Universidade de Lisboa
Faculdade de Farmácia

Amplification and Titration of Stocks of Clinical Strains of BK Virus

Ana Rita Abrunhosa Silva

Trabalho de Campo orientado pelo Doutor Baptiste Demey, Assistente Hospitalar Universitário da Université Picardie Jules Verne e coorientado pelo Professor Doutor José Miguel Azevedo Pereira, Professor Associado com Agregação da Faculdade de Farmácia da Universidade de Lisboa.

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Trabalho Final de Mestrado Integrado em Ciências Farmacêuticas apresentado à Universidade de Lisboa através da Faculdade de Farmácia

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Resumo

Contextualização: O poliomavírus BK (BKPyV) infeta cerca de 80% da população mundial, estabelecendo uma infeção persistente sobretudo nas células epiteliais do trato renal e nas células epiteliais de transição do trato urinário. O BKPyV replica-se de forma discreta, não apresentando quaisquer manifestações clínicas em indivíduos imunocompetentes. No entanto, em situações de transplante renal, a replicação do BKPyV pode conduzir a casos de nefropatia. Até à data, não existe no mercado nenhum fármaco eficaz contra o BKPyV, pelo que a estratégia clínica utilizada para impedir o desenvolvimento da nefropatia consiste na redução da terapia imunossupressora. Neste enquadramento, a pesquisa de um antiviral eficaz é fundamental. De forma a assegurar a eficácia global do fármaco é necessário que sejam realizados estudos pré-clínicos utilizando as mais variantes estirpes do BKPyV.

Objetivo: Desenvolvimento de um modelo *in vitro* mais eficaz para a replicação e amplificação de estirpes isoladas do BKPyV, em comparação com os modelos já desenvolvidos.

Métodos: As células Vero e as células MRC-5 foram cultivadas e incubadas com a estirpe 216x2X durante um mês. De seguida, as partículas virais foram extraídas e purificadas. Estes modelos foram avaliados quanto à sua eficácia através do cálculo da titulação dos stocks produzidos e quanto à sua infectividade através da infeção de células epiteliais tubulares proximais renais humanas (RPTEC).

Resultados: Os resultados demonstram que ambas as linhas celulares são permissíveis à estirpe 216x2X. No entanto, quando utilizando as células Vero, estas não foram capazes de produzir novos viriões, ao contrário do que aconteceu quando se usou as células MRC-5. Assim, podemos aferir que a estirpe 216x2X pode ser adaptada a um modelo *in vitro* utilizando as células MRC-5.

Conclusão: A falta de antivirais no combate à infeção pelo BKPyV apresenta-se como um desafio na gestão de doentes em condições pós-transplante. A procura deste fármaco ideal continua a ser condicionada pela falta de informação consequente da inexistência de bons modelos *in vitro* e *in vivo*. Além disso, a utilização da estirpe Dunlop apresenta como problema adjacente a raridade da mesma na população humana. A investigação deve continuar a rumar no sentido de conseguir-se não só isolar-se estirpes presentes em doentes como também no desenvolvimento de um fármaco eficaz para a população em geral.

Palavras-chave: Poliomavírus BK, modelo *in vitro*, amplificação, estirpes, transplante renal

Abstract

Background: The BK polyomavirus (BKPyV) infects approximately 80% of the world's population, establishing a persistent infection mostly in renal tubular epithelial cells and the urinary tract transitional epithelial cells. BKPyV replicates discreetly, causing no clinical manifestations in immunocompetent individuals. However, BKPyV replication can cause nephropathy in kidney transplant recipients. To date, there is no effective drug on the market to combat BKPyV, so the clinical strategy for preventing nephropathy is to reduce immunosuppressive therapy. In this context, the search for an effective antiviral is critical. Pre-clinical investigations using the most diverse strains of BKPyV are required to ensure the overall efficiency of the antiviral.

Objective: Development of a more effective *in vitro* model for the replication and amplification of isolated BKPyV strains, compared to models already developed.

Methods: For one month, Vero and MRC-5 cells were cultivated and incubated with the 216x2X strain. After that, the virus particles were isolated and purified. These models were evaluated for their efficacy by calculating the titration of the stocks produced and for their infectivity by infecting human renal proximal tubular epithelial cells (RPTEC).

Results: The results show that both cell lines are permissible to the 216x2X strain. However, when Vero cells were used, they were unable to produce new virions, unlike MRC-5 cells. We can therefore see that the 216x2X strain can be adapted to an *in vitro* model using MRC-5 cells.

Conclusion: The lack of antivirals to combat BKPyV infection is a challenge in the management of post-transplant patients. The search for this ideal antiviral continues to be conditioned by the lack of information resulting from the absence of good *in vitro* and *in vivo* models. Furthermore, the use of the Dunlop strain presents the adjacent problem of its rarity in the human population. Continued research is required not just to isolate strains present in patients, but also to develop an effective drug for the general population.

Keywords: BK polyomavirus, *in vitro* models, amplification, strains, kidney transplant

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Abbreviations

Agno – Agnoprotein

BKPyV – BK polyomavirus

BKVAN – BK virus-associated nephropathy

bp – base-pairs

c/mL – copies/mL

CSK – cytoskeletal buffer

DAPI -4’, 6-dia-midino-2-phenylindole

DMEM – Dulbecco’s modified Eagle medium

dpi – days post-infection

dsDNA – double-stranded DNA genome

ER – Endoplasmic reticulum

ERAD -ER-associated degradation

EVGR – Early viral gene region

FBS – fetal bovine serum

FITC – Fluorescein isothiocyanate

GA-100 – gentamicin and amphotericin B

HEK293 -human embryonic kidney 293 cells

HPyVs – human polyomaviruses

hTERT – human telomerase reverse transcriptase

JCPyV – JC polyomavirus

LVGR – Late viral gene region

MCPyV – Merkel cell polyomavirus

miRNA – microRNA

MPyV – murine polyomavirus

NCCR – Non-coding control region

NLS -Nuclear localization sequences

ori – origin of replication

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PFA - paraformaldehyde

PML – progressive multifocal leukoencephalopathy

pre-miRNA – precursor – miRNA

qPCR – quantitative real-time PCR

REGM – renal epithelial growth medium

rpm – rotation per minute

RPTEC – human renal proximal tubular epithelial cells

RPTEC/TERT1 – hTERT immortalized RPTEC

SV40 – simian vacuolating virus 40

TAg – large tumor antigen

tAg – small tumor antigen

$TCID_{50}$ – 50% tissue culture infectious dose

truncTAg – truncated TAg

TSPyV- Trichodysplasia spinulosa polyomavirus

VLPs – virus-like particles

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1. Introduction

a. Polyomaviruses

Polyomaviridae is a non-enveloped virus family with a double-stranded DNA genome (dsDNA)(1). This family was named after its founding member, murine polyomavirus (MPyV). Ludwig Gross described this virus for the first time in 1953, after observing that MPyV could induce adenocarcinomas in exposed mice, hence the name (poly and oma mean many and tumor in Greek) (2).

About ten years later, simian vacuolating virus 40 (SV40) was identified as an abundant contaminant in early poliovirus vaccines (3). Since this virus can cause tumors in animals, extensive research was conducted to determine if SV40 might have the same effect on humans (4), although no evidence of SV40 being oncogenic in humans was found to date (5).

