve	-ve
MMEBV-0026	10.91025	-ve	-ve	-ve
MMEBV-0027	10.93198	-ve	-ve	-ve
MMEBV-0027	10.93706	-ve	-ve	-ve
MMEBV-0027	10.93768	-ve	-ve	-ve
MMEBV-0027	10.91867	-ve	-ve	-ve
MMEBV-0027	10.9105	-ve	-ve	-ve
MMEBV-0027	10.9105	-ve	-ve	-ve
MMEBV-0027	10.92813	-ve	33.6;4.22	-ve
MMEBV-0027	11.91563	37; 3.25	-ve	-ve
MMEBV-0028	10.95206	-ve	-ve	-ve
MMEBV-0028	10.93543	-ve	-ve	-ve
MMEBV-0028	10.92517	-ve	-ve	-ve
MMEBV-0028	10.91306	-ve	-ve	-ve
MMEBV-0028	11.90832	-ve	-ve	-ve
MMEBV-0028	11.91917	-ve	-ve	-ve

MMEBV-0029	10.95206	-ve	-ve	-ve
MMEBV-0029	10.91354	-ve	-ve	-ve
MMEBV-0030	10.92423	-ve	-ve	-ve
MMEBV-0030	11.90061	-ve	-ve	-ve
MMEBV-0030	10.93617	-ve	-ve	-ve
MMEBV-0030	10.91059	-ve	-ve	-ve
MMEBV-0031	10.9106	-ve	-ve	-ve
MMEBV-0031	11.90806	-ve	-ve	-ve
MMEBV-0032	10.9538	-ve	-ve	-ve
MMEBV-0032	10.9105	-ve	-ve	-ve
MMEBV-0033	10.95328	-ve	-ve	-ve
MMEBV-0033	10.92424	-ve	-ve	-ve
MMEBV-0033	10.91073	-ve	-ve	-ve
MMEBV-0033	11.90806	-ve	-ve	-ve
MMEBV-0034	10.92666	-ve	-ve	-ve
MMEBV-0034	10.93769	-ve	-ve	-ve
MMEBV-0034	10.91073	-ve	-ve	-ve
MMEBV-0034	11.91347	-ve	-ve	-ve
MMEBV-0035	10.93449	-ve	-ve	-ve
MMEBV-0035	10.91074	-ve	-ve	-ve
MMEBV-0035	11.9154	-ve	-ve	-ve
MMEBV-0036	11.90049	-ve	-ve	-ve
MMEBV-0036	10.91073	-ve	-ve	-ve
MMEBV-0036	11.92617	-ve	-ve	-ve
MMEBV-0037	10.91073	-ve	-ve	-ve
MMEBV-0037	10.93667	-ve	-ve	-ve
MMEBV-0037	10.94926	-ve	-ve	-ve
MMEBV-0037	11.92628	-ve	-ve	-ve
MMEBV-0038	10.92479	-ve	-ve	-ve
MMEBV-0038	10.91073	-ve	-ve	-ve
MMEBV-0039	10.95197	-ve	-ve	-ve
MMEBV-0039	10.93769	-ve	-ve	-ve
MMEBV-0039	10.92661	-ve	-ve	-ve
MMEBV-0039	10.91073	-ve	-ve	-ve
MMEBV-0040	10.93869	-ve	-ve	-ve
MMEBV-0040	10.92908	-ve	-ve	-ve
MMEBV-0040	10.92015	-ve	-ve	-ve
MMEBV-0040	11.90774	-ve	-ve	-ve
MMEBV-0040	11.92199	-ve	-ve	-ve
MMEBV-0041	10.91091	-ve	-ve	-ve
MMEBV-0041	10.91091	-ve	-ve	-ve
MMEBV-0041	10.9247	-ve	-ve	-ve
MMEBV-0042	11.90048	-ve	-ve	-ve
MMEBV-0042	10.9266	-ve	-ve	-ve
MMEBV-0042	11.91781	-ve	-ve	-ve
MMEBV-0043	10.93728	-ve	-ve	-ve
MMEBV-0043	10.92733	-ve	-ve	-ve
MMEBV-0043	11.91157	-ve	-ve	-ve
MMEBV-0043	11.92812	-ve	-ve	-ve
MMEBV-0044	10.95196	-ve	-ve	-ve
MMEBV-0044	10.91091	-ve	-ve	-ve
MMEBV-0044	10.92573	-ve	-ve	-ve
MMEBV-0044	11.92794	33.7; 4.19	-ve	-ve
MMEBV-0045	10.92798	-ve	-ve	-ve
MMEBV-0045	11.91157	-ve	-ve	-ve
MMEBV-0046	10.91092	-ve	-ve	-ve
MMEBV-0046	10.93354	-ve	-ve	-ve
MMEBV-0047	10.91376	-ve	-ve	-ve
MMEBV-0047	10.91121	-ve	-ve	-ve
MMEBV-0048	10.92709	-ve	-ve	-ve
MMEBV-0048	10.91121	-ve	-ve	-ve
MMEBV-0048	11.91188	-ve	-ve	-ve

MMEBV-0049	10.95296	-ve	-ve	-ve
MMEBV-0049	11.90175	-ve	-ve	-ve
MMEBV-0049	10.91121	-ve	-ve	-ve
MMEBV-0049	11.91948	-ve	-ve	-ve
MMEBV-0050	10.9112	-ve	-ve	-ve
MMEBV-0051	10.9112	-ve	-ve	-ve
MMEBV-0052	10.9123	-ve	-ve	-ve
MMEBV-0052	10.93551	-ve	29.9; 5.27	-ve
MMEBV-0052	10.9247	-ve	-ve	-ve
MMEBV-0052	11.90831	-ve	-ve	-ve
MMEBV-0053	10.91706	-ve	-ve	-ve
MMEBV-0054	11.90061	-ve	-ve	-ve
MMEBV-0054	10.91403	-ve	-ve	-ve
MMEBV-0054	10.91121	-ve	-ve	-ve
MMEBV-0054	11.91578	-ve	-ve	-ve
MMEBV-0055	10.91121	-ve	-ve	-ve
MMEBV-0055	11.91587	-ve	-ve	-ve
MMEBV-0056	11.90164	-ve	-ve	-ve
MMEBV-0056	10.9112	-ve	-ve	-ve
MMEBV-0056	11.91916	-ve	-ve	-ve
MMEBV-0057	10.95261	-ve	-ve	-ve
MMEBV-0057	10.92735	-ve	-ve	-ve
MMEBV-0057	11.91488	-ve	-ve	-ve
MMEBV-0058	10.95508	-ve	-ve	-ve
MMEBV-0058	10.92975	-ve	-ve	-ve
MMEBV-0058	11.91488	-ve	-ve	-ve
MMEBV-0059	10.92995	-ve	-ve	-ve
MMEBV-0059	10.91283	-ve	-ve	-ve
MMEBV-0059	10.91282	-ve	-ve	-ve
MMEBV-0060	10.93808	-ve	-ve	-ve
MMEBV-0060	10.91155	-ve	-ve	-ve
MMEBV-0061	10.91155	33.5; 4.25	-ve	-ve
MMEBV-0062	10.93504	-ve	-ve	-ve
MMEBV-0062	10.91725	-ve	-ve	-ve
MMEBV-0062	10.91154	-ve	-ve	-ve
MMEBV-0063	10.91155	-ve	-ve	-ve
MMEBV-0063	10.92731	-ve	-ve	-ve
MMEBV-0063	11.91187	-ve	-ve	-ve
MMEBV-0064	10.93168	-ve	-ve	-ve
MMEBV-0064	10.91155	-ve	-ve	-ve
MMEBV-0064	11.92726	-ve	-ve	-ve
MMEBV-0065	10.91155	-ve	-ve	-ve
MMEBV-0065	10.92731	-ve	-ve	-ve
MMEBV-0065	11.91488	-ve	-ve	-ve
MMEBV-0066	10.93169	-ve	-ve	-ve
MMEBV-0066	10.91155	-ve	-ve	-ve
MMEBV-0067	10.93617	-ve	35.9; 3.56	-ve
MMEBV-0067	10.91155	-ve	34.8; 3.88	-ve
MMEBV-0067	10.92731	-ve	30.7; 5.04	-ve
MMEBV-0067	11.90715	-ve	-ve	-ve
MMEBV-0067	11.91803	-ve	-ve	-ve
MMEBV-0068	10.92974	-ve	-ve	-ve
MMEBV-0068	10.91182	-ve	-ve	-ve
MMEBV-0068	11.90739	-ve	-ve	-ve
MMEBV-0070	10.9332	-ve	-ve	-ve
MMEBV-0070	10.91183	-ve	-ve	-ve
MMEBV-0071	10.92661	-ve	-ve	-ve
MMEBV-0071	10.91182	-ve	-ve	-ve
MMEBV-0071	11.91347	-ve	-ve	-ve
MMEBV-0072	10.9289	-ve	-ve	-ve
MMEBV-0072	10.91182	-ve	-ve	-ve
MMEBV-0073	10.93748	-ve	-ve	-ve

MMEBV-0073	10.91182	-ve	-ve	-ve
MMEBV-0074	10.93687	-ve	-ve	-ve
MMEBV-0075	10.95346	-ve	-ve	-ve
MMEBV-0075	10.93543	-ve	-ve	-ve
MMEBV-0075	10.92479	-ve	-ve	-ve
MMEBV-0075	10.91183	-ve	-ve	-ve
MMEBV-0075	11.92199	-ve	-ve	-ve
MMEBV-0076	10.92472	28.2; 5.75	-ve	-ve
MMEBV-0076	10.91354	17.5; 8.80	-ve	-ve
MMEBV-0076	10.91182	26.7; 6.18	-ve	-ve
MMEBV-0076	11.91563	-ve	-ve	-ve
MMEBV-0076	11.90716	34.5; 3.96	-ve	-ve
MMEBV-0077	11.90048	-ve	-ve	-ve
MMEBV-0077	10.92645	-ve	-ve	-ve
MMEBV-0077	10.9353	-ve	-ve	-ve
MMEBV-0077	10.92478	-ve	-ve	-ve
MMEBV-0077	10.91182	-ve	-ve	-ve
MMEBV-0078	10.92995	-ve	-ve	-ve
MMEBV-0078	10.91204	-ve	-ve	-ve
MMEBV-0078	11.90832	-ve	-ve	-ve
MMEBV-0079	10.92709	-ve	-ve	-ve
MMEBV-0079	11.9102	-ve	-ve	-ve
MMEBV-0079	11.92546	-ve	-ve	-ve
MMEBV-0080	10.92995	-ve	-ve	-ve
MMEBV-0080	10.93793	-ve	-ve	-ve
MMEBV-0080	10.91204	38; 2.97	-ve	-ve
MMEBV-0081	10.95176	-ve	35.4; 3.70	-ve
MMEBV-0081	10.93617	-ve	-ve	-ve
MMEBV-0081	10.91403	-ve	-ve	-ve
MMEBV-0081	11.91749	-ve	-ve	-ve
MMEBV-0082	11.90634	-ve	-ve	-ve
MMEBV-0082	10.91282	-ve	-ve	-ve
MMEBV-0082	11.91875	-ve	-ve	-ve
MMEBV-0083	10.93504	-ve	-ve	-ve
MMEBV-0083	10.93277	-ve	-ve	-ve
MMEBV-0084	10.91204	-ve	-ve	-ve
MMEBV-0084	10.93792	-ve	-ve	-ve
MMEBV-0084	11.90716	-ve	-ve	-ve
MMEBV-0084	11.92649	-ve	-ve	-ve
MMEBV-0085	10.92889	-ve	-ve	-ve
MMEBV-0085	10.91204	-ve	-ve	-ve
MMEBV-0085	11.91656	-ve	-ve	-ve
MMEBV-0086	10.93645	-ve	-ve	-ve
MMEBV-0086	10.92661	-ve	-ve	-ve
MMEBV-0086	10.91204	-ve	-ve	34.5;1.25
MMEBV-0086	11.90715	-ve	-ve	-ve
MMEBV-0086	11.90987	-ve	-ve	-ve
MMEBV-0087	10.91229	-ve	-ve	-ve
MMEBV-0087	10.95328	-ve	-ve	-ve
MMEBV-0087	10.93465	-ve	-ve	-ve
MMEBV-0087	11.91163	-ve	-ve	-ve
MMEBV-0088	10.9521	-ve	-ve	-ve
MMEBV-0088	10.9123	-ve	-ve	-ve
MMEBV-0088	10.93357	-ve	-ve	-ve
MMEBV-0089	10.95308	-ve	-ve	-ve
MMEBV-0089	10.91229	-ve	-ve	-ve
MMEBV-0089	10.93769	-ve	-ve	-ve
MMEBV-0090	10.92843	-ve	-ve	-ve
MMEBV-0090	10.9123	-ve	-ve	-ve
MMEBV-0090	10.93513	-ve	-ve	-ve
MMEBV-0090	11.91488	-ve	-ve	-ve
MMEBV-0091	10.92813	-ve	-ve	-ve

MMEBV-0091	10.93705	-ve	-ve	-ve
MMEBV-0091	10.91259	-ve	-ve	-ve
MMEBV-0091	11.92589	-ve	-ve	-ve
MMEBV-0091	11.91854	-ve	-ve	-ve
MMEBV-0092	10.92816	-ve	-ve	-ve
MMEBV-0092	10.91259	-ve	-ve	-ve
MMEBV-0092	11.91447	-ve	-ve	-ve
MMEBV-0093	10.93801	-ve	-ve	-ve
MMEBV-0093	10.93031	-ve	-ve	-ve
MMEBV-0093	10.91259	-ve	-ve	-ve
MMEBV-0093	11.91188	-ve	-ve	-ve
MMEBV-0094	10.93028	-ve	-ve	-ve
MMEBV-0094	10.93706	-ve	-ve	-ve
MMEBV-0094	10.91259	-ve	-ve	-ve
MMEBV-0094	11.91188	-ve	-ve	-ve
MMEBV-0095	10.95233	-ve	-ve	-ve
MMEBV-0095	10.92821	-ve	-ve	-ve
MMEBV-0095	10.9326	-ve	-ve	-ve
MMEBV-0095	10.91259	-ve	-ve	-ve
MMEBV-0097	10.93808	-ve	-ve	-ve
MMEBV-0097	10.91455	-ve	-ve	-ve
MMEBV-0097	11.91948	-ve	-ve	-ve
MMEBV-0098	10.93031	-ve	-ve	-ve
MMEBV-0098	10.91258	-ve	-ve	-ve
MMEBV-0098	11.9215	32.6; 4.50	-ve	-ve
MMEBV-0099	10.93592	-ve	-ve	-ve
MMEBV-0099	10.91258	-ve	-ve	-ve
MMEBV-0100	10.95207	-ve	-ve	-ve
MMEBV-0100	10.93687	-ve	-ve	-ve
MMEBV-0100	10.92472	-ve	-ve	-ve
MMEBV-0100	10.91282	-ve	-ve	-ve
MMEBV-0100	11.90855	-ve	-ve	-ve
MMEBV-0101	10.95434	-ve	-ve	-ve
MMEBV-0101	10.91283	-ve	-ve	-ve
MMEBV-0102	10.934	-ve	-ve	-ve
MMEBV-0102	10.91282	-ve	-ve	-ve
MMEBV-0102	11.90927	-ve	-ve	-ve
MMEBV-0103	10.93853	-ve	-ve	-ve
MMEBV-0103	10.92517	-ve	-ve	-ve
MMEBV-0103	10.91306	-ve	-ve	-ve
MMEBV-0103	11.9215	-ve	-ve	-ve
MMEBV-0104	10.92777	-ve	-ve	-ve
MMEBV-0104	10.9345	-ve	-ve	-ve
MMEBV-0104	10.93792	-ve	-ve	-ve
MMEBV-0104	10.91306	-ve	-ve	-ve
MMEBV-0105	10.91305	-ve	-ve	-ve
MMEBV-0105	11.91648	-ve	-ve	-ve
MMEBV-0105	11.90684	-ve	-ve	-ve
MMEBV-0106	10.93853	-ve	-ve	-ve
MMEBV-0106	10.91305	-ve	-ve	-ve
MMEBV-0106	11.90716	-ve	-ve	-ve
MMEBV-0106	11.92588	-ve	-ve	-ve
MMEBV-0107	10.9332	-ve	-ve	-ve
MMEBV-0107	10.91305	-ve	-ve	-ve
MMEBV-0107	11.90716	-ve	-ve	-ve
MMEBV-0108	10.92798	-ve	-ve	-ve
MMEBV-0108	10.92568	-ve	-ve	-ve
MMEBV-0108	10.9133	-ve	-ve	-ve
MMEBV-0109	11.90148	-ve	-ve	-ve
MMEBV-0109	10.92798	-ve	-ve	-ve
MMEBV-0109	10.91354	-ve	-ve	-ve
MMEBV-0110	10.93357	-ve	-ve	-ve

MMEBV-0110	10.92661	-ve	27.7; 5.89	-ve
MMEBV-0110	10.91354	-ve	26.4; 6.26	-ve
MMEBV-0110	11.90857	-ve	-ve	-ve
MMEBV-0111	11.91532	-ve	-ve	-ve
MMEBV-0112	10.91354	-ve	-ve	-ve
MMEBV-0112	11.91564	-ve	-ve	-ve
MMEBV-0113	10.92645	-ve	-ve	-ve
MMEBV-0113	10.91354	-ve	-ve	-ve
MMEBV-0113	11.91157	-ve	-ve	-ve
MMEBV-0114	10.95176	-ve	-ve	-ve
MMEBV-0114	10.93489	-ve	-ve	-ve
MMEBV-0114	10.92709	-ve	-ve	-ve
MMEBV-0114	10.91707	-ve	-ve	-ve
MMEBV-0115	10.95197	-ve	-ve	-ve
MMEBV-0115	10.93465	-ve	-ve	-ve
MMEBV-0115	10.9247	-ve	-ve	-ve
MMEBV-0115	10.91204	-ve	-ve	-ve
MMEBV-0115	11.92506	-ve	-ve	-ve
MMEBV-0116	10.95222	-ve	-ve	-ve
MMEBV-0116	10.93801	-ve	-ve	-ve
MMEBV-0116	10.9112	-ve	-ve	-ve
MMEBV-0116	11.91294	-ve	-ve	-ve
MMEBV-0116	11.92472	-ve	-ve	-ve
MMEBV-0117	10.91376	-ve	-ve	-ve
MMEBV-0118	10.91375	-ve	-ve	-ve
MMEBV-0118	11.92151	-ve	-ve	-ve
MMEBV-0119	10.95272	-ve	-ve	-ve
MMEBV-0119	10.93534	-ve	-ve	-ve
MMEBV-0119	10.92708	-ve	-ve	-ve
MMEBV-0119	10.91375	-ve	-ve	-ve
MMEBV-0119	11.91366	-ve	-ve	-ve
MMEBV-0120	10.92709	-ve	-ve	-ve
MMEBV-0121	10.95508	-ve	-ve	-ve
MMEBV-0121	10.93031	-ve	-ve	-ve
MMEBV-0121	10.91376	-ve	-ve	-ve
MMEBV-0121	10.9192	-ve	-ve	-ve
MMEBV-0122	10.93031	-ve	-ve	-ve
MMEBV-0122	10.91375	-ve	-ve	-ve
MMEBV-0122	11.92688	-ve	-ve	-ve
MMEBV-0123	10.93449	-ve	-ve	-ve
MMEBV-0123	10.91376	-ve	-ve	-ve
MMEBV-0123	11.90717	-ve	-ve	-ve
MMEBV-0123	11.91447	-ve	-ve	-ve
MMEBV-0123	11.91042	-ve	-ve	-ve
MMEBV-0124	10.93372	-ve	-ve	-ve
MMEBV-0124	10.914	-ve	-ve	-ve
MMEBV-0125	10.95369	-ve	-ve	-ve
MMEBV-0125	10.93448	-ve	-ve	-ve
MMEBV-0125	10.91403	-ve	-ve	-ve
MMEBV-0125	11.92199	-ve	-ve	-ve
MMEBV-0126	10.91403	-ve	-ve	-ve
MMEBV-0127	10.91402	-ve	-ve	-ve
MMEBV-0127	11.90855	-ve	-ve	-ve
MMEBV-0128	10.95261	32.2; 4.61	-ve	-ve
MMEBV-0128	10.9273	28.1; 5.78	-ve	-ve
MMEBV-0128	10.91403	28.7; 5.61	-ve	-ve
MMEBV-0128	10.91403	27.5; 5.95	-ve	-ve
MMEBV-0129	10.93215	-ve	-ve	-ve
MMEBV-0129	11.91381	-ve	34.5; 3.96	-ve
MMEBV-0130	10.92732	-ve	-ve	-ve
MMEBV-0131	10.91432	-ve	-ve	-ve
MMEBV-0131	10.93793	-ve	-ve	-ve