In 1971, the first two polyomaviruses capable of infecting humans were identified. The BKPyV was isolated from a nephropathic kidney transplant patient's urine, and the JC polyomavirus (JCPyV) was isolated from the brain tissue of a patient with progressive multifocal leukoencephalopathy (PML). Both viruses were named after the patients from whom they were isolated (6).

The discovery of new human polyomaviruses (HPyVs) was only possible at the beginning of the XXI century due to significant technological advances in DNA sequencing, which enabled the identification of twelve new HPyVs (7). The study of these viruses is critical because, while the infection is usually asymptomatic, immunocompromised individuals can develop a specific pathology (8). The most relevant HPyVs in this situation is JCPyV, which is associated with PML, BKPyV, that can cause nephropathy and hemorrhagic cystitis, Merkel cell polyomavirus (MCPyV), which was isolated from a patient with Merkel cell carcinoma, and trichodysplasia spinulosa polyomavirus (TSPyV), that can lead to trichodysplasia spinulosa (1).

b. BKPyV viral particles

BKPyV virions share structural similarities with other polyomaviruses as such SV40 and MPyV (9). The capsid has a T=7d icosahedral structure composed of 360 copies of the major capsid protein, VP1, organized into 72 pentamers, on the outside, and two additional viral proteins, VP2 and VP3, on the inside of the capsid. (Fig. 1). Recent studies suggest that VP2 and VP3

could link VP1 (10,11) and the viral genome packed with cell histones (H2A, H2B, H3, and H4) (12).

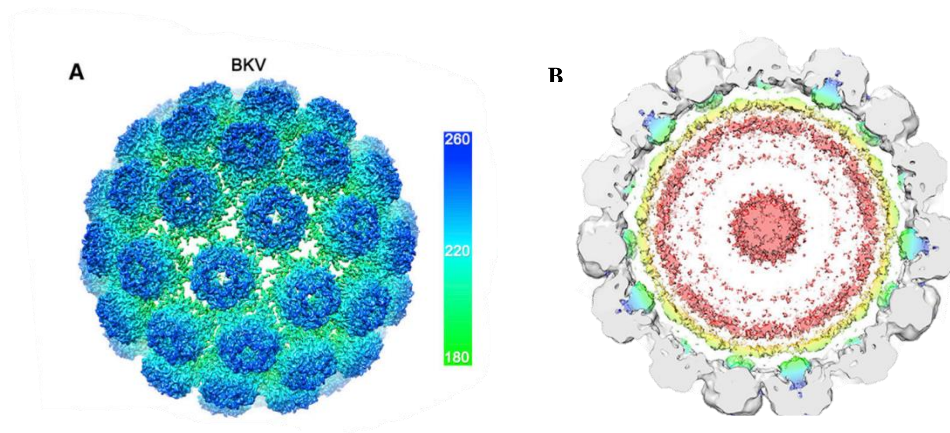

Figure 1. Cryo-electron microscopy structure of BKPyV virions. (A) Isosurface representation of the 3.8 A° structure of BKV viewed down the icosahedral 2-fold and colored according to the radial coloring scheme shown.(Adapted from Hurdiss, et al., 2018) (B) View of a 40-Å thick slab through the unsharpened/unmasked virion map shown at a contour level of 0.0034. Pyramidal density below each VP1 penton and two shells of electron density adjacent to the inner capsid layer can be seen. The density within 6 Å of the fitted coordinates for SV40 VP1 is colored gray. The remaining density is colored in a radial color scheme. Densities for VP2 and VP3 are colored blue and green, and for packaged dsDNA yellow and pink (Adapted from Hurdiss, et al., 2016).

c. BKPyV genome

BKPyV is a virus with 40-45nm in diameter of circular double-stranded DNA molecule comprising approximately 5000 bp genome. Its genome contains three regions: the early viral gene region (EVGR), the non-coding control region (NCCR), and the late viral gene region (LVGR).

After infection, EVGR undergoes primary transcription, which results in the production of three distinct proteins: the small tumor antigen (tAg), the large tumor antigen (TAg), and the truncated TAg (truncTAg), that are required to initiate viral replication (15).

The NCCR is split into five blocks: the O block, that contains the TATA-box, and the origin of replication (ori), from which the virus replicates bidirectionally and the P, Q, R, and S blocks, which include TATA-like elements and regulatory regions of EVGR and LVGR.

The LVGR, which is involved in the formation of the virion structure, is transcribed after genome replication has begun. This region encodes four proteins: VP1, VP2, VP3, and Agnoprotein (Agno), all of which are derived from the same pre-mRNA via alternative splicing

(12). Due to the non-coding transcription of pre-miRNA that is perfectly complementary to a portion 5p (16).

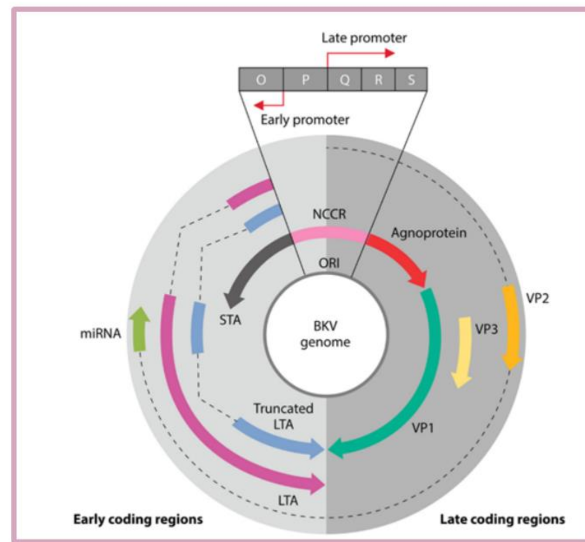

Figure 2. BKPyV genome. The transcription of both early and late viral gene regions proceeds in a bidirectional way from the origin of replication (ORI) within the non-coding control region (NCCR). The transcriptional splicing regions are represented by dashed lines. The late viral gene region encodes three structural proteins (VP1, VP2, and VP3) and the agnoprotein, while the early viral gene region transcribes the large tumor antigen (LTA), the small tumor antigen (STA) and the truncated TAg (truncated LTA). The expression of miRNA complementary to the 3' end of LTA has been shown to be involved in the replication control of BKV. (Adapted from Ambalathingal, et al., 2017).

d. BKPyV genotypes and variants

BKPyV isolates can be categorized into four main genotypes (I through IV), due to significant variations in the VP1 region. The most common in the human population is genotype I, dispersed throughout the world, followed by genotype IV, which is more prevalent in East Asia and Europe (17,18). Because genotypes II and III infect a small percentage of the population, they are rarely detected (10). Using serological and genotyping methods, genotype I is further subdivided into four subgroups Ia, Ib-1, Ib-2, and Ic, while genotype IV can be subdivided into six subgroups IVa-1, IVa-2, IVb-1, IVb-2, IVc-1, and IVc-2. Furthermore, BKPyV genotypes II, III, and IV and subgroups Ib-1 and Ib-2 functioned as five completely distinct serotypes (19).

Due to the high variability of the NCCR, BKPyV also presents two other forms: the archetype form and the rearranged form (20). The structure of this region in archetype forms is made up of five blocks: O, P, Q, R, and S, as mentioned in the previous point. The archetype variants

are assumed to be the transmissible form of the virus since they are found in immunocompetent and immunocompromised people, while the rearranged variants are only detected in patients with disease (21). The rearrangement variants result from duplicating or deleting certain blocks, except block O, which is required for viral replication. According to some research, the rearranged variants are generated during reactivation to allow the virus to adapt to changes in the host cell environment (17).

e. BKPyV life cycle

The replicative cycle of BKPyV consists of four major steps: the attachment of the virus to the host cell, the entry, the genome expression and replication and the assembly and release of new virions.

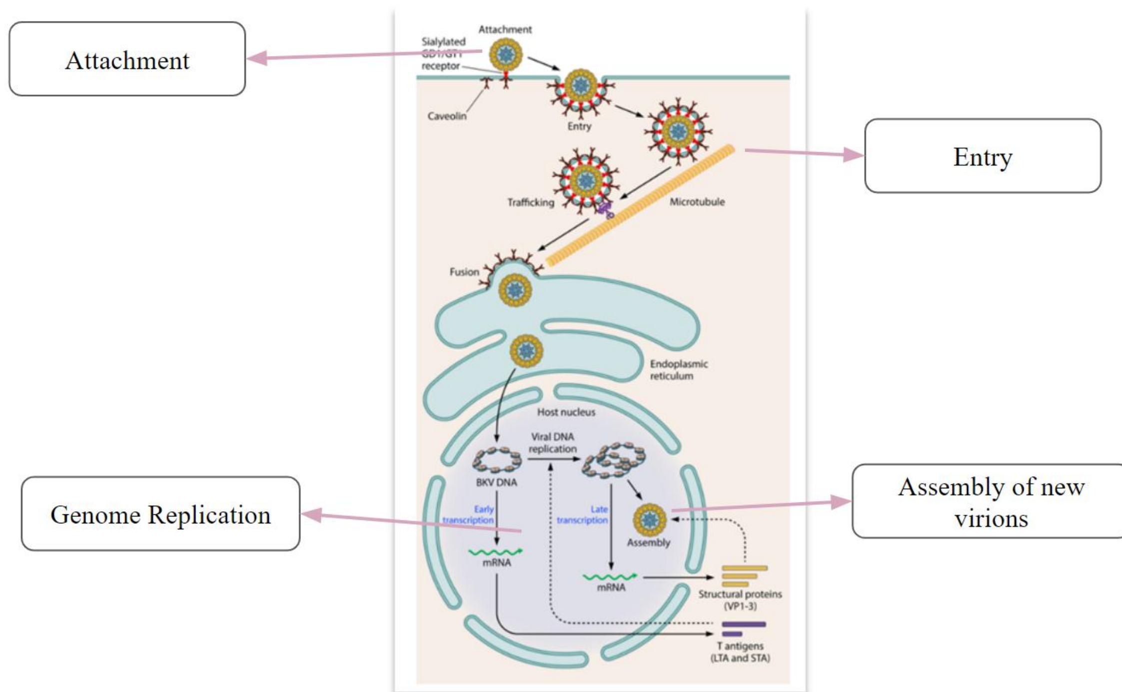

Figure 3. BKPyV life cycle. BKPyV initiates infection by binding to ganglioside receptors and entering host cells via caveolin-mediated endocytosis. BKPyV travels through endosomes along microtubules to the ER. The proteins found in the lumen of the ER are essential for capsid disassembly and exposure to the hydrophobic VP2 and VP3 proteins. The BKPyV enters the cytosol via the ERAD pathway after VP2 and VP3 insert themselves into the ER membrane. The NLS of VP2 and VP3 in the cytosol leads the viral particles into the nucleus via the importin α/β route. The early genes of BKPyV are transcribed in the nucleus and translated in the cytosol. Viral DNA is replicated, which is followed by the expression of late genes. Translocation of VP1, VP2, and VP3 into the nucleus, where they form capsomers around newly synthesized viral genomes. Finally, progeny virions are released from infect cells via a lytic way and/or via a non-lytic pathway.(Adapted from Ambalathingal, et al., 2017)

Attachment

As with the vast majority of circulating viruses, the life cycle of the BKPyV, as mentioned above, begins with the BKPyV attaching itself to the receptors on the host cells. Since the capsid of the BKPyV consists only of VP1 on the outside, it is these viral proteins that will connect to the cell receptors (11).

The primary receptors are gangliosides, which are glycosphingolipids composed of a ceramide section anchored in the lipid bilayer and an oligosaccharide chain containing one or more sialic acids in the terminal region that allows the BKPyV to bind to the host cell (22,23).

According to recent studies, the α2,8-disialic acid motif is required for BKPyV to bind to host cells. This motif is associated with the right arm of b-series gangliosides such as GD3, GD2, GD1b, and GT1b (11,12). Is has also been proposed, following a study involving enzymatic removal of α(2,3)-linked sialic acid from Vero cells and inhibition of N-linked glycosylation that an N-linked glycoprotein containing α(2,3)-linked sialic acid is a potential receptor for BKPyV infection (24).

Entry of the virus

After connecting to receptors, the viral particles are internalized via caveolae-mediated endocytosis (25). Caveolae are plasma membrane invaginations that are rich in cholesterol and sphingolipids and are coated with a protein called caveolin (26).

Based on the structure of caveolae, studies have been conducted in order to better understand the endocytosis mechanism used by BKPyV. While some research have indicated that caveolae mediate the mechanism in both Vero (27) and RPTE cells (28), a more recent study has revealed that the virus may be internalized in RPTE cells without the help of caveolin (29).

BKPyV enters the cell and is carried to the endoplasmic reticulum (ER) by an intact network of microtubules (30). It is critical that acidification and maturation of the endosomes occur during this transport in order to ensure infection (11).

Once within the ER, the BKPyV exploits proteins found there, such as chaperones, disulfide isomerases and reductases, to initiate the process of capsid disassembly via conformational changes in the VP1, exposing the hydrophobic VP2 and VP3 proteins (11,31). Once exposed, the will embed themselves into the ER membrane by passing through a misfolded protein, allowing them to enter the cytosol via ER-associated degradation (ERAD) pathway (32).

The viral particles must be delivered to the nucleus before the replication process can begin. Nuclear localization sequences (NLS) of VP2 and VP3 interact with importins α and β_1 at the C-terminus of VP2 and VP3 to convey the viral genome into the nucleus via the nuclear pore complex (33).

Genome replication

TAg is the primary protein in charge of starting viral DNA replication. So, when the viral genome enters the nucleus, the early TAg and tAg genes are transcribed first, then translated in the cytosol and brought back to the nucleus. TAg is also responsible for activating the DNA synthesis machinery through its binding to the tumor suppressor proteins pRb and p53, as well as preventing apoptosis (25).

The binding of TAg to ORI in the NCCR initiates replication by generating two hexamers with an ATP-dependent helicase that unwinds the DNA bidirectionally. The complex formed by TAg and DNA will subsequently recruit the rest of the necessary machinery for viral genome replication (34).

Once viral replication has begun, LVGR is encoded and the structural proteins essential for assembling virions are produced.

Virion assembly and viral progeny release

After being imported into the nucleus, the structural proteins form capsomeres around newly synthesized viral genomes (25). Two days post-infection, new viral particles begin to concentrate in the nucleus, but the knowledge about their release is still very limited. It is thought that the release of these particles may occur in a lytic and/or non-lytic manner, after a study showed that a small percentage of BKPyV progeny exit via this route (35,36).

f. Epidemiology and Transmission

According to different serological studies on diverse populations throughout the world, BKPyV infection appears to be ubiquitous. These studies show that over 80% of the adult population carries antibodies against BKPyV. Furthermore, they indicate that the primary infection occurs mainly during childhood since the seroprevalence was around 75% between the ages of 5 and 10 (37).