MMEBV-0131	10.91885	-ve	-ve	-ve
MMEBV-0131	11.90882	-ve	-ve	-ve
MMEBV-0132	10.91432	-ve	-ve	-ve
MMEBV-0133	10.91447	-ve	-ve	-ve
MMEBV-0136	10.9324	-ve	-ve	-ve
MMEBV-0136	10.91455	-ve	-ve	-ve
MMEBV-0136	11.91446	-ve	-ve	-ve
MMEBV-0137	10.9289	-ve	-ve	-ve
MMEBV-0137	10.91455	-ve	-ve	-ve
MMEBV-0137	11.91803	29.5; 5.38	-ve	-ve
MMEBV-0138	10.93616	-ve	-ve	-ve
MMEBV-0138	10.91455	-ve	-ve	-ve
MMEBV-0139	10.95567	-ve	-ve	-ve
MMEBV-0139	10.91456	-ve	-ve	-ve
MMEBV-0139	11.91446	-ve	-ve	-ve
MMEBV-0140	10.93667	-ve	-ve	-ve
MMEBV-0140	10.91456	-ve	-ve	-ve
MMEBV-0140	11.91949	-ve	-ve	-ve
MMEBV-0140	11.90832	-ve	-ve	-ve
MMEBV-0141	10.93802	-ve	-ve	-ve
MMEBV-0141	10.91456	34.6; 3.93	-ve	-ve
MMEBV-0142	11.90635	-ve	-ve	-ve
MMEBV-0142	10.91455	-ve	-ve	-ve
MMEBV-0142	10.9273	-ve	-ve	-ve
MMEBV-0142	11.91347	-ve	-ve	-ve
MMEBV-0143	10.93728	-ve	-ve	-ve
MMEBV-0143	10.92573	-ve	-ve	-ve
MMEBV-0143	11.92199	-ve	-ve	-ve
MMEBV-0144	10.95472	-ve	-ve	-ve
MMEBV-0144	10.93705	-ve	-ve	-ve
MMEBV-0144	10.91474	-ve	-ve	-ve
MMEBV-0144	10.92813	-ve	-ve	-ve
MMEBV-0144	11.90717	-ve	-ve	-ve
MMEBV-0144	11.91716	-ve	-ve	-ve
MMEBV-0145	10.93241	-ve	-ve	-ve
MMEBV-0145	10.91474	-ve	-ve	-ve
MMEBV-0145	11.90717	-ve	-ve	-ve
MMEBV-0146	10.91474	-ve	-ve	-ve
MMEBV-0146	11.92648	-ve	-ve	-ve
MMEBV-0147	10.95308	-ve	-ve	-ve
MMEBV-0147	11.9012	-ve	-ve	-ve
MMEBV-0147	10.9247	-ve	-ve	-ve
MMEBV-0147	10.91474	-ve	-ve	-ve
MMEBV-0148	10.95206	-ve	-ve	-ve
MMEBV-0148	10.93014	-ve	-ve	-ve
MMEBV-0148	10.9147	-ve	-ve	-ve
MMEBV-0148	11.92649	-ve	-ve	-ve
MMEBV-0149	10.92339	-ve	-ve	-ve
MMEBV-0149	10.91474	-ve	-ve	-ve
MMEBV-0149	11.92427	-ve	-ve	-ve
MMEBV-0149	11.90711	-ve	-ve	-ve
MMEBV-0149	11.91839	-ve	-ve	-ve
MMEBV-0150	10.93015	-ve	-ve	-ve
MMEBV-0150	10.9147	-ve	-ve	-ve
MMEBV-0150	11.91519	-ve	-ve	-ve
MMEBV-0150	11.92648	-ve	-ve	-ve
MMEBV-0151	10.93513	-ve	-ve	-ve
MMEBV-0151	10.92661	-ve	-ve	-ve
MMEBV-0151	11.91347	-ve	-ve	-ve
MMEBV-0152	10.91479	-ve	-ve	-ve
MMEBV-0152	11.91697	-ve	-ve	-ve
MMEBV-0153	10.93014	-ve	-ve	-ve

MMEBV-0153	10.9148	-ve	-ve	-ve
MMEBV-0154	10.92813	-ve	-ve	-ve
MMEBV-0154	11.92241	-ve	-ve	-ve
MMEBV-0155	10.9303	-ve	-ve	-ve
MMEBV-0155	11.91716	-ve	-ve	-ve
MMEBV-0156	10.92813	-ve	-ve	-ve
MMEBV-0156	11.92472	-ve	-ve	-ve
MMEBV-0156	11.90687	-ve	35; 3.82	-ve
MMEBV-0157	10.95222	-ve	-ve	-ve
MMEBV-0157	10.93031	-ve	-ve	-ve
MMEBV-0158	10.9332	-ve	-ve	-ve
MMEBV-0158	11.91437	-ve	-ve	-ve
MMEBV-0158	11.92688	-ve	-ve	-ve
MMEBV-0159	10.95308	-ve	-ve	-ve
MMEBV-0159	10.93211	-ve	-ve	-ve
MMEBV-0159	11.91716	-ve	-ve	-ve
MMEBV-0160	10.92925	-ve	-ve	-ve
MMEBV-0160	11.91727	-ve	-ve	-ve
MMEBV-0161	11.90123	-ve	-ve	-ve
MMEBV-0161	10.91183	-ve	-ve	-ve
MMEBV-0161	11.90832	-ve	-ve	-ve
MMEBV-0162	10.92661	-ve	-ve	-ve
MMEBV-0162	11.92126	-ve	-ve	-ve
MMEBV-0164	10.95229	-ve	-ve	-ve
MMEBV-0170	10.95321	-ve	-ve	-ve
MMEBV-0170	11.90163	-ve	-ve	-ve
MMEBV-0171	10.95285	-ve	-ve	-ve
MMEBV-0171	10.92516	-ve	-ve	-ve
MMEBV-0172	10.9538	-ve	-ve	-ve
MMEBV-0172	10.93372	-ve	26.1; 6.35	-ve
MMEBV-0172	11.92303	-ve	-ve	-ve
MMEBV-0172	11.90774	-ve	-ve	-ve
MMEBV-0174	10.95308	-ve	-ve	-ve
MMEBV-0174	10.9289	-ve	-ve	-ve
MMEBV-0174	10.91576	-ve	-ve	-ve
MMEBV-0175	10.93667	-ve	-ve	-ve
MMEBV-0175	10.91576	-ve	-ve	-ve
MMEBV-0175	11.92091	-ve	-ve	-ve
MMEBV-0176	11.90164	-ve	-ve	-ve
MMEBV-0176	10.91601	-ve	-ve	-ve
MMEBV-0176	11.92649	-ve	-ve	-ve
MMEBV-0177	10.93794	-ve	-ve	-ve
MMEBV-0177	10.91602	-ve	-ve	37.5; 0.41
MMEBV-0178	10.95328	-ve	-ve	-ve
MMEBV-0178	10.93687	-ve	-ve	-ve
MMEBV-0178	10.92565	-ve	-ve	-ve
MMEBV-0178	10.91601	-ve	-ve	-ve
MMEBV-0178	11.90716	-ve	-ve	-ve
MMEBV-0178	11.92126	-ve	-ve	-ve
MMEBV-0179	10.92595	27.1; 6.07	-ve	-ve
MMEBV-0179	10.91601	26.1; 6.35	-ve	-ve
MMEBV-0179	11.91612	-ve	-ve	-ve
MMEBV-0180	10.91601	-ve	-ve	-ve
MMEBV-0181	10.91602	-ve	-ve	-ve
MMEBV-0182	11.90319	-ve	-ve	-ve
MMEBV-0182	10.93356	-ve	-ve	-ve
MMEBV-0182	10.91601	-ve	-ve	-ve
MMEBV-0183	10.95206	-ve	-ve	-ve
MMEBV-0183	10.91683	-ve	-ve	-ve
MMEBV-0183	11.91971	-ve	-ve	-ve
MMEBV-0184	10.91682	-ve	-ve	-ve
MMEBV-0184	11.92812	-ve	-ve	-ve

MMEBV-0185	10.91683	-ve	-ve	-ve
MMEBV-0185	11.9188	-ve	-ve	-ve
MMEBV-0186	10.92908	-ve	-ve	-ve
MMEBV-0186	10.91682	-ve	-ve	-ve
MMEBV-0187	10.95285	-ve	-ve	-ve
MMEBV-0187	10.91682	-ve	-ve	-ve
MMEBV-0189	10.93031	-ve	-ve	-ve
MMEBV-0189	10.91707	-ve	-ve	-ve
MMEBV-0190	10.91707	-ve	-ve	-ve
MMEBV-0190	11.91993	-ve	-ve	-ve
MMEBV-0191	10.91706	-ve	-ve	-ve
MMEBV-0191	11.91716	-ve	-ve	-ve
MMEBV-0192	10.92764	-ve	-ve	-ve
MMEBV-0192	10.91706	-ve	-ve	-ve
MMEBV-0192	11.92144	-ve	-ve	-ve
MMEBV-0193	10.9303	-ve	-ve	-ve
MMEBV-0193	11.9215	-ve	-ve	-ve
MMEBV-0194	10.93372	-ve	-ve	-ve
MMEBV-0194	10.93257	-ve	-ve	-ve
MMEBV-0194	10.91707	-ve	-ve	-ve
MMEBV-0194	11.91858	-ve	-ve	-ve
MMEBV-0195	10.93372	-ve	-ve	-ve
MMEBV-0196	10.91725	-ve	-ve	-ve
MMEBV-0196	11.90774	-ve	-ve	-ve
MMEBV-0196	11.92199	37.8; 3.02	-ve	-ve
MMEBV-0197	10.93721	-ve	-ve	-ve
MMEBV-0198	10.91725	-ve	-ve	-ve
MMEBV-0198	11.92198	-ve	-ve	-ve
MMEBV-0199	10.93728	-ve	-ve	-ve
MMEBV-0199	10.91725	-ve	-ve	-ve
MMEBV-0200	10.91725	-ve	-ve	-ve
MMEBV-0200	11.91471	-ve	-ve	-ve
MMEBV-0201	10.93277	-ve	-ve	-ve
MMEBV-0201	11.91613	-ve	-ve	-ve
MMEBV-0202	10.95243	-ve	-ve	-ve
MMEBV-0202	10.93163	-ve	-ve	-ve
MMEBV-0202	11.91078	-ve	-ve	-ve
MMEBV-0203	11.90272	-ve	-ve	-ve
MMEBV-0203	10.93168	-ve	-ve	-ve
MMEBV-0203	11.92342	-ve	28.3; 5.72	-ve
MMEBV-0204	10.93214	-ve	-ve	-ve
MMEBV-0204	11.90955	-ve	-ve	-ve
MMEBV-0205	10.95182	-ve	-ve	-ve
MMEBV-0205	10.93352	-ve	-ve	-ve
MMEBV-0205	10.91791	-ve	-ve	-ve
MMEBV-0205	11.92028	-ve	-ve	-ve
MMEBV-0205	11.90684	-ve	-ve	-ve
MMEBV-0206	10.91806	-ve	-ve	-ve
MMEBV-0206	11.92292	-ve	-ve	-ve
MMEBV-0207	10.93265	-ve	-ve	-ve
MMEBV-0207	10.93706	-ve	-ve	-ve
MMEBV-0207	10.91813	-ve	-ve	-ve
MMEBV-0208	10.9278	-ve	-ve	-ve
MMEBV-0208	10.93257	-ve	-ve	-ve
MMEBV-0208	11.91993	-ve	-ve	-ve
MMEBV-0209	10.91813	33.7; 4.19	-ve	-ve
MMEBV-0211	10.9182	-ve	-ve	-ve
MMEBV-0211	11.90891	-ve	-ve	-ve
MMEBV-0212	10.93265	-ve	-ve	-ve
MMEBV-0213	10.95379	-ve	-ve	-ve
MMEBV-0213	10.93278	-ve	-ve	-ve
MMEBV-0213	10.91839	-ve	-ve	-ve

MMEBV-0214	10.92731	-ve	-ve	-ve
MMEBV-0214	10.91839	-ve	-ve	-ve
MMEBV-0215	10.91839	-ve	-ve	-ve
MMEBV-0215	11.90716	-ve	-ve	-ve
MMEBV-0215	11.91781	-ve	-ve	-ve
MMEBV-0216	10.95056	-ve	-ve	-ve
MMEBV-0216	10.91844	-ve	-ve	-ve
MMEBV-0216	10.9529	-ve	-ve	-ve
MMEBV-0217	10.91843	-ve	-ve	-ve
MMEBV-0217	10.93279	-ve	-ve	-ve
MMEBV-0217	11.90927	-ve	-ve	-ve
MMEBV-0217	11.92199	-ve	-ve	-ve
MMEBV-0218	10.92731	-ve	-ve	-ve
MMEBV-0218	11.91627	-ve	-ve	-ve
MMEBV-0219	11.90634	-ve	-ve	-ve
MMEBV-0219	10.93277	-ve	-ve	-ve
MMEBV-0220	10.91844	-ve	-ve	-ve
MMEBV-0220	10.93032	-ve	-ve	-ve
MMEBV-0220	10.93356	-ve	-ve	-ve
MMEBV-0221	10.9342	-ve	-ve	-ve
MMEBV-0221	10.91863	-ve	-ve	-ve
MMEBV-0222	10.91862	-ve	-ve	-ve
MMEBV-0223	10.91863	-ve	-ve	-ve
MMEBV-0223	10.95181	-ve	-ve	-ve
MMEBV-0223	11.92063	-ve	-ve	-ve
MMEBV-0224	10.91868	-ve	-ve	-ve
MMEBV-0225	10.91896	-ve	-ve	-ve
MMEBV-0226	10.93543	-ve	-ve	-ve
MMEBV-0226	11.91294	-ve	-ve	-ve
MMEBV-0227	10.9324	30.6; 5.07	-ve	-ve
MMEBV-0228	10.91924	-ve	-ve	-ve
MMEBV-0228	10.93366	-ve	-ve	-ve
MMEBV-0228	11.91847	-ve	-ve	-ve
MMEBV-0229	10.92974	-ve	33.7; 4.19	-ve
MMEBV-0229	10.9192	-ve	-ve	-ve
MMEBV-0229	11.92587	-ve	-ve	-ve
MMEBV-0231	10.93265	-ve	-ve	-ve
MMEBV-0231	10.9192	-ve	-ve	-ve
MMEBV-0232	10.93513	-ve	-ve	-ve
MMEBV-0232	10.9192	-ve	-ve	-ve
MMEBV-0232	11.91786	-ve	-ve	-ve
MMEBV-0233	10.9329	-ve	-ve	-ve
MMEBV-0233	10.9192	-ve	-ve	-ve
MMEBV-0233	11.92726	-ve	-ve	-ve
MMEBV-0234	10.93372	-ve	-ve	-ve
MMEBV-0234	10.9192	-ve	-ve	-ve
MMEBV-0234	11.92148	-ve	-ve	-ve
MMEBV-0235	10.91924	-ve	-ve	-ve
MMEBV-0235	10.93356	-ve	-ve	-ve
MMEBV-0237	10.93031	-ve	-ve	-ve
MMEBV-0237	10.9194	-ve	-ve	-ve
MMEBV-0237	11.90887	-ve	-ve	-ve
MMEBV-0237	11.92143	-ve	-ve	-ve
MMEBV-0238	10.9194	-ve	-ve	-ve
MMEBV-0238	11.91894	-ve	-ve	-ve
MMEBV-0238	11.92706	-ve	-ve	-ve
MMEBV-0239	10.95242	-ve	-ve	-ve
MMEBV-0239	10.93728	-ve	-ve	-ve
MMEBV-0239	11.90855	-ve	-ve	-ve
MMEBV-0240	10.91949	-ve	-ve	-ve
MMEBV-0240	11.91079	-ve	-ve	-ve
MMEBV-0241	10.93198	-ve	-ve	-ve

MMEBV-0241	10.91949	-ve	-ve	-ve
MMEBV-0243	10.95473	-ve	-ve	-ve
MMEBV-0243	11.91315	-ve	-ve	-ve
MMEBV-0244	10.95064	-ve	-ve	-ve
MMEBV-0244	10.91991	-ve	-ve	-ve
MMEBV-0244	11.90924	-ve	-ve	-ve
MMEBV-0244	11.92535	-ve	-ve	-ve
MMEBV-0245	11.90634	-ve	30.5; 5.10	-ve
MMEBV-0245	10.9332	-ve	25.4; 6.55	-ve
MMEBV-0245	10.91992	-ve	-ve	-ve
MMEBV-0246	10.91992	-ve	-ve	-ve
MMEBV-0247	10.93059	30.1; 5.21	-ve	-ve
MMEBV-0247	10.91992	30.3; 5.16	-ve	-ve
MMEBV-0248	10.93367	-ve	-ve	-ve
MMEBV-0248	10.92016	-ve	-ve	-ve
MMEBV-0249	10.92997	-ve	-ve	-ve
MMEBV-0249	10.92472	-ve	-ve	-ve
MMEBV-0249	10.92019	-ve	-ve	-ve
MMEBV-0249	11.91999	-ve	-ve	-ve
MMEBV-0250	10.93353	-ve	-ve	-ve
MMEBV-0250	10.92016	-ve	-ve	-ve
MMEBV-0251	10.92842	-ve	-ve	-ve
MMEBV-0251	11.91613	-ve	-ve	-ve
MMEBV-0252	10.92016	-ve	-ve	-ve
MMEBV-0252	11.92688	-ve	-ve	-ve
MMEBV-0253	10.93794	-ve	-ve	-ve
MMEBV-0253	11.92649	-ve	-ve	-ve
MMEBV-0254	10.93372	32.2; 4.61	-ve	-ve
MMEBV-0255	10.95206	-ve	-ve	-ve
MMEBV-0255	10.92974	32.2; 4.61	-ve	-ve
MMEBV-0255	10.92757	37.8; 3.02	-ve	-ve
MMEBV-0255	10.93616	-ve	-ve	-ve
MMEBV-0255	11.90855	-ve	-ve	-ve
MMEBV-0256	10.9375	-ve	-ve	-ve
MMEBV-0256	10.92063	-ve	-ve	-ve
MMEBV-0257	10.92661	-ve	-ve	-ve
MMEBV-0257	10.92097	-ve	-ve	-ve
MMEBV-0258	10.93687	-ve	-ve	-ve
MMEBV-0258	10.92097	-ve	-ve	-ve
MMEBV-0258	11.90685	-ve	-ve	-ve
MMEBV-0259	10.93728	-ve	-ve	-ve
MMEBV-0259	11.9284	-ve	-ve	-ve
MMEBV-0260	10.93241	-ve	-ve	-ve
MMEBV-0260	11.92126	-ve	-ve	-ve
MMEBV-0260	11.90717	-ve	-ve	-ve
MMEBV-0261	10.95488	-ve	-ve	-ve
MMEBV-0261	10.93465	-ve	-ve	-ve
MMEBV-0261	11.91803	-ve	-ve	-ve
MMEBV-0262	10.93241	-ve	-ve	-ve
MMEBV-0263	10.93592	-ve	-ve	-ve
MMEBV-0263	11.90739	-ve	-ve	-ve
MMEBV-0263	11.92839	-ve	-ve	-ve
MMEBV-0264	10.93667	-ve	-ve	-ve
MMEBV-0264	11.90988	-ve	-ve	-ve
MMEBV-0264	11.92812	-ve	-ve	-ve
MMEBV-0265	10.93706	-ve	-ve	-ve
MMEBV-0265	11.91861	-ve	-ve	-ve
MMEBV-0266	11.91346	-ve	-ve	-ve
MMEBV-0267	10.93617	-ve	-ve	-ve
MMEBV-0267	11.91623	-ve	-ve	-ve
MMEBV-0269	11.91613	-ve	-ve	-ve
MMEBV-0269	11.90774	29.3; 5.44	-ve	-ve