These studies also reveal that there is no difference in antibody levels against the BKPyV between males and females, and that seroprevalence for the BKPyV decreases after the age of 50, indicating that significant re-infection does not occur in immunocompetent individuals (38).

The route of transmission of the BKPyV is still mostly unknown. BKPyV DNA has been found in tonsil tissue of infants with respiratory disease, suggesting that transmission most likely occurs through the respiratory tract (39). Several different transmission routes, including as the fecal-oral route, blood transfusion, organ transplants, and vertical transmission, have been proposed over the years (40,41).

g. Pathogenicity of BK Polyomavirus

Primary infection is typically asymptomatic or associated with subclinical symptoms. Following infection, the BKPyV establishes a persistent infection in the renal tubular epithelial cells and the urinary tract transitional epithelial cells (17). Throughout its life, the BKPyV replicates discreetly, alternating with episodes of intense replication. In immunocompetent individuals, this persistent replication has no manifestations, while in immunocompromised individuals it can lead to nephropathy.

The majority of BKPyV reactivation occurs in immunocompromised patients, such as those undergoing kidney transplantation or hematopoietic stem cell transplants, or those with HIV, lupus erythematosus, or other pathologies that affect the immune system (42). In these cases, the excretion of BKPyV in the urine increases to values above 7log10 c/mL, resulting in the presence of decoy cells in the urine of these patients.

Hemorrhagic cystitis and BK virus-associated nephropathy (BKVAN) are the most common consequences associated with this reactivation in patients who have had a kidney transplant or hematopoietic stem cell transplant. In conditions when the immune system is severely compromised, this reactivation can manifest as interstitial pneumonitis, retinitis, renal nephritis, and meningoencephalitis (43).

Unlike in immunocompromised people, reactivation in healthy people not only has received less attention but also the BKPyV's behavior appears to be unclear in this group. Egli et al. collected 400 samples from healthy blood donors in order to assess the behavior of both the BK and JC viruses in these people. According to this study, approximately 7% of healthy people excrete the BKPyV through their urine, with a viral load of less than 5log10 copies/mL. This finding indicates that reactivation happens asymptomatically in this population (38).

h. BK polyomavirus – associated nephropathy

The fundamental goal after a kidney transplant is to remove the chance of organ rejection. As a result, it is critical, according to international guidelines, to use immunosuppressive agents to achieve this purpose. Since the risk of rejection is higher during the first three months, the dosages will be higher during this time, then decrease to maintenance doses when the risk of rejection is reduced (44).

Immunosuppressive therapy, on the other hand, has been associated with an increased risk of BKPyV reactivation in these individuals. The first evidence of BKPyV reactivation is viruria, which can develop in up to 50% of cases and progress to viremia in 10 to 30% of patients. During the first year following kidney transplantation, 1 to 10% of patients may develop BKVAN, after present viremia. BKVAN can cause tubular atrophy, interstitial fibrosis, and function loss, which can ultimately result in graft loss (17,25,45).

Although immunosuppressive therapy is the major risk factor for BKPyV reactivation, several other risk factors that potentially augment the likelihood of BKPyV reactivation have been found. These risk factors can be classified into three categories: donor-associated risk factors, recipient-associated risk factors, and transplant-associated risk factors.

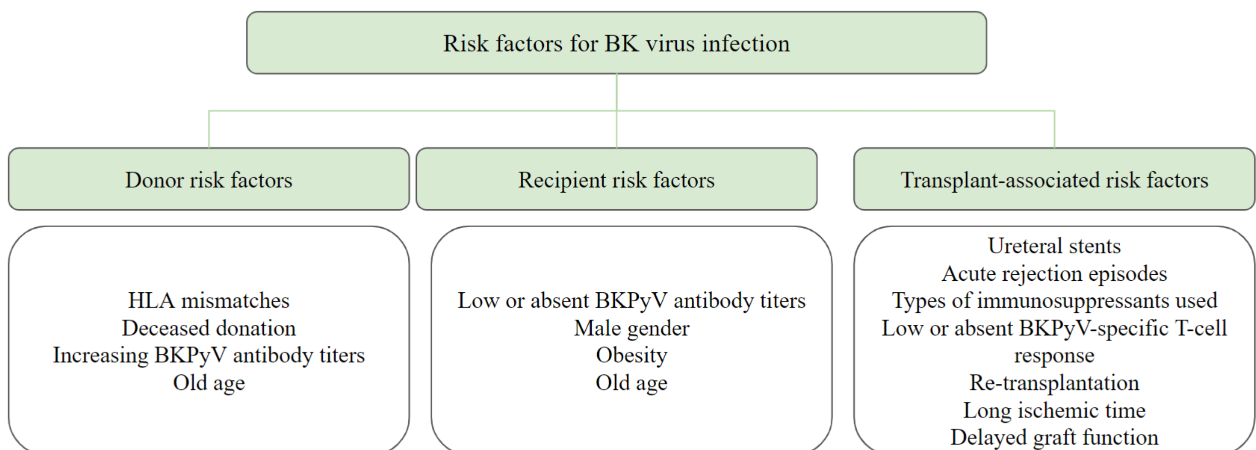

Figure 4. Risk factors for BK polyomavirus-associated nephropathy in kidney transplant recipients. Each of these risk factors can directly affect or indirectly increase the risk of BKVAN (Adapted from Suwelack, et al., 2012; Hirsch et al., 2013; Sawinski et al., 2015)

Early screening and diagnosis

BKVAN causes no specific clinical signs or symptoms. Currently, no effective antiviral therapies against BKPyV are available on the market. Hence, the strategy found rests on early detection and subsequent therapy for/of BKVAN. This involves prior monitoring of viral DNA detection and quantification through quantitative techniques based on polymerase chain reaction (PCR) (44,49).

According to The Kidney Disease Improving Global Outcomes Clinical Practice and The American Society of Transplantation guidelines, plasma viral load quantification should be carried out monthly for the first 6 months and then every 3 months for 2 years (44,49).

Although guidelines recommend testing for the BKPyV in plasma, urine testing is also a possibility. In this situation, screening can be done using urine cytology to detect decoy cells or by quantifying the viral load above 7log10 c/mL in the urine, the virus should be tested for in the plasma (44,49).

The findings of screening the virus in the patient's plasma can indicate whether it is likely to evolve into BKVAN when the values are between 3log10 c/mL and 4log10 c/mL, or whether it is presumed to evolve into BKVAN when values above 4log10 c/mL are obtained. Although a value of viral DNA in plasma above 4log10 c/mL suggests BKVAN, a kidney biopsy is required to confirm the diagnosis (49).

Therapeutic approaches

As previously mentioned, there is currently no effective antiviral treatment against the BKPyV. Therefore, in order to combat the replication of BKPyV and/or BKVAN, the strategy found is to reduce/modify/interrupt immunosuppressive treatment. This method seeks to reestablish the immune response in order to manage the infection. The guideline, however, do not specify the essential therapy adjustments (47).

To minimize the possibility of acute rejection owing to immunosuppressive reduction, these modifications should be implemented gradually while closely monitoring viral load levels in the plasma. This monitoring is critical since, in certain cases, viremia values remain altered for lengthy periods of time with no major consequences for the patient, and there is no need to reduce immunosuppression further (16).

Despite this approach presenting some risks, multiple studies present results which indicate that infection control is achieved in more than 85% of cases (50–52) .

In addition to this strategy, the off-label use of medications with potential activity against the BKPyV, such as cidofovir, fluoroquinolones, and intravenous immunoglobulins, has been described. However, there is still no solid evidence of their effectiveness (53,54).

More recently, cellular immunotherapy has been gaining prominence as a viable hypothesis for treating complications associated with BKPyV. This hypothesis has already been studied as a possible treatment for complications associated with other viruses, such as Epstein-Barr virus (55) and cytomegalovirus (56), where it proved to be effective.

Some studies have already been carried out in this area in relation to BKPyV. Blyth et al. reported the *in vitro* expansion of BKPyV-reactive T cells with mixtures of overlapping peptides from the five BKPyV proteins: VP1, VP2, VP3, TAg, and tAg. With this method, they demonstrated that immune reconstitution is possible after transplantation, satisfying the need for effective therapy against BKPyV-associated complications (57).

One case report showed very positive results after the adoptive transfer of BKPyV-reactive T cells to a patient who developed severe hemorrhagic cystitis after a hematopoietic stem cell transplant (58).

These results lead us to believe that this approach may have great potential for treating complications associated with BKPyV.

i. *In vitro* and *in vivo* models systems for BKPyV

In recent years, the BKPyV has gained prominence due to its association with infections that can arise mostly in kidney and hematopoietic stem cell transplant patients. Despite being identified in 1971, there is little specific knowledge about the BKPyV and much of the existing information has been inferred from another virus in the same family, SV40.

As a result, careful selection of *in vitro* and/or *in vivo* models is required to better understand the structure, transmission, replicative cycle, and pathogenicity as well as discovery of drugs with potent antiviral activity against BKPyV.

Vero cells are the most widely utilized cells in basic research into the BKPyV. This cell line from the kidney of an African green monkey was used to isolate BKPyV for the first time and is therefore widely used to replicate it (6).

RPTE cells are the most suitable culture system because they most closely resemble the BKPyV's natural target. These cells, however, have multiple limitations. In addition to being difficult to manage, RPTE cells experience replicative senescence quickly, which limits the number of passages of these cells. To address this limitation, the RPTEC/TERT1 cell line has been developed, which involves immortalizing RPTE cells with human telomerase reverse transcriptase (hTERT), preventing the cells from entering replicative senescence (59,60).

Within the renal cells lines, human embryonic kidney 293 cells (HEK293) and CV-1 cells are also used as *in vitro* models. In addition to kidney cells, BKPyV replication has been reported in human salivary gland cells and human embryonic lung fibroblast cells (MRC-5) (61–64).

As previously mentioned, the BKPyV has two forms: the archetype form and the rearranged form. The rearranged forms are able to replicate in the *in vitro* models described above. However, due to limited TAg production, the archetype forms do not replicate well in these models. SV40 TAg was introduced into HEK293 and CV-1 cells to achieve efficient replication of these forms, yielding two new cell lines, 293TT and COS-7 (65).

Whatever model you choose to use, will always have its limitations. The preceding models have limitations in terms of time and the quantity of new viral load created (66). As a result, alternative models have been developed that can be employed when studying viral structure or interactions with cell surface receptors on host cells, for example.

These models use virus-like particles (VLPs). These are capsid-like particles that spontaneously self-assemble after overexpression of the major capsid protein, VP1, but are devoid of genetic material. VLPs can be produced in a variety of systems, including insect cells, mammalian cells, yeast, and Escherichia coli. The advantage of these models is that they can be co-transfected with vectors encoding a reporter gene, allowing the particles that enter the target cell to be quantified (67).

While *in vitro* models are important for obtaining knowledge about the structure and replicative cycle of the virus, *in vivo* models are required not just to better understand the pathogenicity of the BKPyV, but also to test potential new drugs. As far as the BKPyV is concerned, there are no such models, since BKPyV is strictly human. It is therefore essential to develop an *in vitro* model that can portray the pathology *in vivo* (63).

In order to identify an effective treatment for the majority of patients, it is necessary to carry out pre-clinical studies against entire genotypes, as has been done for HCV. In other words, it is important that the efficacy of the drug is tested against the most varied strains in order to

have a more global view of its performance (68). However, the strain most often used in these studies is the Dunlop strain, which represents a small proportion of the viruses circulating worldwide, making it difficult to verify the total effectiveness of the treatment (69). To date, few studies have described ways of isolating clinical strains of BKPyV, so finding an *in vitro* model capable of isolating and replicating the most varied strains is essential.

2. Aim

In individuals who have had kidney transplants, BKPyV infection can lead to up to 10% cases of nephropathy and consequently graft loss. To date, there is no preventive or curative therapy against this viral infection. Many studies have been undertaken in recent years in an attempt to develop an efficient antiviral agent to block the replication of BKPyV. However, the majority of these studies use the Dunlop strain, which, while extremely successful in different assays, represents just a small portion of the viruses circulating worldwide. This makes it unclear whether the potential drugs discovered will be effective in the general population.

With this in mind, the Agents Infectieux, Résistance et Chimiothérapie (AGIR) laboratory has devised a study to isolate various genotypes of the BKPyV for use in antiviral research. This project is divided into three phases. Phase 2, in which I was involved, has the following objectives:

- ➔ Adapt one of the previously isolated strains to an *in vitro* model, using Vero cells and MRC-5 cells instead of RPTEC cells which are less suitable for long-term culture due to the time and cost involved.
- ➔ Amplify the infection through long-term culture
- ➔ Evaluate the efficacy of the produced stocks in terms of their infectivity
- ➔ Compare the efficacy of this method to others already developed.

3. Materials and Methods

a. Cell Culture

Vero and the MRC-5 cells were obtained from the ATCC (ATCC-CCL-81 and ATCC-CCL-171, respectively). Both cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen). RPTEC cells (Evercyte, CHT-003-0002) were maintained in renal epithelial growth medium (REGM CC-3191, Lonza, Bale, Switzerland), and supplemented with 0.1% human epidermal growth factor, 0.1% hydrocortisone, 0.1% epinephrine, 0.1% insulin, 0.1% triiodothyronine, 0.1% transferrin, 0.1% GA-100, and 0.5% FBS. All cells were grown at 37°C in a humidified environment with 5% CO2. All the cells were passed when cell confluence was above 80%, the Vero cells were passed twice a week and MRC-5 cells and RPTEC cells were passed once a week.

b. Immunostaining

Cells were washed with phosphate-buffered saline (PBS) sterile, fixed with 7.4% paraformaldehyde (PFA) in PBS for 10 minutes, and permeabilized with 0.5% Triton X-100 in filtered CKS (cytoskeletal buffer, 10 mM PIPES (piperazine-N-N'-bis(2-etanesulfic acid)), 300mM sucrose, 3mM MgCl2, 100mM NaCl and 1mM EDTA) and H20 (1:1) for 15 minutes, both at room temperature.

Then, fixed cells were incubated with the primary antibody (mouse monoclonal anti-SV40 Tag-Pab416) for 1 hour at room temperature, and the secondary antibody (Thermofisher Alexa Plus 488-conjugated) for 30 minutes at room temperature, in the dark. The nuclei of the cells were stained with DAPI (4', 6-dia-midino-2-phenylindole). Both DAPI and the two antibodies were diluted in PBS/Tween 0.1%.