MMEBV-0270	10.92201	-ve	-ve	-ve
MMEBV-0270	10.93808	-ve	-ve	-ve
MMEBV-0270	11.91532	-ve	-ve	-ve
MMEBV-0271	10.93706	-ve	-ve	-ve
MMEBV-0271	11.91372	-ve	-ve	-ve
MMEBV-0272	10.95296	23.3; 7.15	-ve	-ve
MMEBV-0272	10.93551	24.9; 6.69	-ve	-ve
MMEBV-0272	11.92648	32.8; 4.44	-ve	-ve
MMEBV-0273	10.92254	-ve	-ve	-ve
MMEBV-0273	10.93705	-ve	-ve	-ve
MMEBV-0274	10.92254	-ve	-ve	-ve
MMEBV-0274	10.93258	-ve	-ve	-ve
MMEBV-0274	11.91846	-ve	-ve	-ve
MMEBV-0275	10.93667	-ve	-ve	-ve
MMEBV-0275	11.90832	-ve	-ve	-ve
MMEBV-0277	10.95243	-ve	-ve	-ve
MMEBV-0277	10.92288	-ve	-ve	-ve
MMEBV-0277	10.93277	-ve	-ve	-ve
MMEBV-0278	11.90271	-ve	-ve	-ve
MMEBV-0278	10.93489	-ve	-ve	-ve
MMEBV-0278	11.91627	-ve	-ve	-ve
MMEBV-0279	10.93616	35.4; 3.70	-ve	-ve
MMEBV-0279	10.9353	33.6; 4.22	-ve	-ve
MMEBV-0279	10.92757	35.4; 3.70	-ve	-ve
MMEBV-0280	10.95307	-ve	-ve	-ve
MMEBV-0280	10.92307	-ve	-ve	-ve
MMEBV-0281	10.93751	-ve	-ve	-ve
MMEBV-0281	10.92307	-ve	-ve	-ve
MMEBV-0282	10.95072	-ve	-ve	-ve
MMEBV-0282	10.92757	-ve	-ve	-ve
MMEBV-0282	10.9232	-ve	-ve	-ve
MMEBV-0283	10.92325	-ve	-ve	-ve
MMEBV-0283	10.93335	-ve	-ve	-ve
MMEBV-0285	10.92339	-ve	-ve	-ve
MMEBV-0285	10.9324	-ve	-ve	-ve
MMEBV-0286	10.93165	-ve	-ve	-ve
MMEBV-0287	10.93827	-ve	-ve	-ve
MMEBV-0287	10.95503	-ve	-ve	-ve
MMEBV-0287	10.92616	-ve	-ve	-ve
MMEBV-0287	11.91471	-ve	-ve	-ve
MMEBV-0288	10.92398	-ve	-ve	-ve
MMEBV-0290	10.93258	-ve	-ve	-ve
MMEBV-0290	10.93616	-ve	-ve	-ve
MMEBV-0290	10.92395	-ve	-ve	-ve
MMEBV-0291	10.92397	-ve	-ve	-ve
MMEBV-0292	10.93401	-ve	-ve	-ve
MMEBV-0292	10.92403	-ve	-ve	-ve
MMEBV-0292	11.92782	-ve	-ve	-ve
MMEBV-0293	10.92424	-ve	-ve	-ve
MMEBV-0293	10.93265	-ve	-ve	-ve
MMEBV-0293	11.92304	-ve	-ve	-ve
MMEBV-0295	10.92472	-ve	-ve	-ve
MMEBV-0296	10.93667	-ve	-ve	-ve
MMEBV-0296	10.9247	-ve	-ve	-ve
MMEBV-0296	11.90987	-ve	-ve	-ve
MMEBV-0297	10.9247	-ve	-ve	-ve
MMEBV-0298	10.93241	-ve	-ve	-ve
MMEBV-0298	10.9247	-ve	-ve	-ve
MMEBV-0299	10.93241	-ve	-ve	-ve
MMEBV-0300	11.90635	-ve	-ve	-ve
MMEBV-0300	10.92472	-ve	-ve	-ve
MMEBV-0300	11.92126	-ve	-ve	-ve

MMEBV-0301	10.9247	-ve	-ve	-ve
MMEBV-0301	11.91381	-ve	-ve	-ve
MMEBV-0302	11.91564	-ve	-ve	-ve
MMEBV-0303	11.9012	-ve	-ve	-ve
MMEBV-0303	10.93356	-ve	-ve	-ve
MMEBV-0303	10.9247	-ve	-ve	-ve
MMEBV-0303	11.92812	-ve	-ve	-ve
MMEBV-0304	10.9247	-ve	-ve	-ve
MMEBV-0304	11.91923	-ve	-ve	-ve
MMEBV-0304	11.92649	-ve	-ve	-ve
MMEBV-0305	11.90163	-ve	-ve	-ve
MMEBV-0307	10.95369	28.8; 5.58	-ve	-ve
MMEBV-0307	10.934	29.9; 5.27	-ve	-ve
MMEBV-0307	10.92517	27.7; 5.89	-ve	-ve
MMEBV-0307	11.91749	-ve	-ve	-ve
MMEBV-0308	10.95542	-ve	-ve	-ve
MMEBV-0308	10.92517	-ve	-ve	-ve
MMEBV-0308	11.92506	-ve	-ve	-ve
MMEBV-0309	10.93265	-ve	-ve	-ve
MMEBV-0309	11.92684	-ve	-ve	-ve
MMEBV-0310	10.93465	-ve	-ve	-ve
MMEBV-0310	11.90855	-ve	-ve	-ve
MMEBV-0311	10.92516	-ve	-ve	-ve
MMEBV-0311	11.9284	-ve	-ve	-ve
MMEBV-0313	10.93801	-ve	-ve	-ve
MMEBV-0313	10.92573	-ve	-ve	-ve
MMEBV-0314	10.95196	-ve	-ve	-ve
MMEBV-0314	10.92565	-ve	-ve	-ve
MMEBV-0314	11.91011	-ve	-ve	-ve
MMEBV-0315	11.91117	-ve	-ve	-ve
MMEBV-0316	10.93465	-ve	-ve	-ve
MMEBV-0316	10.92566	-ve	-ve	-ve
MMEBV-0316	11.92104	-ve	-ve	-ve
MMEBV-0316	11.90711	-ve	-ve	-ve
MMEBV-0317	10.93465	-ve	-ve	-ve
MMEBV-0317	10.92568	-ve	-ve	-ve
MMEBV-0318	10.92584	-ve	-ve	-ve
MMEBV-0318	11.91347	-ve	-ve	-ve
MMEBV-0319	10.95308	-ve	-ve	-ve
MMEBV-0319	10.93216	-ve	-ve	-ve
MMEBV-0319	10.9289	-ve	-ve	-ve
MMEBV-0319	10.93336	-ve	-ve	-ve
MMEBV-0319	10.92763	-ve	-ve	-ve
MMEBV-0319	11.90717	-ve	-ve	-ve
MMEBV-0319	11.92812	-ve	-ve	-ve
MMEBV-0320	10.95176	-ve	-ve	-ve
MMEBV-0320	10.92588	-ve	-ve	-ve
MMEBV-0320	11.91438	-ve	-ve	-ve
MMEBV-0321	10.92591	-ve	-ve	-ve
MMEBV-0322	10.9526	-ve	-ve	-ve
MMEBV-0322	10.93265	-ve	-ve	-ve
MMEBV-0322	10.92591	-ve	-ve	-ve
MMEBV-0322	11.91187	-ve	-ve	-ve
MMEBV-0324	10.95196	-ve	-ve	-ve
MMEBV-0324	10.93687	-ve	-ve	-ve
MMEBV-0324	10.92638	-ve	-ve	-ve
MMEBV-0324	11.91396	-ve	-ve	-ve
MMEBV-0325	10.95222	-ve	-ve	-ve
MMEBV-0325	10.93706	-ve	-ve	-ve
MMEBV-0325	10.92757	-ve	-ve	-ve
MMEBV-0326	10.93667	-ve	-ve	-ve
MMEBV-0326	10.92763	-ve	-ve	-ve

MMEBV-0326	11.90808	-ve	-ve	-ve
MMEBV-0327	10.93794	-ve	-ve	-ve
MMEBV-0327	10.92643	-ve	-ve	-ve
MMEBV-0328	10.93215	-ve	-ve	-ve
MMEBV-0328	10.93543	-ve	-ve	-ve
MMEBV-0328	11.90806	-ve	-ve	-ve
MMEBV-0328	11.92589	-ve	-ve	-ve
MMEBV-0329	10.92639	-ve	-ve	-ve
MMEBV-0330	10.93449	-ve	-ve	-ve
MMEBV-0331	10.93551	-ve	-ve	-ve
MMEBV-0331	11.91532	-ve	-ve	-ve
MMEBV-0332	10.95261	-ve	-ve	-ve
MMEBV-0332	10.92661	-ve	-ve	-ve
MMEBV-0334	10.93827	-ve	-ve	-ve
MMEBV-0334	10.93616	-ve	-ve	-ve
MMEBV-0334	10.9266	-ve	-ve	-ve
MMEBV-0334	11.9102	-ve	-ve	-ve
MMEBV-0336	11.90121	-ve	-ve	-ve
MMEBV-0336	11.9215	-ve	-ve	-ve
MMEBV-0337	10.92573	-ve	-ve	-ve
MMEBV-0337	10.9105	-ve	-ve	-ve
MMEBV-0338	10.92709	-ve	-ve	-ve
MMEBV-0338	11.91847	-ve	-ve	-ve
MMEBV-0339	10.93258	24.7; 6.75	-ve	-ve
MMEBV-0340	10.92709	-ve	-ve	-ve
MMEBV-0340	11.9215	-ve	-ve	-ve
MMEBV-0341	10.92708	-ve	-ve	-ve
MMEBV-0342	10.92708	-ve	-ve	-ve
MMEBV-0343	10.92708	-ve	-ve	-ve
MMEBV-0344	10.95229	-ve	-ve	-ve
MMEBV-0344	10.93489	-ve	-ve	-ve
MMEBV-0344	10.92708	-ve	-ve	-ve
MMEBV-0345	10.93465	-ve	-ve	-ve
MMEBV-0345	10.92708	-ve	-ve	-ve
MMEBV-0345	11.91838	-ve	-ve	-ve
MMEBV-0346	10.92731	-ve	-ve	-ve
MMEBV-0346	11.91564	-ve	-ve	-ve
MMEBV-0347	10.92731	-ve	-ve	-ve
MMEBV-0347	11.91613	-ve	-ve	-ve
MMEBV-0348	10.93728	-ve	-ve	-ve
MMEBV-0348	10.92731	-ve	-ve	-ve
MMEBV-0349	10.92757	-ve	-ve	-ve
MMEBV-0350	10.92763	-ve	-ve	-ve
MMEBV-0350	11.91917	-ve	-ve	-ve
MMEBV-0351	10.92763	-ve	-ve	-ve
MMEBV-0352	10.92763	-ve	-ve	-ve
MMEBV-0352	11.91372	-ve	-ve	-ve
MMEBV-0353	10.95284	-ve	-ve	-ve
MMEBV-0353	10.92763	-ve	-ve	-ve
MMEBV-0353	11.90806	-ve	-ve	-ve
MMEBV-0353	11.92447	-ve	-ve	-ve
MMEBV-0354	10.92974	-ve	-ve	-ve
MMEBV-0355	10.92763	-ve	-ve	-ve
MMEBV-0355	11.91187	-ve	-ve	-ve
MMEBV-0356	10.9278	-ve	-ve	-ve
MMEBV-0356	11.9215	-ve	-ve	-ve
MMEBV-0357	10.9278	-ve	-ve	-ve
MMEBV-0357	11.90635	-ve	-ve	-ve
MMEBV-0358	10.953	-ve	-ve	-ve
MMEBV-0358	10.9278	-ve	-ve	-ve
MMEBV-0358	11.90684	-ve	-ve	-ve
MMEBV-0359	10.928	-ve	-ve	-ve

MMEBV-0359	11.92535	-ve	-ve	-ve
MMEBV-0360	10.92798	-ve	-ve	-ve
MMEBV-0361	10.92798	-ve	-ve	-ve
MMEBV-0361	11.91564	-ve	-ve	-ve
MMEBV-0362	10.95567	-ve	-ve	-ve
MMEBV-0362	10.92798	-ve	-ve	-ve
MMEBV-0364	10.9369	-ve	-ve	-ve
MMEBV-0364	10.92797	-ve	-ve	-ve
MMEBV-0365	10.92816	-ve	-ve	-ve
MMEBV-0365	11.90684	-ve	-ve	-ve
MMEBV-0365	11.92516	-ve	-ve	-ve
MMEBV-0366	10.93367	-ve	-ve	-ve
MMEBV-0366	10.92812	-ve	-ve	-ve
MMEBV-0367	10.92813	-ve	-ve	-ve
MMEBV-0367	11.91612	-ve	-ve	-ve
MMEBV-0368	11.91424	-ve	-ve	-ve
MMEBV-0369	10.92821	-ve	-ve	-ve
MMEBV-0369	11.91997	-ve	-ve	-ve
MMEBV-0370	10.92821	-ve	-ve	-ve
MMEBV-0370	10.93616	-ve	-ve	-ve
MMEBV-0371	11.90808	-ve	-ve	-ve
MMEBV-0372	10.95261	-ve	-ve	-ve
MMEBV-0372	10.92843	-ve	-ve	-ve
MMEBV-0372	10.93728	-ve	-ve	-ve
MMEBV-0372	11.91605	-ve	-ve	-ve
MMEBV-0373	10.92843	-ve	-ve	-ve
MMEBV-0373	11.92225	-ve	-ve	-ve
MMEBV-0374	10.92842	-ve	-ve	-ve
MMEBV-0374	11.92224	-ve	-ve	-ve
MMEBV-0375	10.92842	-ve	-ve	-ve
MMEBV-0375	11.92341	-ve	-ve	-ve
MMEBV-0376	10.92842	-ve	-ve	-ve
MMEBV-0378	10.92843	-ve	-ve	-ve
MMEBV-0379	10.92842	-ve	-ve	-ve
MMEBV-0379	11.90833	-ve	-ve	-ve
MMEBV-0380	11.92072	-ve	-ve	-ve
MMEBV-0381	10.9513	-ve	-ve	-ve
MMEBV-0381	10.92842	-ve	-ve	-ve
MMEBV-0381	10.934	-ve	-ve	-ve
MMEBV-0382	10.95433	-ve	-ve	-ve
MMEBV-0382	10.92889	-ve	-ve	-ve
MMEBV-0382	11.91398	-ve	-ve	-ve
MMEBV-0383	10.95327	-ve	-ve	-ve
MMEBV-0383	10.93353	30.1; 5.21	-ve	-ve
MMEBV-0384	10.92886	-ve	-ve	-ve
MMEBV-0384	11.9101	-ve	-ve	-ve
MMEBV-0385	10.92889	-ve	-ve	-ve
MMEBV-0386	11.9012	-ve	-ve	-ve
MMEBV-0386	10.92889	-ve	-ve	-ve
MMEBV-0386	11.91812	-ve	-ve	-ve
MMEBV-0387	10.93904	-ve	-ve	-ve
MMEBV-0387	10.9345	-ve	-ve	-ve
MMEBV-0388	10.92908	-ve	-ve	-ve
MMEBV-0388	11.91424	-ve	-ve	-ve
MMEBV-0389	10.9342	-ve	-ve	-ve
MMEBV-0389	10.92925	-ve	-ve	-ve
MMEBV-0389	11.90717	-ve	-ve	-ve
MMEBV-0390	10.9383	-ve	-ve	-ve
MMEBV-0390	10.92925	-ve	-ve	-ve
MMEBV-0391	10.93448	-ve	-ve	-ve
MMEBV-0392	10.92925	-ve	-ve	-ve
MMEBV-0393	10.95434	-ve	-ve	-ve

MMEBV-0393	10.92974	-ve	-ve	-ve
MMEBV-0393	11.91447	-ve	-ve	-ve
MMEBV-0395	10.95434	-ve	-ve	-ve
MMEBV-0395	10.92994	-ve	-ve	-ve
MMEBV-0395	10.93215	-ve	-ve	-ve
MMEBV-0395	11.90987	-ve	-ve	-ve
MMEBV-0396	10.95307	-ve	-ve	-ve
MMEBV-0396	11.90062	-ve	-ve	-ve
MMEBV-0396	10.92994	-ve	-ve	-ve
MMEBV-0396	10.93728	-ve	-ve	-ve
MMEBV-0396	11.92002	-ve	-ve	-ve
MMEBV-0397	10.9521	-ve	-ve	-ve
MMEBV-0397	10.92994	-ve	-ve	-ve
MMEBV-0397	10.93792	-ve	-ve	-ve
MMEBV-0397	11.91488	-ve	-ve	-ve
MMEBV-0399	11.91563	-ve	-ve	-ve
MMEBV-0400	10.95308	-ve	-ve	-ve
MMEBV-0401	10.93014	-ve	-ve	-ve
MMEBV-0402	10.93014	-ve	-ve	-ve
MMEBV-0403	10.93015	-ve	-ve	-ve
MMEBV-0405	10.95412	-ve	-ve	-ve
MMEBV-0405	11.92685	-ve	-ve	-ve
MMEBV-0406	11.91288	-ve	-ve	-ve
MMEBV-0407	10.95229	-ve	-ve	-ve
MMEBV-0407	10.93031	-ve	-ve	37.2; 0.49
MMEBV-0408	10.93258	29.4; 5.41	-ve	-ve
MMEBV-0408	11.92782	19; 8.37	-ve	-ve
MMEBV-0408	11.90832	33.7; 4.19	-ve	-ve
MMEBV-0409	10.93031	-ve	-ve	-ve
MMEBV-0410	10.95434	-ve	-ve	-ve
MMEBV-0410	10.93059	-ve	-ve	-ve
MMEBV-0411	11.90634	-ve	-ve	-ve
MMEBV-0411	10.93722	-ve	-ve	-ve
MMEBV-0411	11.92706	-ve	-ve	-ve
MMEBV-0413	10.93059	-ve	-ve	-ve
MMEBV-0413	11.91128	-ve	-ve	-ve
MMEBV-0413	11.92688	-ve	-ve	-ve
MMEBV-0415	10.93059	-ve	-ve	-ve
MMEBV-0416	10.93062	-ve	-ve	-ve
MMEBV-0416	10.934	-ve	-ve	-ve
MMEBV-0417	10.95567	24.4; 6.83	-ve	-ve
MMEBV-0417	10.93062	18.6; 8.48	-ve	-ve
MMEBV-0418	10.93166	-ve	-ve	-ve
MMEBV-0418	11.91579	-ve	-ve	-ve
MMEBV-0419	10.93165	-ve	-ve	-ve
MMEBV-0419	11.92707	-ve	-ve	-ve
MMEBV-0420	10.95124	-ve	-ve	-ve
MMEBV-0420	11.92706	-ve	-ve	-ve
MMEBV-0421	10.93168	-ve	-ve	-ve
MMEBV-0423	10.93198	-ve	-ve	-ve
MMEBV-0423	11.92072	-ve	-ve	-ve
MMEBV-0424	11.92104	-ve	-ve	-ve
MMEBV-0425	10.93187	-ve	-ve	-ve
MMEBV-0425	11.90684	-ve	-ve	-ve
MMEBV-0426	10.93197	-ve	-ve	-ve
MMEBV-0427	10.93211	-ve	-ve	-ve
MMEBV-0427	11.92688	-ve	-ve	-ve
MMEBV-0428	10.93241	-ve	-ve	-ve
MMEBV-0428	11.91287	-ve	-ve	-ve
MMEBV-0429	10.93241	-ve	-ve	-ve
MMEBV-0429	11.92688	-ve	-ve	-ve
MMEBV-0430	10.93241	-ve	-ve	-ve

MMEBV-0430	11.9215	-ve	-ve	-ve
MMEBV-0430	11.90739	-ve	-ve	-ve
MMEBV-0431	10.95187	-ve	-ve	-ve
MMEBV-0431	10.9324	-ve	-ve	-ve
MMEBV-0432	10.93258	-ve	-ve	-ve
MMEBV-0433	10.93257	-ve	-ve	-ve
MMEBV-0434	10.93265	-ve	-ve	-ve
MMEBV-0435	10.93265	-ve	-ve	-ve
MMEBV-0436	11.91846	-ve	-ve	-ve
MMEBV-0437	10.9332	-ve	-ve	-ve
MMEBV-0437	11.92583	-ve	-ve	-ve
MMEBV-0438	10.93354	-ve	-ve	-ve
MMEBV-0439	10.93354	-ve	-ve	-ve
MMEBV-0440	10.93354	-ve	-ve	-ve
MMEBV-0440	11.90716	-ve	-ve	-ve
MMEBV-0441	10.95567	-ve	-ve	-ve
MMEBV-0441	10.93372	-ve	-ve	-ve
MMEBV-0442	10.93366	-ve	-ve	-ve
MMEBV-0443	10.93372	-ve	-ve	-ve
MMEBV-0444	10.93367	-ve	-ve	-ve
MMEBV-0444	11.92143	-ve	-ve	-ve
MMEBV-0445	10.93371	-ve	-ve	-ve
MMEBV-0445	11.91937	-ve	-ve	-ve
MMEBV-0446	11.90739	-ve	-ve	-ve
MMEBV-0447	10.93398	-ve	-ve	-ve
MMEBV-0447	11.91716	35.6; 3.65	35; 3.82	35.7; 0.91
MMEBV-0448	10.93401	-ve	-ve	-ve
MMEBV-0449	10.934	-ve	-ve	-ve
MMEBV-0450	10.93401	-ve	-ve	-ve
MMEBV-0450	11.91697	-ve	-ve	-ve
MMEBV-0451	10.9342	-ve	-ve	-ve
MMEBV-0451	10.93687	-ve	-ve	-ve
MMEBV-0451	11.92216	-ve	-ve	-ve
MMEBV-0451	11.90716	-ve	-ve	-ve
MMEBV-0452	10.93465	-ve	-ve	-ve
MMEBV-0453	10.93504	-ve	-ve	-ve
MMEBV-0454	10.93489	-ve	-ve	-ve
MMEBV-0454	11.92292	-ve	-ve	-ve
MMEBV-0455	10.93489	-ve	-ve	-ve
MMEBV-0455	11.92849	-ve	-ve	-ve
MMEBV-0456	10.93489	-ve	-ve	-ve
MMEBV-0456	11.90987	-ve	-ve	-ve
MMEBV-0456	11.92688	-ve	-ve	-ve
MMEBV-0457	10.93592	-ve	-ve	-ve
MMEBV-0458	10.93596	-ve	-ve	-ve
MMEBV-0458	11.91716	-ve	-ve	-ve
MMEBV-0459	10.93598	-ve	28.4; 5.70	-ve
MMEBV-0459	11.90883	-ve	-ve	-ve
MMEBV-0459	11.92688	-ve	-ve	-ve
MMEBV-0460	11.91159	-ve	-ve	-ve
MMEBV-0461	10.95296	-ve	-ve	-ve
MMEBV-0461	10.93616	-ve	-ve	-ve
MMEBV-0461	11.90831	-ve	-ve	-ve
MMEBV-0461	11.92617	-ve	-ve	-ve
MMEBV-0462	10.95197	-ve	-ve	-ve
MMEBV-0462	10.93687	-ve	-ve	-ve
MMEBV-0462	11.91838	-ve	-ve	-ve
MMEBV-0463	10.93687	31.4; 4.84	-ve	-ve
MMEBV-0464	10.95206	-ve	-ve	-ve
MMEBV-0464	10.93686	-ve	-ve	-ve
MMEBV-0464	11.91294	-ve	-ve	-ve
MMEBV-0465	11.90272	-ve	-ve	-ve