Immunostained cells were observed on an Axio vert.A1 microscope equipped with Colibri 7 light source (Zeiss, Germany). To determine the percentage of infected cells, two random pictures were taken per well and DAPI and TAg stained nuclei were automatically counted using the QuantIF macro.

c. Infectivity of 216x2X strain

Two 24-well plates were prepared, one for each cell line type: Vero cells, and MRC-5 cells. In each plate, three wells were used, one with non-infected cells (negative control), another with cells plus 50µL of Dunlop (positive control), and other with cells plus 50µL 216x 2X strain.

At 7 days post-infection (dpi), cells culture's supernatants from infected cells and non-infected cells were harvested and stored at 4°C until amplification by qPCR. On the same day, cells were washed, fixed, and immunostained as described above.

d. Quantitative real-time PCR (qPCR)

Cell culture supernatants of infected and non-infected cells were used to amplify the BKPyV DNA. Custom primers and probes were obtained from Invitrogen. A BKPyV-negative control (H20) was added to each run. Quantities (number of copies of DNA per mL) were obtained by comparison to a standard calibration curve. Reactions were performed in a 25 µL volume containing 20 µL of amplification premix and 5 µL of standard or sample, using the following amplification profile: 1 cycle at 95ºC for 10 min, and 45 two-step cycles at 95ºC for 15s and 60ºC for 1 min on 7900HT thermocycler (Applied Biosystems).

e. BKPyV stock production

MRC-5 cells and Vero cells were incubated into a T25 flask with 125 µL of 216x 2X strain for approximately 4 weeks. At 3, 7, 10, 16, 21, 24, and 28 dpi, Vero cells were washed with PBS, detached with Trypsin-EDTA solution (Sigma Aldrich), and cultured in DMEM. On the same days, cell culture's supernatants of infected cells and non-infected cells were harvested and stored at 4ºC until amplification by qPCR.

The medium in the T25 flask containing MRC-5 cells was replaced with a new medium at 5 and 15 dpi. The same protocol as previously described for Vero cells was used at 9, 12, 19, and 23 dpi.

The infected cells were then moved to a T75 flask for a week, before being transferred into three T75 flasks and kept until further cytopathic effects were noticed.

f. Extraction and purification of viral particles

BKPyV stocks were produced in the way indicated above. One week, after the last passage, the supernatants were harvested and the infected cells were scraped. The resulting supernatant was used to wash the cells, ensuring that no cells were lost in the process. All supernatants and infected cells were stored in a 50mL tube with 5mL of chloroform (1:10). Next, the tube was vortex for 5 minutes and then centrifuged for 5 minutes at 1000 rpm. Several phases may be seen in the tube, but the phase that was kept was the upper phase (aqueous).

g. BKV infection of RPTEC

In a 96-well plate, 15.000 cells were cultured in each well with 150 µL of REGM and grown at 37ºC in a humidified environment with 5% CO_2. Three days later, the cells were infected with cell culture supernatants, previously stored, plus Dunlop and 216x 2X strain. 24h after the infection, the supernatants were removed and supplied with appropriate cell growth media, REGM. At 6 dpi, the cells were washed, fixed, and immunostained.

h. Virus stock titration

BKPyV stock titration was performed in RPTEC cells. Cells were plated and infected with viruses in 10-fold serial dilutions ranging from 10^{-1} to 10^{-8}. After 24h of viral adsorption incubation, the supernatants were removed and supplied with appropriate cell growth media (REGM). Cells were washed, fixed, and immunostained at 7 dpi. The titer of the stocks was calculated using Spearman & Kärber algorithm and expressed in 50% tissue culture infectious dose (TCID50)/mL.

All of the procedures described above were carried out in accordance with good laboratory practice. Except for qPCR, which was performed in a level I laboratory, the remaining operations were performed in a level II laboratory using a vertical laminar flow chamber. During the numerous processes, personal protective equipment such as gloves, gowns, and disposable gowns were employed to protect the operators.

4. Results

a. Infectivity of 216x2X strain

In order to test the 216x 2X strain's infectivity in different types of cells, two 24-well plates were prepared, one for each cell line type: Vero cells and MRC-5 cells. In each plate, three wells were used, one with non-infected cells (negative control), another with cells plus 50µL of Dunlop (positive control), and other with cells plus 50µL 216x 2X strain.

The cells were kept for a week, and at 7 dpi, cell culture's supernatants from infected and non-infected cells were harvested and stored at 4ºC until amplification by qPCR. On the same day, cells were washed, fixed, and immunostained.

The qPCR amplification results show that there was an infection in both cell lines since BKPyV DNA was amplified, which is in contrast to what happened in our negative control group, as shown in the results below.

When comparing these results to the images obtained with an Axio vert.A1 microscope equipped with Colibri 7 light source, we may conclude that despite the presence of BKPyV DNA, there is not much production of new virions, since the TAg was barely detected, on the wells that the cells were infected with 216x2X strain.

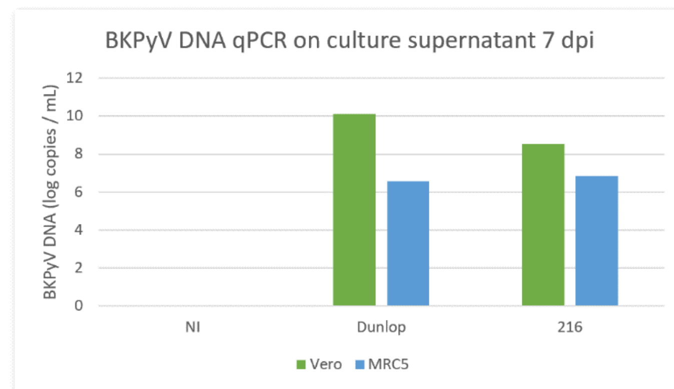

Figure 5. Results of qPCR amplification. MRC-5 cells and Vero cells were infected with Dunlop and 216x 2x strain. Supernatants were harvested at 7 dpi and BKPyV DNA was measured by qPCR. The results of qPCR amplification show that we have an infection when we infect both cell lines with Dunlop and the 216x 2X strain. The results of non-infected cell samples assure us that the cells were not previously infected.