MMEBV-0465	10.9369	-ve	-ve	-ve
MMEBV-0465	11.92036	-ve	-ve	-ve
MMEBV-0466	11.9101	-ve	-ve	-ve
MMEBV-0467	10.93721	-ve	-ve	-ve
MMEBV-0470	10.93801	-ve	-ve	-ve
MMEBV-0470	11.91117	-ve	-ve	-ve
MMEBV-0471	10.9375	-ve	-ve	-ve
MMEBV-0471	11.92688	-ve	-ve	-ve
MMEBV-0472	10.95285	-ve	-ve	-ve
MMEBV-0472	10.93748	-ve	-ve	-ve
MMEBV-0472	11.91425	-ve	-ve	-ve
MMEBV-0473	10.95206	-ve	-ve	-ve
MMEBV-0473	10.9375	-ve	-ve	-ve
MMEBV-0476	11.90272	-ve	-ve	-ve
MMEBV-0476	11.90634	-ve	-ve	-ve
MMEBV-0476	11.9215	-ve	-ve	-ve
MMEBV-0477	10.95206	-ve	-ve	-ve
MMEBV-0477	10.93793	-ve	-ve	-ve
MMEBV-0478	10.93793	-ve	-ve	-ve
MMEBV-0478	11.90892	-ve	-ve	-ve
MMEBV-0478	11.92848	-ve	-ve	-ve
MMEBV-0479	10.9381	-ve	-ve	-ve
MMEBV-0479	10.95556	-ve	-ve	-ve
MMEBV-0479	11.91532	-ve	-ve	-ve
MMEBV-0480	11.91578	-ve	-ve	-ve
MMEBV-0481	11.92841	-ve	-ve	-ve
MMEBV-0482	10.9381	-ve	-ve	-ve
MMEBV-0482	10.95312	-ve	-ve	-ve
MMEBV-0483	10.9381	-ve	-ve	-ve
MMEBV-0484	10.95247	-ve	-ve	-ve
MMEBV-0484	10.93829	-ve	-ve	-ve
MMEBV-0484	11.91613	-ve	-ve	-ve
MMEBV-0487	10.93828	-ve	-ve	-ve
MMEBV-0487	10.95261	-ve	-ve	-ve
MMEBV-0487	11.90634	-ve	-ve	-ve
MMEBV-0488	10.93828	-ve	-ve	-ve
MMEBV-0489	10.93853	-ve	-ve	-ve
MMEBV-0489	11.90883	-ve	-ve	-ve
MMEBV-0489	11.92688	-ve	-ve	-ve
MMEBV-0490	10.93853	-ve	-ve	-ve
MMEBV-0490	11.91488	-ve	-ve	-ve
MMEBV-0491	10.93853	-ve	-ve	-ve
MMEBV-0491	10.95284	-ve	-ve	-ve
MMEBV-0491	11.91786	-ve	-ve	-ve
MMEBV-0492	11.90738	-ve	-ve	-ve
MMEBV-0492	11.92848	-ve	-ve	-ve
MMEBV-0493	10.93853	-ve	-ve	-ve
MMEBV-0493	11.90924	-ve	-ve	-ve
MMEBV-0494	10.95207	-ve	-ve	-ve
MMEBV-0494	11.92104	-ve	-ve	-ve
MMEBV-0496	11.90891	-ve	-ve	-ve
MMEBV-0497	11.91396	-ve	-ve	-ve
MMEBV-0498	10.95272	-ve	-ve	-ve
MMEBV-0498	11.90715	-ve	-ve	-ve
MMEBV-0499	11.91157	-ve	-ve	-ve
MMEBV-0499	11.92648	-ve	-ve	-ve
MMEBV-0500	10.95284	-ve	-ve	-ve
MMEBV-0500	11.92649	-ve	-ve	-ve
MMEBV-0503	11.90711	-ve	-ve	-ve
MMEBV-0503	11.92426	-ve	-ve	-ve
MMEBV-0504	11.92583	-ve	-ve	-ve
MMEBV-0505	11.92845	-ve	-ve	-ve

MMEBV-0507	10.95488	-ve	-ve	-ve
MMEBV-0507	11.90892	-ve	-ve	-ve
MMEBV-0509	11.90716	-ve	-ve	-ve
MMEBV-0509	11.92471	-ve	-ve	-ve
MMEBV-0511	11.90832	-ve	-ve	-ve
MMEBV-0512	10.934	-ve	-ve	-ve
MMEBV-0512	10.92041	-ve	-ve	-ve
MMEBV-0512	11.92198	-ve	-ve	-ve
MMEBV-0514	11.91579	-ve	-ve	-ve

Cq values were used to interpolate results from the positive control calibrated in $\log_{10}$ International units per mL for BKPyV and JCPyV and in $\log_{10}$ copies/mL for HPyV9 using 'forecast' function within Excel and the calibration data presented in Table 18 for BKPyV, Table 20 for JCPyV and Table 21 for HPyV9. -ve results refelct undetectable polyomavirus and detectable internal control molecule.

Among the 1156 samples tested, 49 (4.2%) were positive for BKPyV, 19 (1.6%) for JCPyV and 4 (0.35%) for HPyV9. A total of 44 patients had at least one positive sample. The age range, sex, and ethnicity of positive patients was similar to those of the population from which the samples were drawn (Table 25).

Table 25: Summary of the study cohort patients.

	Total patients N = 465	Patients with positive samples N = 44 (9.5%)	Patients with negative sample N = 421 (90.5%)
Age in years (mean ± SD)	52 ± 12.8	51.5 ± 13.5	51.7 ± 12.8
Gender			
Male	274 (59.8%)	32 (74.4%)	249 (60%)
Female	164 (35.8%)	11 (25.6%)	149 (35.9%)
Unspecified	20 (4.4%)	0	17 (4.1%)
Ethnicity			
White	395 (86.2%)	33 (76.7%)	362 (87.2%)
Indian	6 (1.3%)	2 (4.7%)	4 (1%)
Pakistani	9 (2%)	0	9 (2.2%)
Bangladesh	1 (0.2%)	1 (2.3%)	0 (0)
Black	8 (1.8%)	1 (2.3%)	7 (1.7%)
Irish	4 (0.9%)	0	4 (1%)
Chinese	2 (0.4%)	1 (2.3%)	1 (0.2%)
Asian (other)	1 (0.2%)	0	1 (0.2%)
Other ethnicity	5 (1.1%)	1 (2.3%)	4 (1%)
Not recorded	27 (5.9%)	4 (9.3%)	23 (5.5%)

The average time from transplant for patients with positive samples differed from those with negative samples (positive 3.79±4.35 years versus 8.95±7.53 years). Among BKPyV positive patients (27) the amount of virus detected ranged between log 2.05 to 8.08 IU/mL (112 to 120X10^6 IU/mL). The mean value was log 5.05 (112,055 IU/mL) suggesting a significant level of the virus were produced in these patients who were on average 4 years post-transplant. The 15 JCPyV positive patients had lower levels of virus ranging between log 3.55 and 6.56 IU/mL (3630 and 3.5X10^6 IU/mL) with an average of log 4.9 IU/mL (79,400

IU/mL). Only very low levels of HPyV9 were detected in the 4 patients testing positive for the virus range log 0.41 to 1.25 copies/ml (2.5 to 18 copies/mL).

One patient sample tested positive at a low levels for all three viruses; BKPyV log 3.65 IU/mL (4466 IU/mL); JCPyV 3.82 IU/mL (6606 IU/mL) and HPyV9 9.8 copies/mL. The sample being obtained from a white male (37 years of age) 19.57 year post transplant indicating that recurrence or reinfection is possible even long after transplant.

BKPyV and JCPyV infection were found in one patient in samples taken 6 months apart.

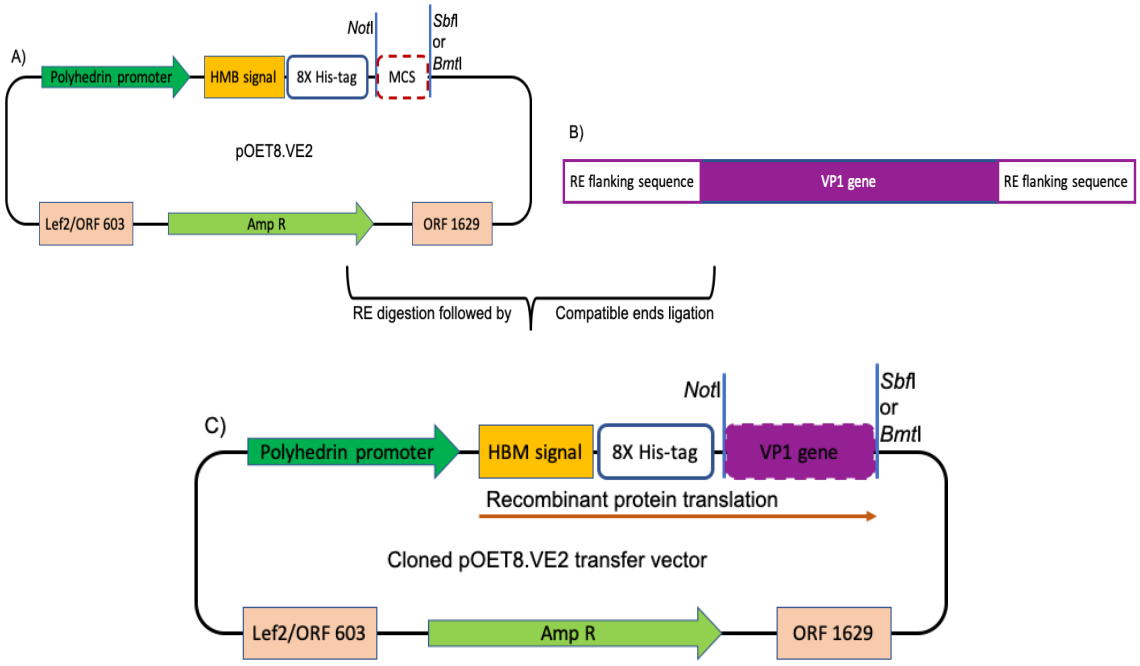
No cases of HPyVan were reported in the limited clinical information available for this patient cohort.

3.7. Protein Expression

3.7.1. Transfer vector assembly

A schematic summary of the cloning procedure of the transfer vector is shown in Figure 23.

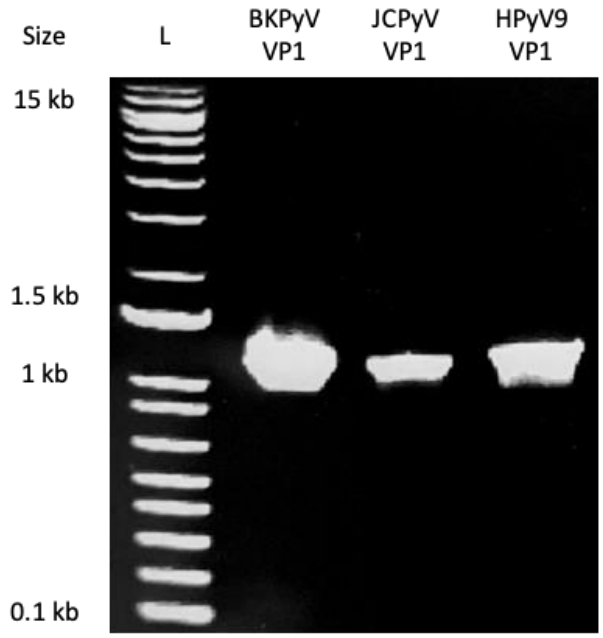
Figure 23: schematic of VP1 gene cloning procedure.



A) pOET8.VE2 plasmid B) VP1 gene PCR amplification product, restriction enzymes (RE) flanking sequences were added using tail sequences in the amplification primers. The RE flanking sequences facilitated compatible RE digestion to permit end ligation. C) the cloned transfer vector map the VP1 gene was inserted by cutting the first end with *Not*I and the second end with *Bmt*I for BKPyV and JCPyV or *Sbf*I for HPyV9. The translated recombinant protein uses the polyhedron promoter and contains; Honey Bee Melittin (HBM) signal sequences, the N-terminal 8X histidine fusion tag (8x His-tag), and the VP1 gene.

Amplification of the viral protein 1 (VP1) genes for each virus (BKPyV, JCPyV, and HPyV9) used primers with an added tail sequence containing restriction enzyme digestion sequences for Not1 flanking the forward primer and *Bmt*I flanking the reverse primer (BKPyV and JCPyV) or *Sbf*I on the reverse primer for HPyV9. The VP1 genes for BKPyV and HPyV9 were amplified from a synthetic DNA construct obtained commercially, while JCPyV was amplified from a full length of a clone of JCPyV held within a plasmid. Products of PCR were analysed by electrophoresis on pre-cast 1.2% agarose gels (Figure 24).

Figure 24: Agarose gel electrophoresis to confirm VP1 PCR amplification.

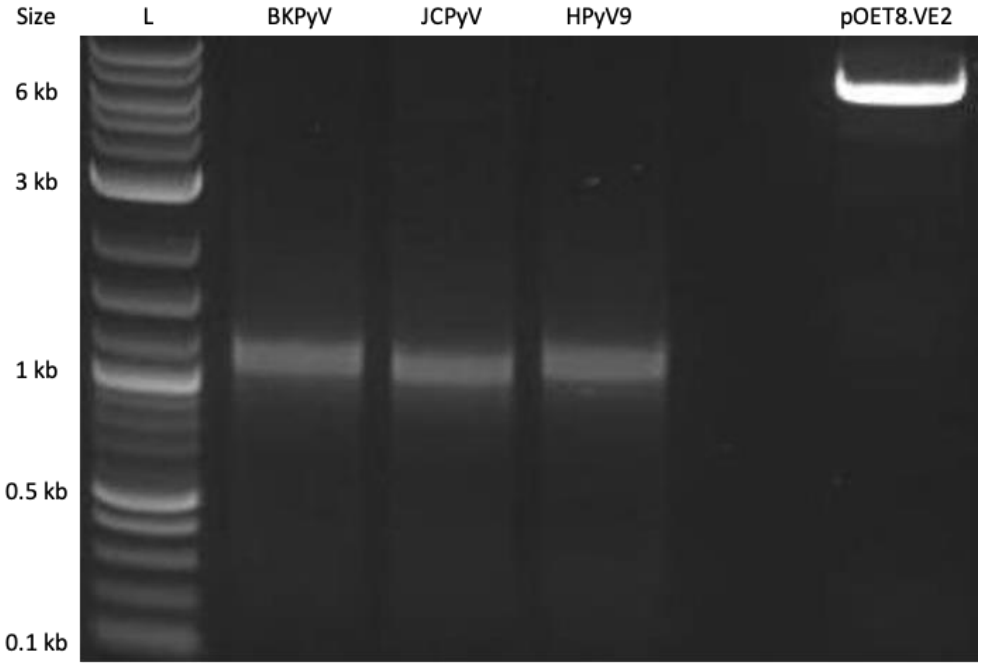


1.2 % E-Gel was run for 30 minutes and stained using SYBR Safe DNA stain and visualised by transillumination (Fastgene blue/green). L: Invitrogen™ 1 Kb Plus DNA Ladder, BKPyV VP1 size 1155 bp, JCPyV VP1 size 1098 bp, and HPyV9 VP1 size 1184 bp.

PCR products were purified using a spin column and the amount of DNA quantified by UV spectrophotometry.

Purified VP1 DNA and vector DNA (pOET8.VE2 plasmid) were subject to restriction enzyme (RE) digestion to produce compatible ends needed for the VP1 gene insertion. Agarose gel electrophoresis was performed to confirm the band sizes (Figure 25).

Figure 25: confirmation of restriction enzyme digestion modification.



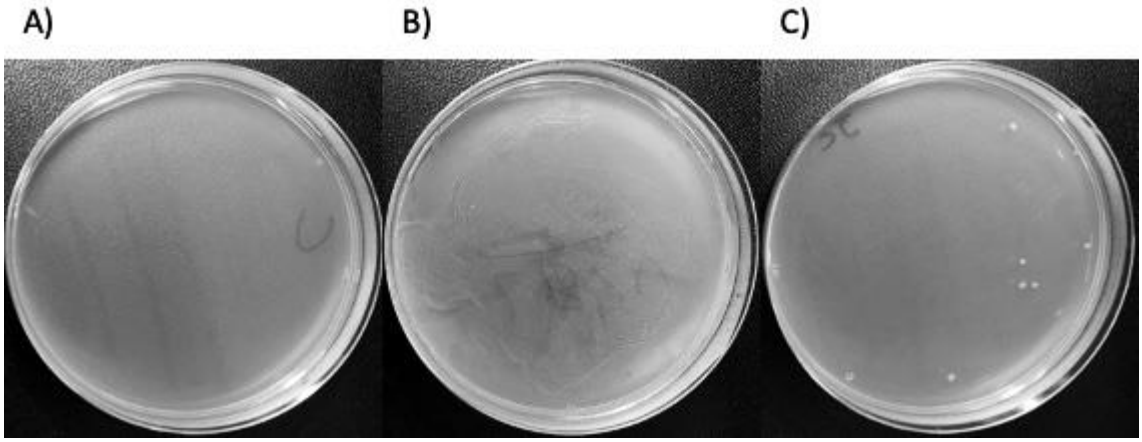
1.2 % E-Gel ran for 30 minutes. L New England Biolabs Quick-Load® 2-Log DNA Ladder, digested BKPyV VP1 size 1140 bp, digested JCPyV VP1 size 1082 bp, digested HPyV9 VP1 size 1168 bp, and the linearised pOET8.VE2 plasmid size 6480 bp.

Following ligation of VP1 DNA and vector DNA, the transfer vector plasmid was used to transform competent *E. coli*.

3.7.2. Transformation of *E. coli*.

Transformation of *E. coli* was performed using heat shock. Transformants were selected using LB agar with ampicillin [100 mg/ml]. Successful transformation was observed by comparing transformant plates with negative and positive controls (Figure 26). *E. coli* transformants colonies were then amplified overnight in liquid LB with ampicillin to be used for plasmid extraction.

Figure 26: *E. coli* transformation.



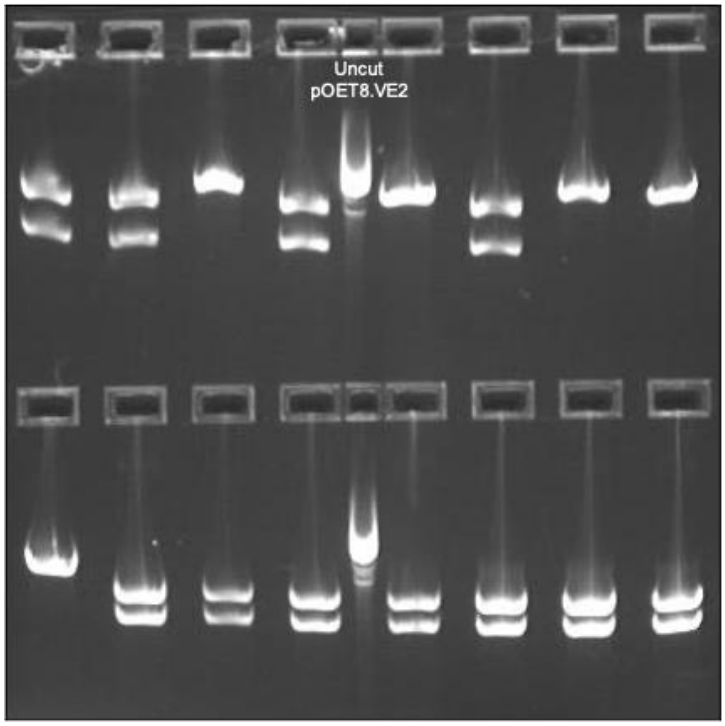
A) negative control, where control competent *E. coli* cells were spread on an LB agar with ampicillin and shows no growth. B) positive control, where control competent *E. coli* cells were spread on an LB agar without ampicillin and shows complete growth in the form of a bacterial lawn. C) Transformed *E. coli* showing few colonies growing in the selective media (LB with ampicillin).

3.7.3. Cloned transfer vector confirmation

The amplified transfer vector plasmids extracted from the transformed *E. coli* overnight cultures were screened. The screening was performed by RE using *Eco*RI, where positive candidates produced two bands. First RE screening was

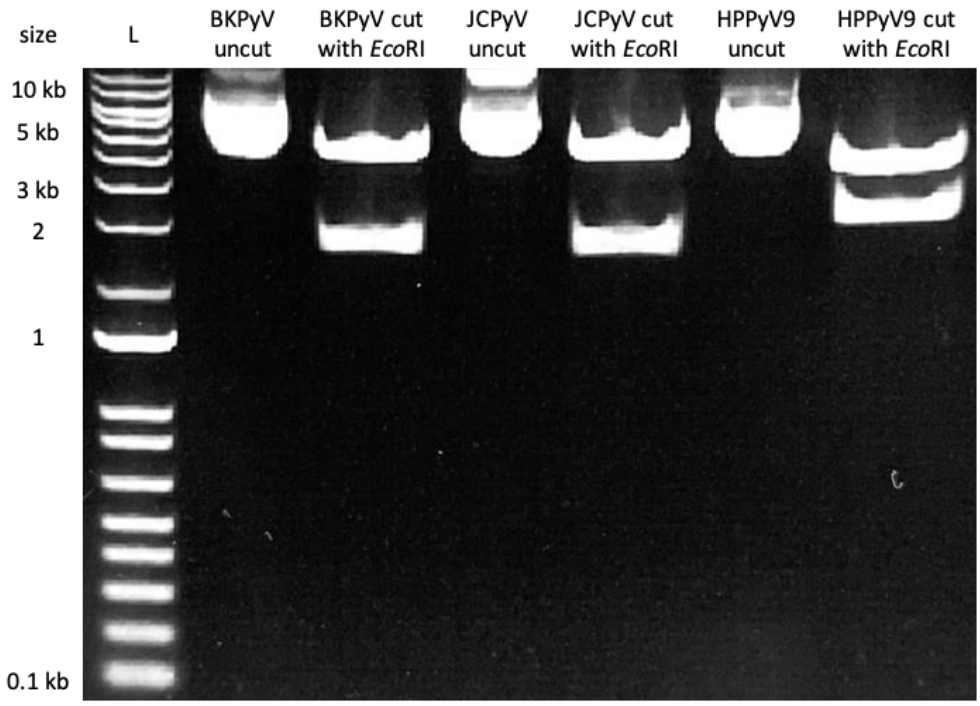
done using dual comp agarose gel, comparing the RE digested plasmids with uncut pOET8.VE plasmid (Figure 27). The positive candidates were subjected to a second RE screening using a single comp agarose gel and a molecular DNA ladder to check the specific band sizes (Figure 28). After screening the positive candidates were confirmed by Sanger sequencing (sequencing results appended as Appendix 1).

Figure 27: RE screening of cloned plasmids using *Eco*R.



2% double comb E-Gel run for 15 minutes. pOET8.VE2 plasmids were used as a marker in the middle column, and several plasmid extracts digested with *Eco*RI, positive candidates produce two bands. Correct band sizes post-digestion were 4995bp and 2625bp for BKPyV; 4976 bp and 2586 bp for JCPyV; and 4490bp and 3165bp for HPyV9.

Figure 28: Second RE screening of cloned plasmids using *Eco*RI.

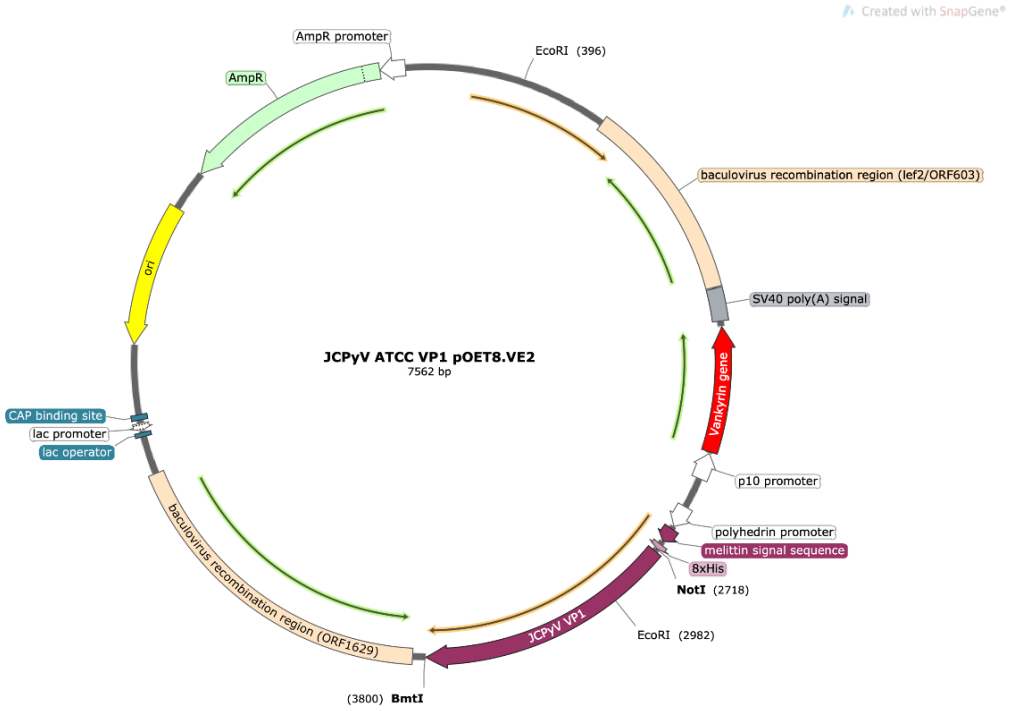


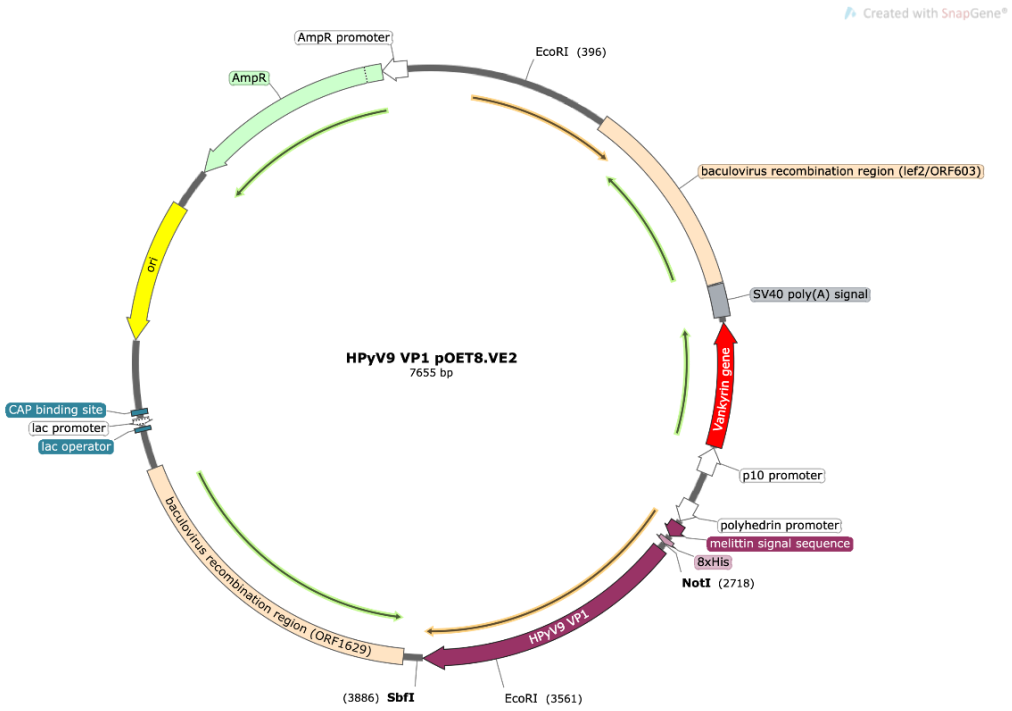
1.2 % E-Gel run for 30 minutes. L: Invitrogen™ 1 Kb Plus DNA Ladder. Positive BKPyV VP1 clone produce two fragments sized 4995 bp and 2625 bp. Positive JCPyV VP1 clone produce two fragments sized 4976 bp and 2586 bp. Positive HPyV9 VP1 clone produces two fragments sized 4490 bp and 3165 bp.

3.7.4. Transfer vectors

The transfer vector maps are shown in Figure 29. Each virus transfer vector was checked for a complete translation of the desired VP1 in the same frame of the polyhedron promoter with the Honey Bee Melittin (HBM) signal sequences used to enhance the secretion of the recombinant protein, and the N-terminal 8X histidine fusion tag (8x His-tag) used to facilitate immobilised metal affinity chromatography (IMAC) purification of the target recombinant protein.

Figure 29: Baculovirus transfer vectors DNA maps for BKPyV, JCPyV, and HPyV9.





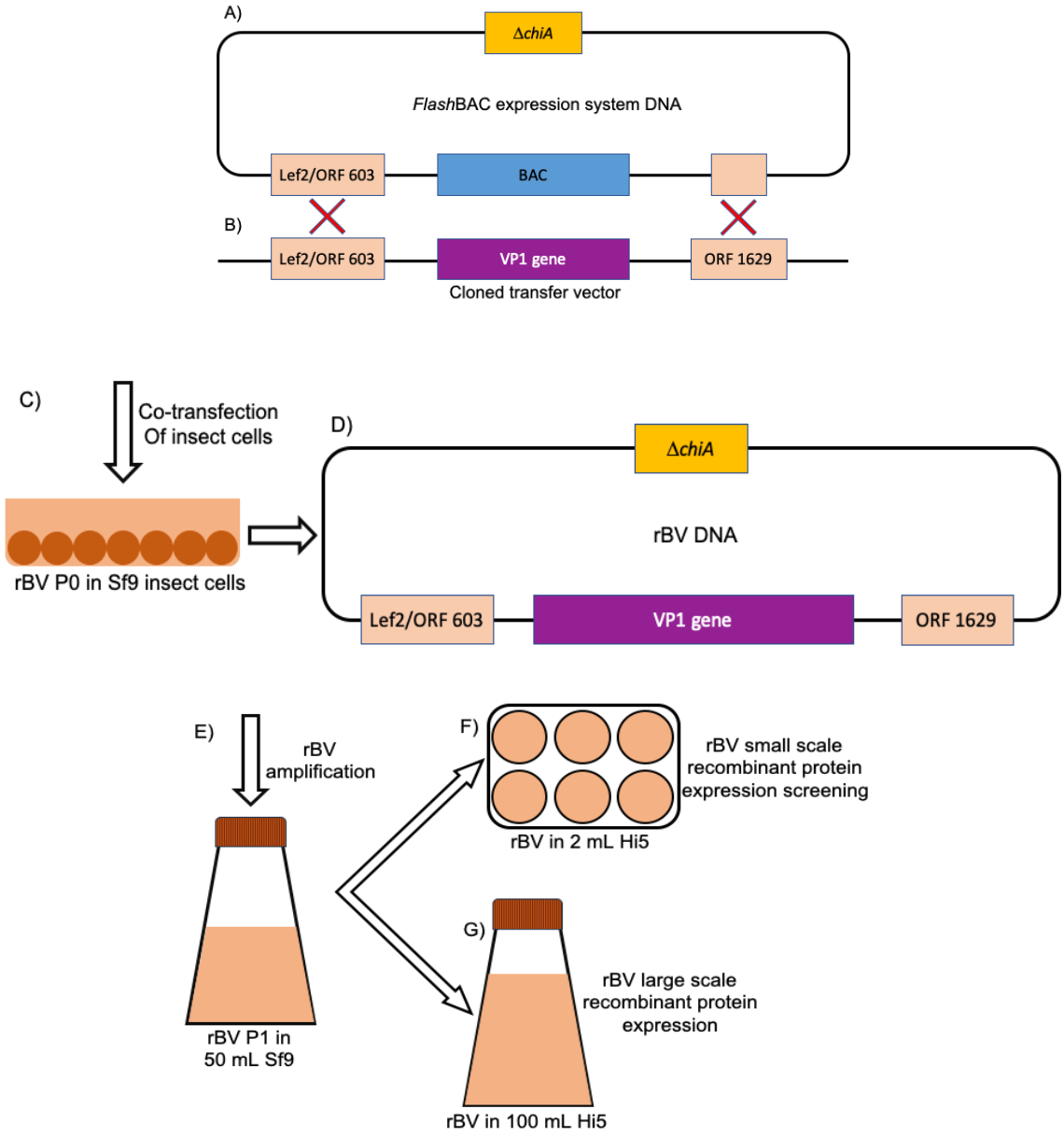
Designs show the insertion of the VP1 genes in the desired positions, achieving proper translation. The translated recombinant protein shown in orange arrow starts by the polyhedron promoter and include the melittin signal, 8XHis and each virus VP1 protein. The vankyrin gene is also shown, which is an insect cell anti-apoptotic protein used to prolong the viability of the cells for increased protein production. BKPyV and JCPyV PCR amplified VP1 genes used *Not*I and *Bmt*I enzymes for insertion. Meanwhile, HPyV9 used *Not*I and *Sbf*I enzymes. Finally, the positions where the restriction enzyme *Eco*RI can dual cut the cloned transfer vectors are shown, one of which is inside the inserted VP1 genes, which was used to screen for the success of gene insertion.

3.7.5. Recombinant baculovirus generation and expression.

Recombinant baculoviruses (rBVs) were produced by co-transfection of Sf9 (*Spodoptera frugiperda*) insect cells with *flash*BAC ULTRA virus DNA and the transfer vectors containing VP1 with the expression cassette. During co-transfection inside the insect cells, homologous recombination occurs. Homologous recombination recovers ORF 1629 and replaces the bacterial

artificial chromosome (BAC) with the VP1 gene under the control of the polyhedrin promoter inside the *flash*BAC ULTRA expression virus from the cloned transfer vector. This recombination allows the recombinant virus to replicate and express the desired protein. Virus recovered from Sf9 co-transfection growth medium was considered (P0) rBV and transferred entirely to an Sf9 suspension for amplification to produce the rBV seeding stock (P1). The rBV seeding stock DNA was extracted and titrated in a baculovirus qPCR. Recombinant protein expression was achieved using Hi5™ (*Trichoplusia ni)* insect cells, first by 6-well plate small scale expression screening, followed by large scale expression in Hi5™ insect cells suspension. A schematic of the recombinant baculovirus generation and expression procedure is shown in (Figure 30).

Figure 30: schematic of recombinant baculovirus generation and expression.

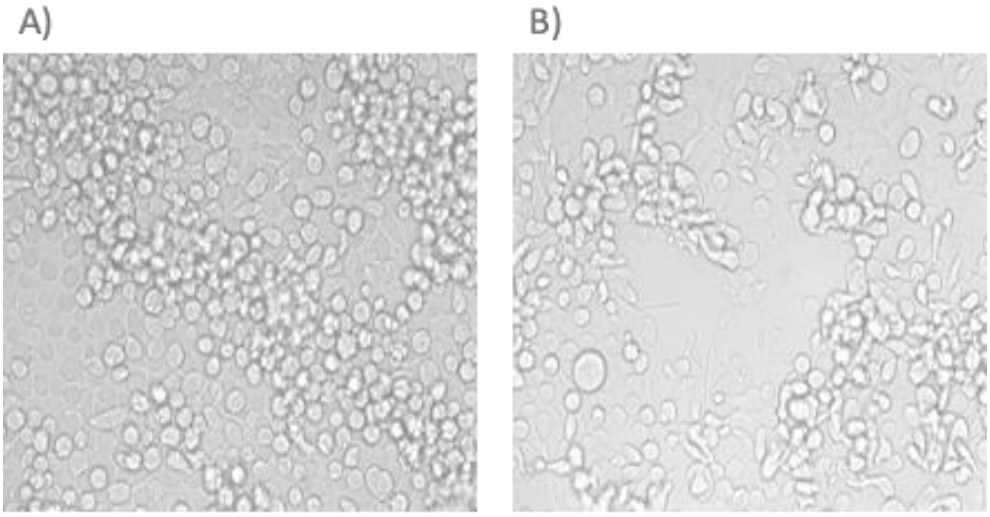


A) *flash*BAC expression system DNA which has bacterial artificial chromosome (BAC) and a defective ORF1629. B) cloned transfer vector DNA containing the viral protein 1 (VP1) at the polyhedron locus and the ORF129. C) co-transfection in Sf9 cells to achieve homologous recombination. D) recombinant baculovirus (rBV) DNA incorporating the desired VP1 gene flanked bylef2 and a functioning ORF1629. E) amplification of rBV P0 in Sf9 suspension to produce the P1 seeding stock. F) small scale recombinant protein expression screening in Hi5™ insect cells 6-well plate. G) Large scale recombinant protein expression screening in Hi5™ insect cells suspension.

3.7.6. Co-transfection of insect cells.

Insect cells Sf9 plates were co-transfected with VP1 cloned transfer vectors and *flash*BAC expression system for a week to produce rBVs. Each virus (BKPyV, JCPyV, and HPyV9) VP1 was inserted separately into a different rBV. During co-transfection, homologous recombination occurs producing rBV capable of replication after restoring ORF1629 and recombinant protein expression as the VP1 gene is inserted in place of the BAC sequence under the control of the polyhedron promoter. After co-transfection, Sf9 cells were visualised under the microscope (Figure 31) and the growth media containing budded rBV passage 0 (P0) was collected and seeded into 50 mL of Sf9 suspension culture for amplification. After one week of rBV amplification, a sample was collected from the rBV seeding stock (P1) for DNA extraction to be used in quantitative PCR (qPCR), while the remainder was stored to be seeded into Hi5™ insect cells for recombinant protein expression.

Figure 31: Sf9 insect cells after co-transfection.



Sf9 insect cells one week post co-transfection X40. A) un-infected Sf9 cells. B) cells co-transfected with *flash*BAC expression system DNA and cloned pOET8.VE2 transfer vectors containing VP1. Cytopathic effect (CPE) observed in the form of few enlarged cells.

3.7.7. Quantitative PCR titration of rBV seeding stock.

Extracted DNA from rBV seeding stock (P1) was quantitated using qPCR before being used for recombinant protein expression (Table 26). Samples were tested in duplicates and quantified using a calibrated standard curve (section 2.9.3).

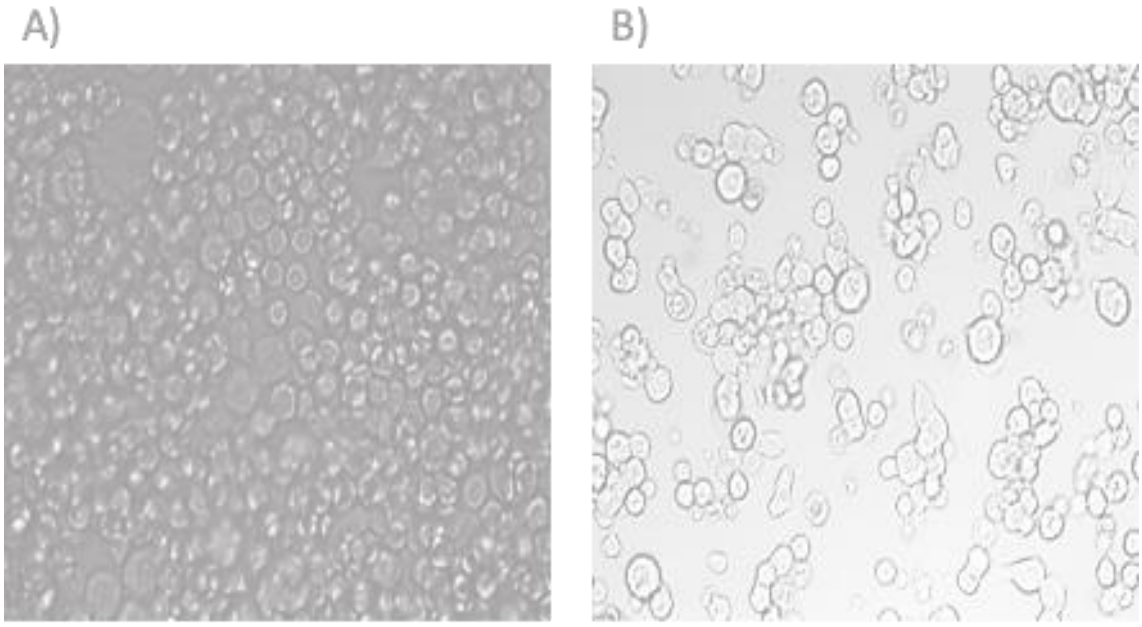
Table 26: Baculovirus qPCR results.

rBV	Average Ct	Log pfu/ml	pfu/ml
BKPyV VP1 P1	13.5	7.45	$2.84x10^7$
JCPyV VP1 P1	12.4	7.85	$7.06 x10^7$
HPyV9 VP1 P1	11.5	8.04	$1.09 x10^8$

3.7.8. Recombinant protein expression

For small scale expression, recombinant baculovirus stock (P1) was seeded in Hi5™ insect cells in a six-well plate and visualised after five days incubation (Figure 32). After screening, large scale expression was performed in 100 mL Hi5™ suspension culture for five days (Figure 33). Recombinant protein expressions were further analysed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and western blot.