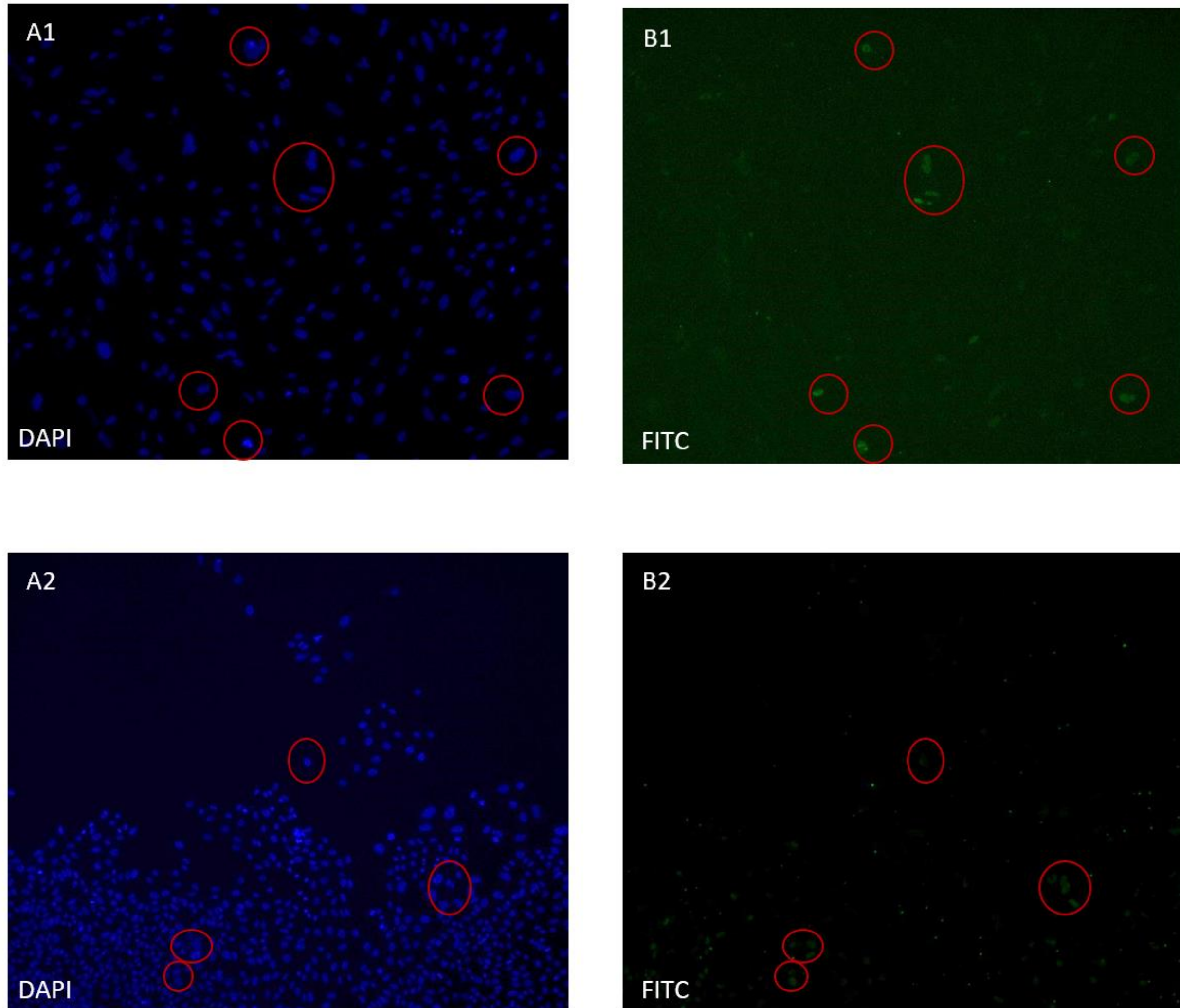

Figure 6. Immunofluorescence of MRC-5 cells and Vero cells infected with 216x2X strain MRC-5 cells (A1 and B1) and Vero cells (A2 and B2) infected with 50 µL of 216x2X strain were fixed at 7 dpi and immunostained for TAg (B1 and B2). Cell nuclei were stained with DAPI (A1 and A2). In (A1 and A2), all the nuclei of the cells present in the well are revealed (infected and non-infected cells). In (B1 and B2), only the nuclei of the infected cells are shown. The nuclei of the infected cells are marked in red.

b. Amplification

Considering the prior results, the next step was to try to amplify the number of infective viral particles for a longer time. For this, MRC-5 cells and Vero cells were cultured with 216x 2X strain for one month and the viral particles were extracted and purify as described in the methods.

Some assays were carried out to determine whether the objective had been achieved. Initially, qPCR amplification of BKPyV DNA was employed to confirm that the cells were infected. Following that, cell culture supernatants were used to infect RPTEC cells to find out if the

stocks produced could infect cells. Finally, two titer assays were performed to determine the concentration of viral particles in each BKPyV stock.

i. qPCR

As previously mentioned, supernatants from the cell cultures were harvested and stored to be used to confirm that both MRC-5 cells and Vero cells were productively infected with the 216x 2X strain. The supernatants corresponding to 3, 7, 10, 16, 21, and 24 dpi for Vero cells and 5, 9, 12, 15, 19, and 23 dpi for MRC-5 cells were used for this purpose. On the same days, supernatants were harvested from non-infected cell cultures to serve as a negative control group.

The qPCR amplification results reveal that there was a productive infection in both cell lines since BKPyV DNA was detected at high copy number levels. When comparing the two graphs, we may observe that in the MRC-5 cells more DNA copies were amplified. In these graphs, we can also see that there was a decline in the last days of culture, which leads us to think that maybe we can keep the cells for 3 weeks instead of 1 month.

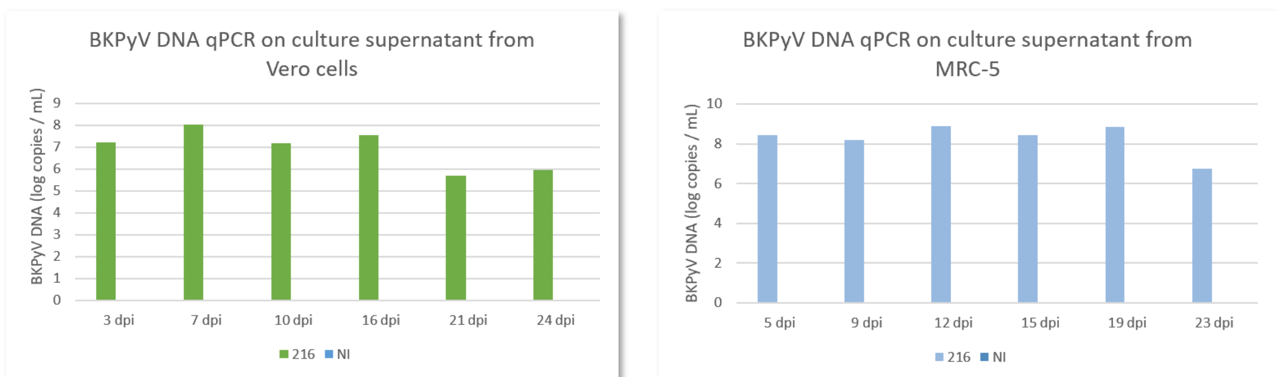

Figure 7. Results of qPCR amplification. MRC-5 cells and Vero cells were infected with 125µL of 216x 2x strain. Supernatants that were harvested at 3, 7, 10, 16, 21, and 24 dpi corresponding to Vero cells and the supernatants at 5, 9, 12, 15, 19, and 23 dpi belonging to MRC-5 cells were used to measure the BKPyV DNA by qPCR.

ii. BKPyV infection of RPTEC

To test the infectivity of the viral stocks produced in Vero and MRC-5 cells, we infected RPTEC cells with the supernatants previously obtained from each cell culture. In addition to using supernatants corresponding to 3, 10, and 21 dpi from Vero cells and supernatants corresponding to 5, 12, and 19 dpi from MRC-5 cells, we also used supernatants harvested on

the same days from non-infected cell cultures, the 216x 2X strain stock and Dunlop genome. The samples were distributed in the 96-well plate as schematically shown in Figure X. Some samples were replicated with different amounts to see if this can influence the infectivity values of the stocks. As a negative control, two wells were left without adding any sample to ensure that RPTEC cells had not previously been infected. Because these are human renal epithelial cells, they may contain the BKPyV in initially undetectable amounts, but the virus can reactivate and infect the cells after manipulation and subsequent passages.