Figure 32: Small scale recombinant protein expression screening in Hi5™ insect cells.



Monolayer plate culture five days after seeding with rBVs X40. A) control un-infected Hi5™ cells. B) Hi5™ plate culture infected with rBV P1 showing shrivel,ed cells and granular nucleus.

Figure 33: Large scale recombinant protein expression in Hi5™ insect cells.

Suspension culture five days after seeding with rBVs X40. A sample from Hi5™ suspension culture infected with rBV P1 showing many shrunken cells.

3.7.9. Recombinant protein analyses

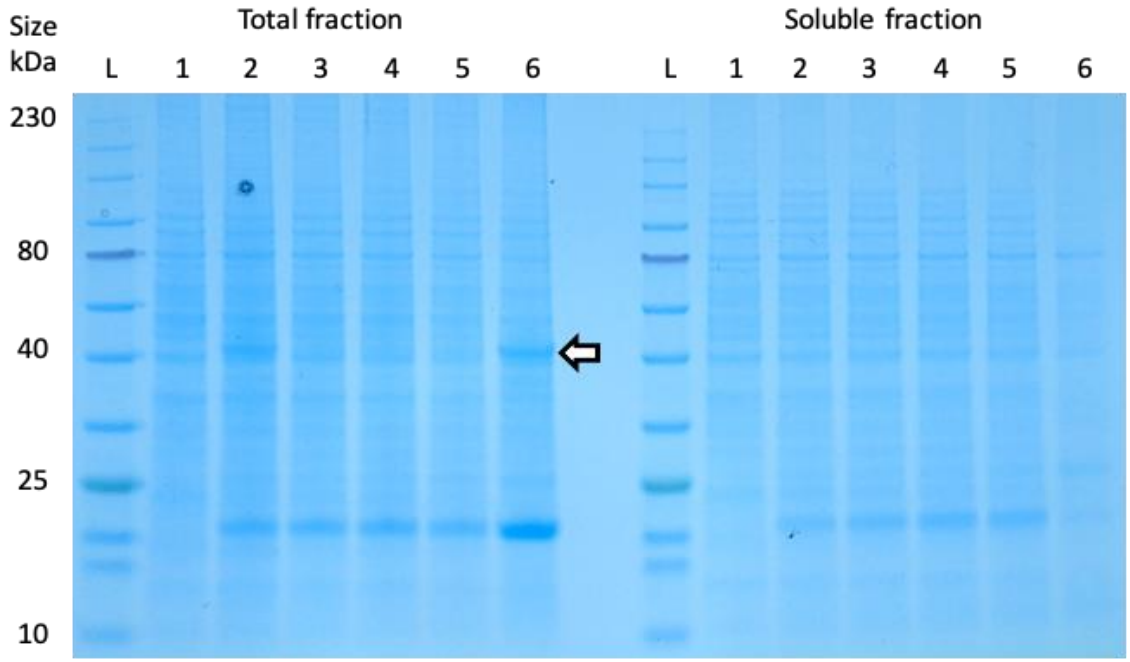
Recombinant baculovirus from Hi5™ insect cell cultures expressing recombinant proteins of BKPyV VP1, JCPyV VP1, and HPyV9 VP1 were extracted and analysed by SDS-PAGE for both the small scale protein expression screening and large scale protein expression. Extracted proteins were checked for solubility by comparing the total fraction (TF) collected directly from the Hi5™ insect cell lysate, and the soluble fraction (SF) collected from the supernatant after pelleting proteins by centrifugation. Expressed proteins were as follows; BKPyV VP1 405 amino acids (aa) and 45.1 kilodaltons (kDa), JCPyV VP1 391 aa and 44 kDa, and HPyV9 VP1 414 aa and 45.3 kDa.

3.7.9.1. Small scale recombinant protein expression

Hi5™ cells in 6-well plate monolayer cultures were infected at differing multiplicities of infection (section 2.10.1; Table 15).

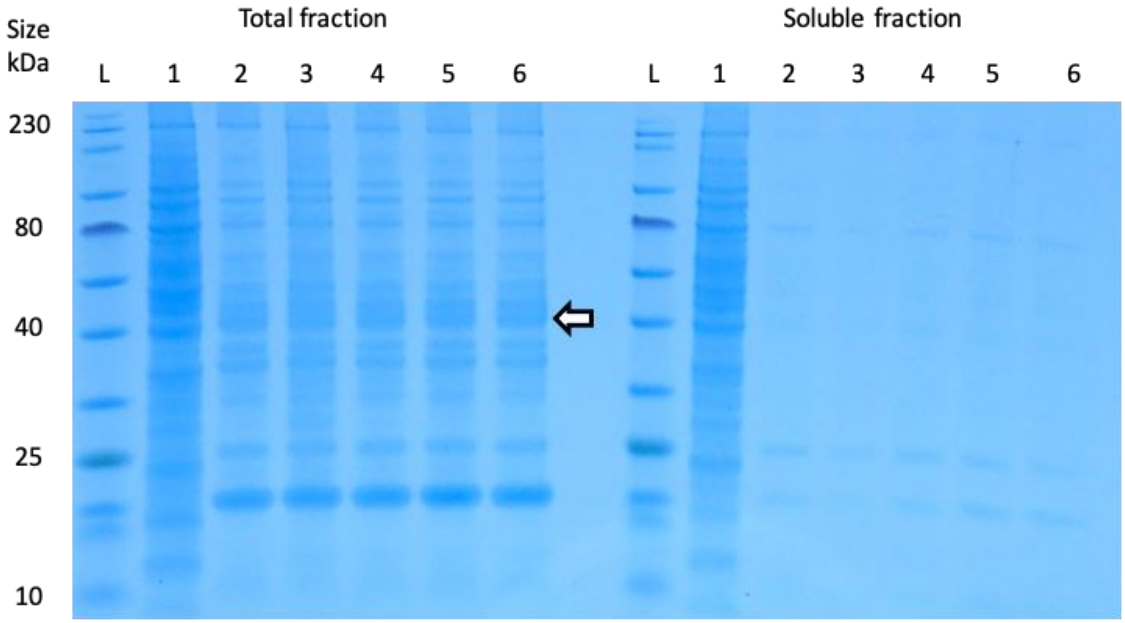
The products of expression were harvested and analysed in SDS PAGE; BKPyV VP1 (Figure 34); JCPyV VP1 (Figure 35), and HPyV9 VP1 (Figure 36).

Figure 34: BKPyV Vp1 recombinant protein expression SDS-PAGE analysis.



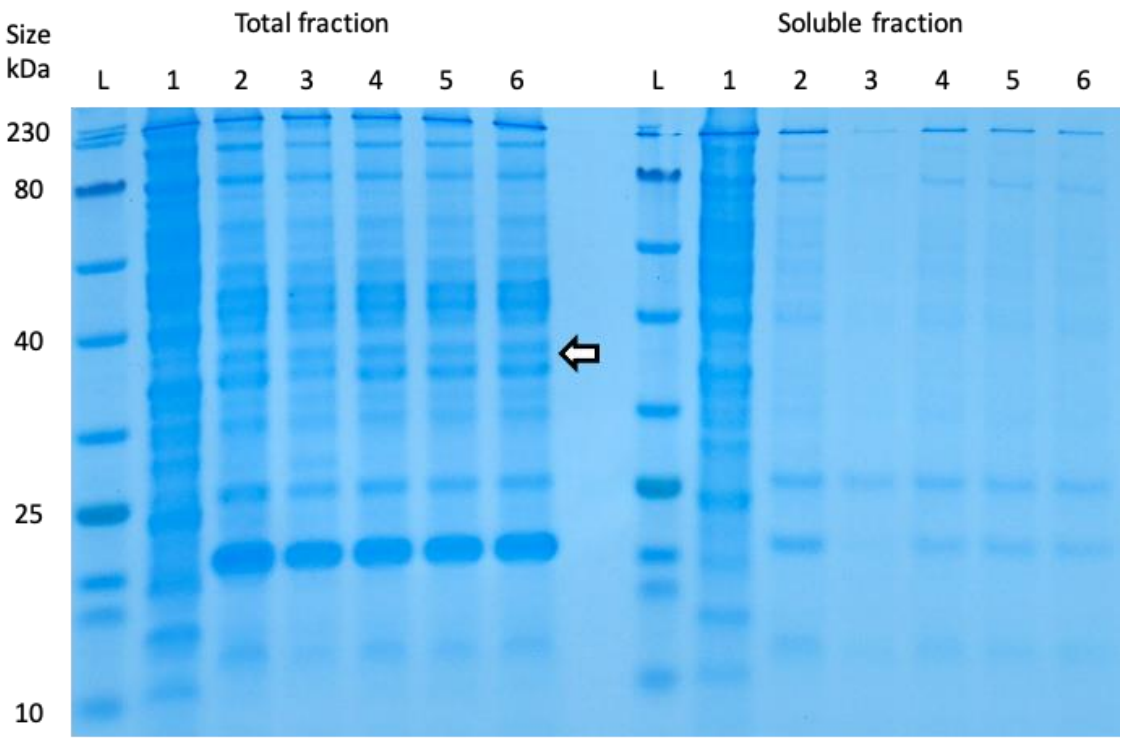
SDS-PAGE using NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.0 mm. Total fraction is cell lysis containing soluble and insoluble proteins. Soluble fraction is the supernatant after centrifugation. L is ColorPlus™ Prestained Protein Ladder. rBV P1 seeding in different volumes; 1 is 0 µl, 2 is 25 µl, 3 is 50 µl, 4 is 100 µl, 5 is 150 µl, and 6 is 200 µl. Expected BKPyV VP1 recombinant protein size is 45.1 kDa shown by the arrow.

Figure 35: JCPyV Vp1 recombinant protein expression SDS-PAGE analysis.



SDS-PAGE NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.0 mm. Total fraction is cell lysis containing soluble and insoluble proteins. Soluble fraction is the supernatant after centrifugation. L is ColorPlus™ Prestained Protein Ladder. rBV P1 seeding in different volumes; 1 is 0 µl, 2 is 25 µl, 3 is 50 µl, 4 is 100 µl, 5 is 150 µl, and 6 is 200 µl. Expected JCPyV VP1 recombinant protein size is 44 kDa shown by the arrow.

Figure 36: HPyV9 Vp1 recombinant protein expression SDS-PAGE analysis.

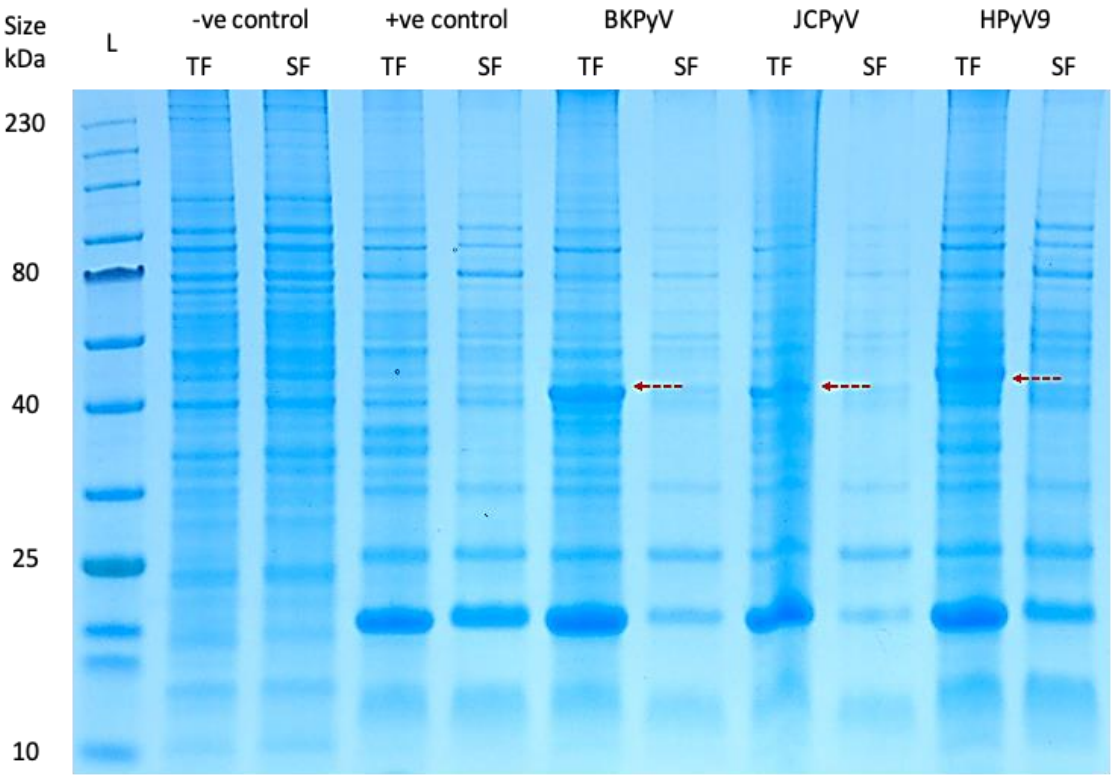


SDS-PAGE NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.0 mm. Total fraction is cell lysis containing soluble and insoluble proteins. Soluble fraction is the supernatant after centrifugation. L is ColorPlus™ Prestained Protein Ladder. rBV P1 seeding in different volumes; 1 is 0 µl, 2 is 25 µl, 3 is 50 µl, 4 is 100 µl, 5 is 150 µl, and 6 is 200 µl. Expected HPyV9 VP1 recombinant protein size is 45.3 kDa shown by the arrow.

3.7.9.2. Large scale recombinant protein expression

Hi5™ insect cell suspension cultures seeded with rBVs P1 were used to produce larger amounts of recombinant protein. The expressed proteins were analysed using SDS-PAGE (Figure 37).

Figure 37: Large scale recombinant protein expression in Hi5™ suspension cultures.



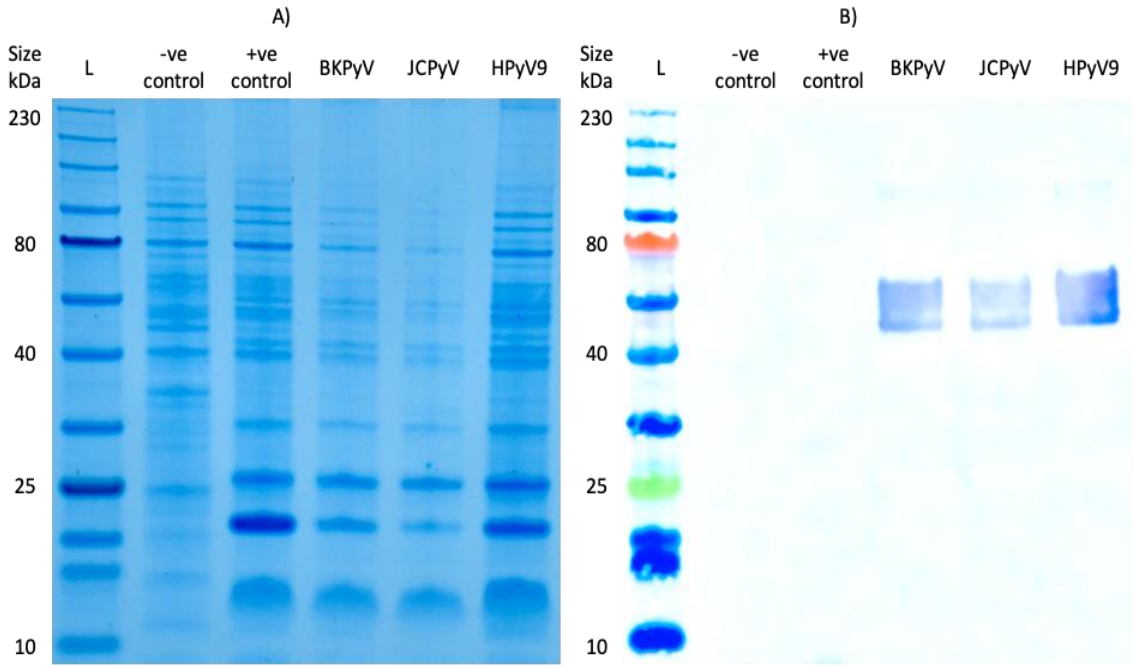
SDS-PAGE NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.0 mm. TF is the total fraction containing soluble and insoluble proteins. SF is soluble fraction which is the supernatant after centrifugation. -ve control is uninfected Hi5™ insect cells. +ve control is insect cells seeded with rBV that does not contain the VP1 gene. BKPyV, JCPyV, and HPyV9 show cells seeded with rBV VP1 containing their respected VP1 gene. Expressed protein bands are highlighted with red arrows at their expected size.

3.7.9.3. Western blot

Western blot was used to confirm the expression of the recombinant VP1 proteins using a monoclonal antibody against the histidine fusion tag (His-Tag). The target VP1 protein was successfully expressed for all three viruses in a soluble form depicted in (Figure 38). The three target proteins have comparable sizes (BKPyV VP1 45.1 kDa, JCPyV 44 kDa, and HPyV9 VP1 45.3 kDa) and appear as similar

bands. There was no expression of reactive proteins of this size in non-infected cells or cell infected with baculovirus without the VP1 protein.

Figure 38: Recombinant protein expression western blot.



Samples of soluble expressed proteins collected from large scale Hi5™ insect cells suspension cultures. -ve control is uninfected Hi5™ insect cells. +ve control is insect cells seeded with rBV that does not contain the VP1 gene. BKPyV, JCPyV, and HPyV9 show cells seeded with rBV VP1 containing their respected VP1 gene. A) SDS-PAGE. B) western blot nitrocellulose membrane confirming proper expression of the recombinant proteins BKPyV VP1 45.1 kDa, JCPyV 44 kDa, and HPyV9 VP1 45.3 kDa.

3.7.10. Recombinant Protein Purification

Recombinant proteins were concentrated and purified Ni-NTA beads. The term Ni-NTA (Nickel NTA) refers to a nickel^{2+} ion that has been coupled to Nitrilotriacetic acid (NTA). The Ni-NTA was coupled to agarose resin and used to

obtain the functional His tagged protein from the mixture of proteins. His tag purification is based on ionic interactions of the coupled metal ion and the Poly His-tag. Nickel is a balanced choice between the specificity and the affinity of the metal ion to the His-tag. Alternates such as Copper have the highest affinity (requiring harsher elution procedures to elute protein) Cobalt provides the purest His-tag purifications. The procedure is described in section 2.12. Optimisation of the conditions of elution is necessary. The finalised procedure washed irrelevantly, and proteins with low affinity form the beads using 20 mM and 30 mM Imidazole in PBS at pH 8.2. Elution was then performed using 250 mM Imidazole in PBS at pH 8.2. The resultant purified protein was examined using SDS-Page, and while the level of irrelevant protein was much reduced, and the protein of interest was concentrated, the preparation was not pure. Unwanted binding of untagged host cell proteins to purification media is a known problem of the His-tag purification procedure as these untagged proteins may elute with the target protein. The binding affinity of these contaminants is often lower than that of the tagged recombinant proteins and removal of these contaminants may require optimisation of buffer and the separation conditions, use of an alternate metal chelate and/or additional purification steps, e.g. ion-exchange or reverse-phase chromatography.

3.7.11. ELISA

Purified antigens were quantified using Invitrogen™ Qubit™ Protein Assay Kit, as described in section 2.13.1. the yield of antigen was between 350 – 500 ug/ml for the different VP1 antigens. Owing to time constraints, ELISA experiments were limited to only a preliminary evaluation of the immunoreactivity of the developed antigens. Plates

were coated with antigen at a concentration of 5 ug/ml in 0.05 M Carbonate-Bicarbonate coating buffer pH9.6 by overnight incubation at +4° C. Results using a single antibody positive serum and are shown in BKPyV and HPyV9 are shown in Table 27.

Table 27: Immune reactivity of BKPyV and HPyV9.

	Negative	BKPyV	HPyV9
OD (450nm)	0.159	0.328	0.466

Clearly once yield of optimisation of production and purification of the VP1 antigens is complete (section 3.7.10) greater concentration of antigens and more highly purified antigens will allow optimisation of the ELISA procedure including optimisation of the conditions for antigen adsorption to plates (and optimisation of antigen concentration), primary and secondary antibody dilutions and their diluent buffers, reaction temperatures and incubation times. Study of potential cross reaction will inform evaluations of sensitivity and specificity of the developed ELISA in comparisons with existing immunoassay techniques.

Chapter 4. Discussion

Improved, more potent, immune suppression therapies used after renal transplantation has resulted in reduced allograft rejection (Gaston, 2006). However, increased immune suppression, particularly using the calcineurin inhibitors and combinations of calcineurin inhibitors (tacrolimus, cyclosporine A) with antiproliferative agents (mycophenolate mofetil, azathioprine) and corticosteroids has been associated with an increase in the incidence of polyomavirus associated nephropathy (PyVAN). PyVAN is now recognised as a significant cause of premature allograft failure (Lamb et al., 2011). As there is currently no effective antiviral treatment for polyomavirus infection modification of maintenance immunosuppression is usually the first course of action (Hardinger et al., 2010). However, as acute rejection and PyVAN may coexist, diagnosis of PyVAN is important. PyVAN is typically diagnosed within the first year post-transplant, although approximately 25% of the cases are seen later (Costa, 2012). A peak in viruria and viraemia was detected at around 3 months in 2 European cohort studies (Alméras et al., 2011b; Koukoulaki et al., 2009). Pre-transplantation screening of polyomaviruses has been proposed for the recipient as well as for donors. Donors may be screened for polyomavirus viruria or VP1 serology. Meanwhile, recipients can be screened for VP1 serology and polyomavirus specific T-cells (Hirsch and Randhawa, 2019). However, there is no overall consensus screening for polyomavirus infection pre-transplant and it is not recommended in Europe (Hirsch et al., 2014). Screening post-transplantation especially, in the early post-transplantation period can detect a significant portion of polyomavirus infection and reduce the development of PyVAN (Hirsch and Randhawa, 2019). However, the duration of screening post-

transplant, after the early post-transplantation period is a matter of debate since information on the incidence of infection in late (>1 year) period post-transplant is sparse.

Two of the human polyomaviruses BKPyV, JCPyV are known to be involved in the pathogenesis of PyVAN, but the discovery of a plethora of new human polyomaviruses including WU, KI, Merkel cell virus raised the possibility of other causes of PyVAN. At the commencement of this work one of these new viruses, HPyV9 emerged as a potential cause of disease including PyVAN in renal transplant recipients and disease in renal dialysis patients (van der Meijden et al., 2014). In 101 kidney transplant patients in the Netherlands for HPyV9 DNA, 21 patients had positive results for HPyV9 DNA; positivity rates peaked at 3 months after transplantation, with the highest viral loads measured just after transplantation. It, therefore, seemed sensible to include this virus in an investigation of the development of PyVAN post-transplant. It is known that the most common aetiological agent of PyVAN is BKPyV, JCPyV has been recognised as the etiologic agent in less than 3% of all reported cases, either alone (Kazory et al., 2003; Wen et al., 2004) or in association with BKPyV (Cavallo et al., 2007). Nevertheless, it is likely that there is under reporting and the frequency of involvement of JCPyV is thus likely to be higher.

Diagnosis of PyVAN using techniques such as urine cytology examination for decoy cells or histopathological assessment of renal biopsies is possible (Randhawa et al., 2005). Diagnosis is, however, readily made by examination or urine or blood for the presence of polyomavirus DNA. The accurate, sensitive

measurement of virus can be achieved using real time qPCR and multiplex qPCR assay, where multiple viral targets are amplified and detected concurrently, reduces test turn-around time and overall cost of diagnosis. This ability to detect multiple viruses in samples is especially useful in renal transplant patients. Another advantage of multiplex qPCR is the ability to detect co-infection with two or more viruses (Ali et al., 2014). Since the first description of the polymerase chain reaction and its rapid adoption in diagnostic virology, there has been progressive improvement in the quality and reliability of methods for extraction of nucleic acids from clinical samples matched by development in the technology and chemistry of the PCR technique culminating in the modern multiplex qPCR assay of today. In the present project, a new internally controlled multiplex qPCR assay was developed to detect the presence of BKPyV, JCPyV, and HPyV9. This assay allowed the distinction between the three polyomaviruses associated with PyVAN, reliable quantitation of each viral target, and detection of co-infection.

The minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines provide a useful framework to ensure the reliability of results and promote consistency between laboratories (Bustin et al., 2009). This report attempts to adopt the guidance and nomenclature of this guidance. To ensure comparability with existing assays for BKPyV and JCPyV DNA, the WHO international standards for BKPyV and JCPyV DNA (Govind et al., 2015a, 2015b) were used to calibrate the assays. A standard positive control was developed by a synthesis of the predicted amplicons of each of the PCR procedures. The sequences were inserted in a plasmid and propagated in *E. coli.* Following purification of plasmid DNA free from bacterial DNA, the inserted sequences were confirmed by sequencing and the DNA quantitated using UV

spectrophotometry. The number of copies of each of the inserted polyomavirus specific sequences was computed from the knowledge that each mole of DNA amplicon contained Avogadro's number of copies of the inserted target sequence. The control was titrated in each of the developed PCRs, and a rough estimate of the limit of detection of the assay was derived for each of the developed PCR procedures. The linearity of each assay was also determined by plotting copies per mL versus Cq (the cycle in which fluorescence can be detected is termed quantitation cycle; Cq) for each dilution of the positive control. The straight-line equation for each PCR was computed, and the correlation coefficient between the two parameters was measured. The same procedure was repeated for the WHO International standards, and the resultant calibration curve (Cq versus International units per mL) was used to calibrate the positive control material as a secondary WHO standard. By interpolation from the WHO standard, the positive control was recalibrated in IU/mL. As there is presently no international standard for HPyV9 DNA, the calibration curve for the HPyV9 curve could only be produced in copies/ml. Nonetheless, the linearity, correlation coefficient and dynamic range of this assay were also good.

Computation of the equation of the straight line of the calibration curve also allowed calculation of the efficiency of each of the developed PCR procedures. The efficiency of a PCR is defined as the fraction of target molecules that are copied in one PCR cycle (Alvarez et al., 2007; Lalam, 2006). The exponential phase of a PCR reaction is known as the geometric phase and is remarkable in that it maintains the original quantitative relationships of the target gene across samples. The efficiency does not change with original gene quantity over a wide range. During PCR, the DNA target may accumulate to a high enough level that

one or more of the PCR reagents will no longer be sufficient to support geometric amplification and amplification changes to a linear phase. In the linear phase, efficiency declines cycle-to-cycle. Therefore, changes in efficiency during the linear phase become less consistent with each cycle number, and data becomes less quantitative. A theoretical relationship exists between a standard curve's slope and efficiency (Section 2.6.1). Each of the assays was shown to achieve high efficiency with values of >90% and in one case, in excess of 100%. A disadvantage of using standard curve slopes to determine assay efficiency is that many factors can cause such errors, including inhibitors, contamination, pipette precision error, dilution mixing problems. These errors can combine and result in values 100% efficiency even though geometric efficiency cannot exceed 100%.

The developed qPCR assay included an internal control molecule in each qPCR reaction. This was in the form of purified plasmid DNA containing a sequence of pumpkin DNA. The presence of PCR inhibitors in a test sample decreases amplification efficiency and the amount of PCR product generated from each target molecule. Addition of an internal control molecule provides assurance that clinical specimens are successfully amplified and detected. Because only 166 copies/mL of the IC are introduced into each test sample, a positive IC signal indicates that amplification was sufficient to generate a positive signal from targets present at the limit of test sensitivity. A set of 4 primer oligonucleotides are used in the multiplex to amplify both the IC and target DNA. These targets also draw from a common pool of nucleotides and polymerase. Thus, IC amplification may be suppressed due to competition in samples containing large amounts of target DNA. The amount of target DNA required to suppress the IC

signal was examined by testing each of the BKPyV, JCPyV and HPyV9 positive controls in the presence of the IC. Reliable amplification of IC was obtained in the presence of log 9.62 IU/mL BKPyV, log 9.01 IU/mL JCPyV and 4.11 X 10^9 copies per mL of HPyV9 conversely samples containing as low as BKPyV log 2.88 IU/mL, JCPyV log 2.92 IU/mL and 4.2X10^3 copies per mL of HPyV9 were amplified in the presence of the IC to see whether these targets were similarly sensitive to competition.

The efficiency of amplification was re-examined using the positive control when all targets were combined in a single multiplex PCR test. The linearity, correlation co-efficient and efficiency of the multiplex PCR in the presence of the IC molecule were equivalent to the corresponding single target PCRs there were only minor differences between the concentrations calculated for the positive control across the entire range of analyte concentrations (Table 22). The data indicated that inter-target competition did not occur and that the addition of the IC molecule had not altered test sensitivity. When the IC was used with patient samples control was found to be consistently positive confirming the utility of this control in eliminating the occurrence of false negative test results due to sample inhibition during the qPCR reaction (McHugh et al., 2015).

The repeatability of the assay and its reproducibility was investigated. Repeatability was estimated by measuring the intra-assay variation of the positive control Cq values. For the multiplex assay; the cumulative variance average was 0.4% for BKPyV 0.39%, for JCPyV, and 0.68% for HPyV9. Reproducibility was

estimated by inter-assay variation of the same control yielding values (CV%) of 1.02% for BKPyV; 0.91% for JCPyV; and 1.07% for HPyV9.

The analytical specificity of the assay was assessed by Blast N comparison of the primer and probe sequences versus the entire EMBL database of genomic sequences to ensure only the desired targets were amplified. Diagnostic specificity was not fully assessed, but the multiplex PCR did not produce any detectable products when control material in the form of the WHO International standards for Epstein-Barr virus (EBV), cytomegalovirus (CMV), hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) nucleic acid quantitation control material were run in the assay (data not shown).

Due to the limited availability of patient samples in the first-year post-transplantation and the unique access to samples collected late post-transplantation. The multiplex qPCR for BKPyV, JCPyV, and HPyV9 was then used to evaluate the incidence of infection in a cohort of renal transplant recipients with samples collected > 1 year after transplant. Information on the incidence of polyomavirus infection > 1 year after transplant is generally lacking, most studies having concentrated on the immediate post-transplant period when the incidence of PyVAN is highest. This may explain our overall lower infection rate in comparison to the literature, which focuses on the first-year post-transplantation. However, it is known that at least 25 % of BKPyV associated cases become apparent > 1 year after transplant and incidence of JCPyV and HPyV9 associated PyVAN in this period is not known. Not surprisingly, in this period so long after transplant, the influence of peri-transplant conditioning and

immediate post-transplant immunosuppressive regimens are diminished. Analysis of the conditioning regimen and immediate post-transplant immunotherapy revealed no significant association between the identification of infection in this cohort (data not shown). In addition, there appeared to be no significant association between the maintenance immunosuppressive therapy and the appearance of infection in these patients.

Molecular techniques such as real time qPCR provide a rapid and dependable method for the diagnosis of viral infection. Detection of polyomavirus by qPCR is the recommended method for the detection of infection in renal transplant recipients (Hirsch et al., 2014). The sensitivity of qPCR allows early detection of polyomavirus infection before the development of PyVAN (Hirsch et al., 2002).

Currently, the guidelines of the British Transplantation Society (Baker et al., 2017) recommend regular qPCR screening for BKPyV in all renal transplant recipients, but do not define the interval for such screening and do not define the duration of such screening post-transplant. European consensus guidance (Hirsch et al., 2014) recommends that all kidney transplant recipients should be regularly screened for BKPyV replication in urine (viruria) or plasma (viraemia) to identify patients at increased risk of PyVAN. Screening plasma for BKPyV is recommended monthly in the first six months post-transplant, followed by 3-monthly screening until 2 years post-transplant. Meanwhile, the American Society of Transplantation guidelines suggests extending the monthly screening to nine months instead of six post-transplantation, followed by 3-monthly screening until 2 years post-transplant. Testing urine for significant BKPyV replication (BKPyV

DNA loads of ≥7 $\log_{10}$ GEq/mL) is suggested, but positive cases should be followed-up by quantifying plasma BKPyV levels (Hirsch and Randhawa, 2019). The other polyomavirus associated with PyVAN is JCPyV which accounts for 5% of PyVAN cases (Delbue et al., 2013; Drachenberg et al., 2007). However, universal screening for JCPyV is not recommended because of the uncertainty around the exact involvement of JCPyV in PyVAN development and the rarity of the condition (Helanterä et al., 2016).

Investigation for polyomaviruses in the late transplant period is rarely done. Taking advantage of a previous study looking at the role of EBV in late after transplant post-transplant lymphoproliferative disease (Morton et al., 2016) samples of blood collected were examined from 466 patients participating in the study. DNA had been extracted from these samples and stored in a Biobank prior to use in the study. The 1156 samples from these patients were tested for BKPyV, JCPyV, and HPyV9 DNA. A total of 72 samples from 44 patients were positive for one or more polyomavirus DNA. Forty nine samples (4.2%) were positive for BKPyV, 19 (1.6%) for JCPyV and 4 (0.35%) for HPyV9. The age range, sex, and ethnicity of positive patients was similar to those of the population from which the samples were drawn. BKPyV was detected most frequently (in 27 of the 466 patients; 5-8%), the BKPyV positive samples tended to be collected earlier after transplantation, with a median of 2.33 years. The detection rate is within the range of reported prevalence in literature, which is 1-10% within the first five years (Bechert et al., 2010). The amount of virus detected in these samples ranged from log 2.97 IU/ml to log 8.48 IU/mL (mean = log 5.20 IU/mL; median log 5.07 IU/mL; mode log 4.61 IU/mL) the higher range positives (up to 120 million IU/mL) indicated very high rates of viral replication.

JCPyV was detected in 19 samples from 15 patients (3.2% of the patient cohort) at a median of 1.37 years post transplantation. This is lower than the rate (6.8%) reported by (Mengelle et al., 2011), however, the latter study collected samples only during the first year post transplantation (median collection time of 0.38 years). The 15 JCPyV positive patients had relatively lower levels of virus ranging between log 3.55 and 6.56 IU/mL with an average of log 4.9 IU/mL (median log 4.69 IU/mL; mode log 5.72 IU/mL).

HPyV9 was only detected in 4 samples from 4 patients. The level of viral DNA was very low in all of these samples ranging from log 0.42 to log 1.25 copies/mL. Such low detection rates and low levels of virus are not consistent with HPyV9 being a major pathogen in renal transplantation and are certainly inconsistent with HPyV9 being a causal agent of PyVAN. While HPyV9 was reported to be found in a significant proportion of renal transplant recipients (van der Meijden et al., 2014) it now seems more likely that the detection of HPyV9 reflects incidental reactivation or reinfection in immunocompromised hosts. The low rate of detection is consistent with other work, for example (Rockett et al., 2013) failed to find any HPyV9 positive samples in 161 routine BKPyV screening samples. However, the constraints of the limit of detection observed in the developed HPyV9 assay should be taken into consideration and validated further in future studies.

In line with previous findings, mixed infection of BKPyV, JCPyV and HPyV9 was found in one patient. The relatively low levels of each of the 3 viruses found in

the blood: - BKPyV log 3.65 IU/mL; JCPyV log 3.82 IU/mL and HPyV9 log 0.91 copies/mL, suggest this finding is likely to be of low clinical significance. One patient was noted to be BKPyV positive in one blood sample, and JCPyV positive in a separate sample collected 6months earlier the relatively low levels of virus detected in these two blood samples (BKPyV log 3.25 IU/mL; JCPyV log 4.22 IU/mL) perhaps suggest transient reactivation/reinfection of these viruses rather than any disease or other interrelation.

The observational design of the study allowed for testing for a substantially wide range of timing after renal transplant between 0.04 to 32.84 years post transplantation (average 9.22 years ±10.66 years; Median 7.01 years). Interestingly, in our study, the median time for polyomavirus detection in the blood was two years post transplantation. This result is consistent with the literature finding where HPyVs detection occurs at or near the time of transplantation during longitudinal follow up (Bialasiewicz et al., 2016).

A limitation of the study is that the samples analysed in this current study were collected for the purpose of another study (Morton et al., 2013). The delay between the collection of the samples and the extraction of nucleic acid from the samples were considerable. Whilst the samples were stored frozen inevitably in their transfer between the original collection site (the virus laboratory in Manchester Royal Infirmary), their transfer to the Manchester Institute for Nephrology and Transplantation Biobank, and subsequent transfer to the University of Manchester freezing and thawing of the samples will have occurred. Deterioration of samples may this have caused a lower rate of detection and

quantification (Ross et al., 1990). The study results should thus be interpreted with caution. One method of monitoring possible sample deterioration on storage could have been the addition of an internal control molecule to the original sample. This would have provided a means of monitoring of the complete handling of samples from extraction to storage and qPCR (Saunders et al., 2013).

Nonetheless, the developed qPCR provided sensitive and robust detection of polyomavirus in blood and should provide a useful tool for future investigation in renal transplant recipients preferably using fresher specimens than were available for this study.

Serological evaluation holds a prominent part in viral infection diagnosis, seroepidemiological studies, and an investigation of the response to infection. Assays for the detection of polyomavirus antibody used to rely upon cell culture propagation of virus to allow plaque reduction, neutralizing antibody, or indirect immunofluorescent assay of antibody. The restricted range of cell types infectable by these viruses, particularly JCV, their lengthy growth cycle, and poor replication capacity, limited the use of these assays. Haemagglutination inhibition (HI) antibody assay became the standard method for measurement of antibody to BKV and JCV. Unfortunately, such biological assays of this type are technically demanding and are poorly reproducible. Most contemporary assays for measuring viral antibodies now employ enzyme immunoassay (EIA) techniques because of their greater sensitivity and precision. The development of EIA techniques for polyomavirus antibody detection was hampered by the difficulty of propagation of these viruses. The advent of molecular biological procedures to

allow the *in vitro* expression of viral proteins (viral antigens) in bacteria, yeasts, mammalian cells or insect cells.

HPyVs are non-enveloped viruses which have an icosahedral nucleocapsid, that holds a supercoiled circular dsDNA. The capsid is mainly made of VP1, which accounts for 80% of capsid protein and holds the antigenic epitopes. VP1 forms the outer part of the capsid, where the viral binding receptors are (Daniels et al., 2007; Jiang et al., 2009). Polyomaviruses have only sufficient genetic information to code for six proteins but are able to expand their functional capabilities by utilizing post-translationally modified versions of their structural proteins for required functions (e.g. cellular attachment and hemagglutination) and the enzymes responsible for these modifications are a property of the host cell (Ludlow and Consigli, 1987). When proteins are expressed in bacteria, usually *E. coli*, the prokaryotic cell cannot perform many of the post translational modifications that are found in eukaryotic cells, including N- and O- linked glycosylation, fatty acid acylation, phosphorylation and disulphide-bond formation. In contrast, the insect cells that serve as hosts for baculovirus vector infection are capable of the post translational modification of mammalian cells and can transfer oligosaccharide side chains (glycans) to the same sites in recombinant proteins as those that are used for native protein N-glycosylation in mammalian cells. However, while mammalian cells produce compositionally more complex N-glycans containing terminal sialic acids, insect cells mostly produce simpler N-glycans with terminal mannose residues.

Ever since the description of baculovirus recombination in 1983 (Smith et al., 1983), continuous improvements have been implemented, making baculovirus expression systems one of the most popular and versatile expression system (Rohrmann, 2019). Baculovirus protein expression system utilises the eukaryotic insect cell cultures to produce recombinant proteins with post translational modifications such as glycosylation, phosphorylation, and acylation similar to mammalian cells (Kost et al., 2005). Another substantial benefit from using baculovirus expression system is the retention of the immunogenic properties of the expressed recombinant protein (Gillock et al., 1997; Li et al., 2003; Zhou et al., 2019). Despite the high degree of similarity between the human polyomaviruses in the amino acid sequence of the VP1, which may cause serological cross-reactivity (Moens et al., 2013), recent serological techniques showed the ability not only to distinguish between different viruses but between the different serotypes of BkPyV (Wunderink et al., 2019).

Recombinant polyomavirus VP1 protein expressed using baculovirus system has shown the ability to self-assemble into a virus like particle (VLP) and retain immunogenicity (Gillock et al., 1997; Li et al., 2015, 2003; Zhou et al., 2019). In order to produce recombinant VP1 for BKPyV, JCPyV, and HPyV9, we used the pOET8.VE2 transfer vector plasmid along with the baculovirus expression system *Flash*BAC Ultra. This system has the advantage of the inability of the non-recombinant parental viral particle to replicate, removing the need for plaque assay purification of recombinant baculovirus (rBV) (Rohrmann, 2019).

The chosen plasmid pOET8.VE2 plasmid has 8X histidine (His) fusion tags (His-tag) at the N terminus of the multiple cloning site (MCS) to allow easy purification of the recombinant protein and an ampicillin resistance gene for *E. coli* colony selection. The pOET8.VE2 plasmid includes a honeybee melittin (HBM) signal sequence that enhances recombinant protein secretion (Tessier et al., 1991). Vankyrin gene expression cassette is also included; this is an antiapoptotic protein to delay cell lysis after infection with rBV, allowing prolonged recombinant protein expression of infected cells (Steele et al., 2017).

Amplification of the VP1 genes was carried out using PCR, using either synthetic polyomavirus gene sequences or purified plasmid DNA as a target. Primers to amplify the VP1 gene sequence included additional bases as a tail sequence bearing a restriction digestion enzyme specific sequences and six extra bases. The six extra bases facilitate optimal activity of the restriction enzyme. These additional sequences also serve to align the amplified VP1 gene within the polyhedrin promoter reading frame, which included including 8 X His-tag sequences and Honey Bee Melittin signal (Figure 9). The restriction enzymes were specific to virus VP1 target as the restriction enzyme had cut inside the pOET8.VE2 MCS region and not cut inside the target VP1 gene sequence. Restriction enzymes were chosen to produce sticky DNA ends to ensure the correct orientation of insertion.

After restriction digestion and end modification in preparation for ligation, both the gene inserts and the linearised pOET8.VE2 plasmid were resolved in a 1% agarose gel electrophoresis this extra step allowed for DNA gel extraction.

The successful VP1 cloned pOET8.VE2 transfer vectors were checked by visualisation of construct using SnapGene software to ensure the VP1 genes were inside the polydedrin locus in the same reading frame as the His-tag sequences with no stop sequences preventing proper translation. The importance of this was shown by previous critical mistakes (Figure 39) which delayed the project when using an alternate transfer vector, pOET1N 6xHis; the error led to minimal recombinant VP1 protein expression and the His-tag not being attached to the VP1 protein. The His-tag became attached to a short truncated protein because it was in a different reading frame to VP1 and had a stop codon (TAA) inhibiting VP1 translation. Unfortunately, this was not discovered until the late stages of western blot characterisation and immobilised metal affinity chromatography (IMAC) protein purification, which is dependent on the proper incorporation of His-tag (data not shown).

Figure 39: Transfer vector pOET1N 6xHis design.

Failed transfer vector pOET1N 6xHis design. A) the VP1 protein shown in yellow is in a different translation frame to 6XHis-tag. B) the stop codon (TAA) early in the translation frame of 6XHis-tag stopping translation of VP1.

Co-transfection of Sf9 insect cells with the cloned transfer vectors and the modified AcMNPV *flash*BAC Ultra was carried out to achieve homologous recombination. Homologous recombination restores the open reading frame (ORF) 1629, allowing multiplication of recombinant baculovirus (rBV) and replacement of the bacterial artificial chromosome (BAC) with the VP1 gene target. Only rBVs can multiply and be released in the supernatant. Sf9 cells were used for rBV production and amplification, while Hi5™ cells were used for protein expression (Zhou et al., 2019).

Quantification of rBV was achieved by qPCR of insect cell culture following the method described by (Hitchman et al., 2007). Titration with qPCR revealed concentrations of 2.84 X 10^7 pfu/ml, 7.06 X 10^7 pfu/ml, 1.09 X10^8 pfu/ml for BKPyV, JCPyV, and HPyV respectively.

Recombinant protein expression was found to reach higher titre at day five after infection of Hi5 ™ cells in accordance with the previous report by (Li et al., 2003). Characterisation of recombinant protein was done on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by anti-8Xhistidine western blot and revealed recombinant protein sizes between 44 and 45.3 kDA in agreement with our designing prediction and earlier publication (Gillock et al., 1997).

Purification of the recombinant protein relied on the affinity between the nickel cation (Ni^{2+}) nitriloacetic acid (NTA) Ni^{2+}-NTA resin beads and the poly-histidine fusion tag to perform IMAC (Bornhorst and Falke, 2011; Nelson et al., 2015). The recombinant VP1 fused to 8 X His-tag was confirmed with the positive anti-8Xhistidine western blot.

In conclusion, we have successfully produced the antigenic major capsid protein VP1 for BKPyV, JCPyV, and HPyV9, using a baculovirus recombinant protein expression in insect cells. Future utilisation of these recombinant proteins may enhance our understanding of the relationship of the serological status of polyomavirus infection in renal transplant patients and the development of PyVAN, building on previous insightful work done by (Bohl et al., 2008; Wunderink et al., 2017).

A multiplex qPCR for BKPyV, JCPyV, and HPyV9 DNA with internal control was developed. The dynamic range of the developed assay was large, and the reproducibility and precision of the developed assay were good. The BKPyV and JCPyV analytes were calibrated in IU/mL by reference to the relevant WHO International standards for RNA quantitation and the HPyV9 DNA calibrated in copies/ml. The development was not without problems in that the original thermocycler (FluoroCycler® 96, Hain Lifescience GmbH, Germany) developed problems with lid sealing leading to failure of PCR runs and the software used could not measure more than 3 dye channels simultaneously (even though designed to measure 5), at a time when the company discontinued support for the instrument. Switch of the assay to a different thermocycler resolved problems

and the developed assay was used to investigate a previously collected series of samples from a cohort of renal transplant recipients. The samples were collected at some time after transplant, and this allowed investigation of polyomavirus infection in the late post-transplant period. Most studies of polyomavirus infection concentrate in the immediate post-transplant period when peak rates of PyVAN are known to occur. It was known that for BKPyV associated PyVAN about 25% of cases occur >1year after transplant. The results of this study provide further evidence of the need for vigilance in the late post-transplant period. Overall, 4.2% of samples had detectable BKPyV and among these most had significant levels (10,000 IU/mL ranging up to >100million IU/mL) suggesting significant BKPyV viraemia consistent with clinical disease. It is unfortunate that due to difficulties in the linkage of clinical information (see below) more detail on outcome of infection, in particular, whether this significant level of viral replication was associated with adverse outcome including PyVAN was not obtained. JCPyV DNA was also detectable in this late post-transplant period. Lower numbers of samples were JCPyV positive, and the amount of virus detected was generally lower than was seen in BKPyV positive samples. JCPyV is thought to be an infrequent cause of PyVAN, and this may be reflected in the generally lower level viraemia seen with JCPyV and the less frequent reactivation/reinfection seen with the virus. At the beginning of this work, HPyV9 was thought to represent a new potential polyomavirus pathogen post renal transplantation. Work in the intervening years has cast doubt upon the relevance of HPyV9 in morbidity following renal transplantation. The results reported here reinforce that view with the very few samples testing positive among the cohort tested and with the extremely low levels of virus detected in those samples suggesting they would not be of clinical relevance.

Serology as a means of predicting the outcome of polyomavirus infection in transplantation has been enjoyed a resurgence of interest since this project commenced. Determining donor and recipient polyomavirus serostatus has been suggested to be predictive of polyomavirus disease and HPyV9. Previous studies were hampered by the lack of assays of adequate sensitivity and reproducibility. Immunoassays utilising recombinant proteins using recombinant VP1, the immunodominant and serotype specific antigen of the viruses, enabled assays of improved sensitivity and reproducibility rekindling interest in the use of serology as a disease predictor. The merits of insect cell expression of protein mediated by baculoviruses were well understood at the commencement of this project and at the time of start only 2 publications had documented the production of BKPyV and JCPyV VP1 in insect cells. To date, the expression of HPyV9 VP1 in insect cells has not been documented. The three VP1 proteins of BKPyV, JCPyV, and HPyV9 were successfully produced in this project. The production was, however, not without difficulty. At the time of inception of the project, there was an intention to re-use a procedure previously used in the successful expression of BKPyV and JCPyV VP1 proteins in insect cell culture, the Novagen® InsectDirect™ (Merck, New Jersey USA) system. The system does not involve a baculovirus life cycle. It uses the transcriptional elements ie1 and Hr5 to promote endogenous insect transcriptional enzymes to express the gene of interest. As no life cycle is involved, the system is extremely rapid in producing protein in as little as 48 hours. Unfortunately, soon after the commencement of the project, supplies of the reagents for this system became unavailable. This necessitated the change of the system for baculovirus expression and led to work using the POET series

of vectors from Oxford Expression technologies. Several versions of the vector were trialled without success before the successful expression of the VP1 proteins using the final pOET8.VE2 vector system. The technical delays reduced the time available for completion of all but very preliminary work on the development of the ELISA assay. Nevertheless, the successful production of VP1 proteins was demonstrated via this procedure and with further optimisation of the cell culture system and purification system to boost production of protein development of ELISA could now proceed.

A major difficulty arose with the re-use of samples in this study. The samples on completion of the original renal unit study had been transferred from the virus laboratory at Manchester Royal Infirmary to the Human Tissue Act approved Biobank within the Renal Unit at Manchester Royal Infirmary so that they could be preserved under the ethical approvals for the Biobank after the ethical approval for the original study lapsed. The clinical information was believed to have been transferred with the samples. It later emerged (at a late stage of the project) that this data had not been transferred and only fragmented data was available from the original study investigator. As outlined above, this reduced the ability to make clinical correlates of the detection of the virus in the stored samples. A further detail related to ethical permission for re-use of samples from the Biobank. The investigators and the curator of the Biobank were absolutely clear that the Biobank ethics allowed the re-use of samples for this work within the ethical permissions of the Biobank. As this was a University of Manchester Research Degree, the ethical permission was reviewed within the Research Office of the University. A requirement for a full application for research ethics be made

through the integrated research ethics approval process and health research approval process. A full application was prepared and submitted and was rejected as being unnecessary by the ethics committee. As it was considered good practise not to rely solely on Biobank Research Ethics as new application was made to the University of Manchester Research Ethics committee who granted further permission for the research. The various processes consumed over 2 years of the researchers' time and when the problem of the lack of linked clinical information emerged, the principal clinical collaborator was unavailable to assist owing to the extreme pressure of work. The process revealed deficiencies in Biobank procedures concerning storage of lined information and serious deficiencies in the level of knowledge within the University Research Office concerning the operation of Biobanks and the grant of ethical approval for research.

Future Work

A frustration of the present work is that time did not permit the application of the developed PCR assay in a prospective study of polyomavirus infection post-renal transplantation. Renal transplants are life saving and for those with end-stage renal disease represent the only opportunity for a return to a good quality of life. Organs for donation are in short supply, and the operative procedure and associated work up are expensive. There is clearly much interest in ensuring the success of transplantation and for polyomaviruses in the absence of preventative or curative antiviral agents' timely diagnosis of infection remains key.

A prospective study in renal transplant recipients where blood and urine specimens were collected and analysed prospectively the immediate post-transplant period and on into the late transplant period would provide information to inform the necessity (or otherwise) of routine screening for polyomavirus infection in renal transplantation as an aid to the detection and prediction of the development of PyVAN. From the data presented in the present work, the involvement of JCPyV and HPyV9 in the causation of PyVAN in the late post-transplant period seems unlikely. However their involvement in the early post-transplant period and the inclusion for the study of infection with these two organisms seems appropriate in a future prospective study.

Accurate serological matching of donor and recipient is an attractive proposition and results to date have produced mixed results in which there is a suspicion that the quality of serological procedures have not been adequate. The successful

expression of VP1 proteins of BKPyV, JCPyV, and HPyV9 in insect cell culture means that with minor further work to optimise expression and purification of these proteins, antigens of the virus closely mimicking the antigens produced by mammalian cells during infection with polyomaviruses will be available to develop improved ELISA test for antibody. Application of these assays in the serological investigation of both donor and recipient pre-transplant may realise predictive measures for polyomavirus disease.

In addition, the diagnostic tools developed herein represent important procedures to aid the development of antiviral drug application in polyomavirus disease in allowing early detection of infection and in monitoring therapeutic responses. Improved antibody assays may also be of value in the development of preventative vaccination not only in assessing response to vaccination but potentially in aiding vaccine production. The VP1 proteins produced within this project has the potential to form self-aggregating structures – virus like particles – which are acknowledged as being highly immunogenic. Virus like particles are used in the highly successful human papillomavirus vaccines. Experiments in mice using MCPyV VLP showed promising degrees of immunity in both immunosuppressed and immune competent mice. Such vaccine prevention of human polyomavirus infection would be a major advance for renal medicine.

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Appendix 1

BKPyV VP1 sequencing

BK-VP1-Seq-F1 TTCCCCTCCCCAATTTAAATGAG Forward

TTCCCCTCCCCAATTTAAATGAGGACCTAACCTGTGGAAATCTACTGATGTGGGAGGCTGT
AACAGTACAAACAGAGGTCATTGGAATAACTAGCATGCTTAACCTTCATGCAGGGTCACAA
AAAGTGCATGAGCATGGTGGAGGTAAACCTATTCAAGGCAGTAATTTCCACTTTTTTGCTGT
TGGTGGAGACCCCTTGGAAATGCAGGGAGTGCTAATGAATTACAGGACAAAGTACCCAGA
AGGTACTATAACCCCAAAAAACCCAACAGCCCAGTCCCAAGTAATGAATACTGACCATAAG
GCCTATTTGGACAAAAACAATGCTTATCCAGTTGAGTGCTGGATTCCTGATCCCAGTAGAA
ATGAAAATACTAGGTATTTTGGGACTTTCACAGGAGGGGAAAATGTTCCCCCAGTACTTCAT
GTGACCAACACAGCTACCACAGTGTTGCTAGATGAACAGGGTGTGGGGCCTCTTTGTAAA
GCTAATAGCCTGTATGTTTCAGCTGCTGATATTTGTGGCCTGTTTACTAACAGCTCTGGAAC
ACAACAGTGGAGAGGCCTTGCAAGATATTTTAAGATTCGCCTGAGAAAAAGATCTGTAAAA
AATCCTTACCCAATTTCCTTTTTGCTAAGTGACCTTATAAACAGGAGAACCCAGAGAGTGGA
TGGGCAGCCTATGTATGGTATGGAATCCCAGGTAGAAGAGGTTAGGGTGTTTGATGGCAC
AGAAAGACTTCCAGGGGACCCAGATATGATAAGATATATTGACAAACAAGGACAATTGCAA
ACTAAAATNGTTTAAGAGCTCTCGAGGTACCGCGGCCGC

BK-VP1-Seq-F2 AGCCCAGTCCCAAGTAATGAA Forward

AGCCCAGTCCCAAGTAATGAATACTGACCATAAGGCCTATTTGGACAAAAACAATGCTTATC
CAGTTGAGTGCTGGATTCCTGATCCCAGTAGAAATGAAAATACTAGGTATTTTGGGACTTTC
ACAGGAGGGGAAAATGTTCCCCCAGTACTTCATGTGACCAACACAGCTACCACAGTGTTGC
TAGATGAACAGGGTGTGGGGCCTCTTTGTAAAGCTAATAGCCTGTATGTTTCAGCTGCTGA
TATTTGTGGCCTGTTTACTAACAGCTCTGGAACACAACAGTGGAGAGGCCTTGCAAGATAT
TTTAAGATTCGCCTGAGAAAAAGATCTGTAAAAAATCCTTACCCAATTTCCTTTTTGCTAAGT
GACCTTATAAACAGGAGAACCCAGAGAGTGGATGGGCAGCCTATGTATGGTATGGAATCC
CAGGTAGAAGAGGTTAGGGTGTTTGATGGCACAGAAAGACTTCCAGGGGACCCAGATATG

ATAAGATATATTGACAAACAAGGACAATTGCAAACTAAAATNGTTTAAGAGCTCTCGAGGTA
CCGCGGCCGC

BK-VP1-Seq-R1 TTTACAAAGAGGCCCCACACC Reverse

TTTACAAAGAGGCCCCACACCCTGTTCATCTAGCAACACTGTGGTAGCTGTGTTGGTCACA
TGAAGTACTGGGGGAACATTTTCCCCTCCTGTGAAAGTCCCAAAATACCTAGTATTTTCATT
TCTACTGGGATCAGGAATCCAGCACTCAACTGGATAAGCATTGTTTTTGTCCAAATAGGCC
TTATGGTCAGTATTCATTACTTGGGACTGGGCTGTTGGGTTTTTTGGGGTTATAGTACCTTC
TGGGTACTTTGTCCTGTAATTCATTAGCACTCCCTGCATTTCCAAGGGGTCTCCACCAACA
GCAAAAAAGTGGAAATTACTGCCTTGAATAGGTTTACCTCCACCATGCTCATGCACTTTTTG
TGACCCTGCATGAAGGTTAAGCATGCTAGTTATTCCAATGACCTCTGTTTGTACTGTTACAG
CCTCCCACATCAGTAGATTTCCACAGGTTAGGTCCTCATTTAAATTGGGGAGGGGAATTCT
TGCTGTGCTGTAACAGGGAAGCATTTTTCTTTCTGGGCTATCACTGCTAAAGTCATTTTCAG
CACTTAGCTTTAGACTAAAGCCCCTAAGGTTTTCATCTGGATCCCCCATTTCTGGGTTTAGG
AAGCATTCTACCTCTGTTATAGCATCTAGCCCAGTTTTAACTTCTAGAACTTCTACTCCTCCT
TTTATTAGTAGTTTTGGCACTTGCACGGGTTCCTTTGGCTTTTTGGGAGCTGCCCCTGGAC
ACTCTCCTTTTCTTTTTGTTGGGGCCATGTCGACTCTAGAAGCTTGGATCCACGCGGAACC
AGACCGGTGTGATGGTGGTGATGATGGACCATGGTCCTAGGAGATCCTATATTTATAGGTT
TTTTTATTACAAAACTGTTACGAAAACAGTAAAATACTTATTTATTTG

JCPyV Sanger Sequencing

JC-VP1-Seq-F1 ATGTGCACTCTAATGGGCAAG Forward

ATGTGCACTCTAATGGGCAAGCAACTCATGACAATGGTGCAGGGAAGCCAGTGCAGGGCA
CCAGCTTTCATTTTTTTTCTGTTGGGGGGGAGGCTTTAGAATTACAGGGGGTGCTTTTTAAT
TACAGAACAAAGTACCCAGATGGAACAATTTTTCCAAAGAATGCCACAGTGCAATCTCAAGT
CATGAACACAGAGCACAAGGCGTACCTAGATAAGAACAAAGCATATCCTGTTGAATGTTGG
GTTCCTGATCCCACCAGAAATGAAAACACAAGATATTTTGGGACACTAACAGGAGGAGAAA
ATGTTCCTCCAGTTCTTCATATAACAAACACTGCCACAACAGTGTTGCTTGATGAATTTGGT
GTTGGGCCACTTTGCAAAGGTGACAACTTATACTTGTCAGCTGTTGATGTCTGTGGCATGT

TTACAAACAGGTCTGGTTCCCAGCAGTGGAGAGGACTCTCCAGATATTTTAAGGTGCAGCT
AAGGAAAAGGAGGGTTNAAAAACCCCTACCNAATTTCTTTCCTTCNTACTGANTNANTTNAC
AGAANNNNTCCTNNNNNNGANGNNNNNCNTATGTNTGGCAT

JC-VP1-Seq-F2 ACAGAGCACAAGGCGTACCT Forward

ACAGAGCACAAGGCGTACCTAGATAAGAACAAAGCATATCCTGTTGAATGTTGGGTTCCTG
ATCCCACCAGAAATGAAAACACAAGATATTTTGGGACACTAACAGGAGGAGAAAATGTTCC
TCCAGTTCTTCATATAACAAACACTGCCACAACAGTGTTGCTTGATGAATTTGGTGTTGGGC
CACTTTGCAAAGGTGACAACTTATACTTGTCAGCTGTTGATGTCTGTGGCATGTTTACAAAC
AGGTCTGGTTCCCAGCAGTGGAGAGGACTCTCCAGATATTTTAAGGTGCAGCTAAGGAAAA
GGAGGGTTNAAAAACCCCTACCNAATTTCTTTCCTTCNTACTGANTNANTTNACAGAANNNN
TCCTNNNNNNGANGNNNNNCNTATGTNTGGCATNGNNGC

JC-VP1-Seq-R1 ATGCCACAGACATCAACAGCT Reverse

ATGCCACAGACATCAACAGCTGACAAGTATAAGTTGTCACCTTTGCAAAGTGGCCCAACAC
CAAATTCATCAAGCAACACTGTTGTGGCAGTGTTTGTTATATGAAGAACTGGAGGAACATTT
TCTCCTCCTGTTAGTGTCCCAAAATATCTTGTGTTTTCATTTCTGGTGGGATCAGGAACCCA
ACATTCAACAGGATATGCTTTGTTCTTATCTAGGTACGCCTTGTGCTCTGTGTTCATGACTT
GAGATTGCACTGTGGCATTCTTTGGAAAAATTGTTCCATCTGGGTACTTTGTTCTGTAATTA
AAAAGCACCCCCTGTAATTCTAAAGCCTCCCCCCCAACAGAAAAAAAATGAAAGCTGGTGC
CCTGCACTGGCTTCCCTGCACCATTGTCATGAGTTGCTTGCCCATTAGAGTGCACATTCAT
CAAACTTGTCACCCCTATAACCTCAGTTTTTAAGGTCACAGCCTCCCACATGAGTATATTTC
CACAGGTTAGATCCTCATTTAGATTGGGTAGTGGAATTCTGGCCACACTGTAACAAGGAAG
CATGTCCCTATTTGGGGAGTCACTTTCAAATGTATCTGATATAGATATTGACTTACTAAAAC
CCCTAAGATGCTCATCTGGGTCACCCATTTCTGGAGTTAAAAAGCATTCTACCTCTGTAATT
GAGTCAACCCCAGTTTTAACTTCTAGANNNNNACTTCTACTCCTCCTNNTTATAAAAGTTTT
GGAACTTGCACGGGGTCCTTCCTTTCTCCTTTTNCTTTTTGTTGGGGCCATGTCGACTCTA
GAAGCTTGGATCCACGCGGAACCAGACCGGTGTGATGGTGGTGATGATGGACCATGGTCC

TAGGAGATCCTATATTTATAGGTTTNTTTATTACAAAACTGTTACGAAAACAGTAAAATACTT

ATTTATTTGNNNGNNNGTTATCATNTNNATTNNNNNCANNATNNCANNA