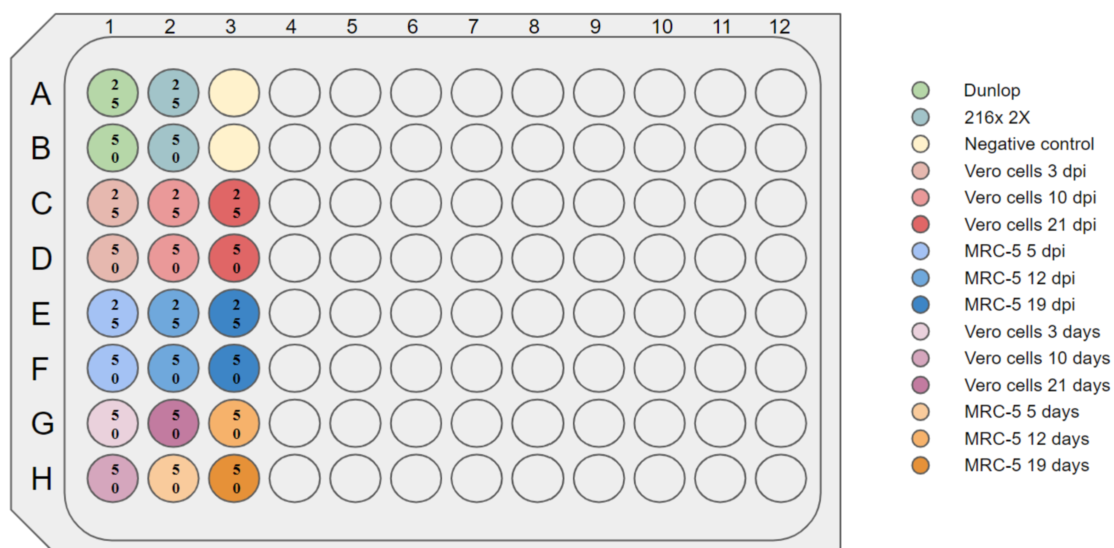

Figure 8. BKPyV infection of RPTEC. RPTEC cells were infected with supernatants corresponding to 3, 10, and 21 dpi from Vero cells and supernatants corresponding to 5, 12, and 19 dpi from MRC-5 cells. The infection with Dunlop was used as positive control and the two wells that were left without any sample were used as a negative control. RPTEC cells were infected with 216x 2X strain to be compared with the other wells. The supernatants from non-infected cell cultures were used as control group Some of the samples were replicated with different amounts, to test if this could influence the infectivity values of the stocks.

One week post-infection, the cells were washed, fixed, and immunostained. Then images were taken to see if the RPTEC cells were infected. The results reveal that only the supernatants belonging to MRC-5 cells were capable to infect the cells. When different wells were compared, we can see that there was a minor rise in RPTEC cells infection with increasing days post-infection of MRC-5 cells, as well as a higher increase in the number of infected cells when originally infected with 50µL of supernatant. However, despite the infection, as seen previously, there was not a high percentage of infected cells in each well when compared to the total number of inoculated cells.

iii. Titration of the BKPyV stocks

The concentration of viral particles in each stock must be determined since these are created with the goal of being used in future investigations.

For each assay, RPTEC cells were infected with the stock in 10-fold serial dilutions ranging from 10^{-1} to 10^{-8}. A week after infection, the cells were washed, fixed, and immunostained before being examined under a microscope.

When we examined the stock produced from Vero cells, we concluded that there was no infected cells in the RPTEC cultures, making it impossible to quantify the concentration of virus particles. This conclusion was possible to be drawn, since in one of the columns, the RPTEC cells were infected with Dunlop in the same conditions, and was possible to observe infection until 10^{-6}.

With the stock produced from MRC-5 cells, four wells were positive. So using the titer calculator by Marco Binder, the titer of this stock was 10^2 viral particles/mL.

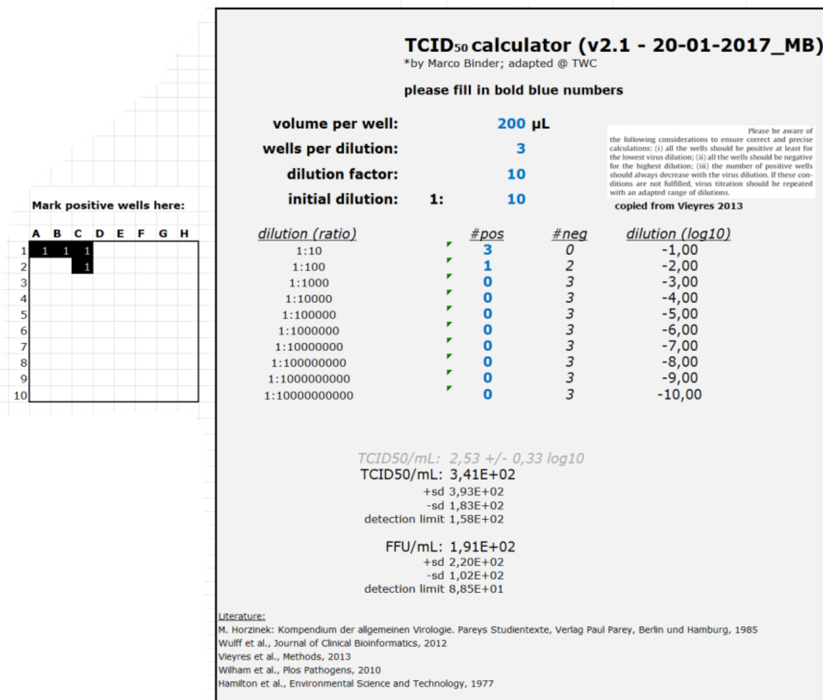

Figure 9. Calculation of titer of stock produced from MRC-5 cells. The titer of stock from MRC-5 cells was calculated using a TCID50 calculator by Marco Binder. This value is calculated using the Spearman & Kärber algorithm.

5. Discussion

The project developed by Agents Infectieux, Résistance et Chimiothérapie (AGIR) laboratory is divided into three phases. The first phase of the project involved isolating different strains of BKPyV from patient's samples to be used in studies for the development of potential antiviral agents to block replication of the BKPyV. As a result of this initial phase, a stock of 216x2X strain with initial volume of 3mL and a concentration of 10^2 viral particles per mL was produced.

The current study was designed with the goal of developing an *in vitro* model capable of amplifying the number of infective viral particles of 216x2X strain for use in a variety of studies.

The first stage of this study was to test the 216x2X strain's infectivity in selected cells in order to adapt this clinical strain to an *in vitro* model. Vero cells are renal epithelial cells extracted from African green monkey and were the first cells used to isolate BKPyV. These cells are currently widely used in research to replicate the virus. MRC-5 cells are human fetal lung fibroblast cells that have been demonstrated in multiple studies that they are permissive to BKPyV. For these reasons, these two types of cells were selected to try to adapt this clinical strain to an *in vitro* model.

The results of assay to test the 216x2X strain's infectivity reveal that this clinical strain was able to infect both types of cells. However, because no significant number of new virions were produced at 7 dpi, the next step in this study was to extend the infection time in both cell lines in an attempt to increase the quantity of infective viral particles.

For one month, the two cell lines infected with 125 µL of the clinical strain were maintained and then evaluated in order to see if the main goal was attained. According to the results obtained, we can conclude that, while the Vero cells were permissible to the 216x2X strain, they were unable to produce new virions and thus did not prove to be effective in producing a stock that could be used in the future.

The results of the stock made from MRC-5 cells reveal that, in addition to being permissible for the 216x2X strain, these cells can also produce new virions. Despite the fact that we were unable to amplify the concentration of viral particles per mL in comparison to the initial stock, we were able to develop a stock of 216x2X strain with the same concentration but a higher volume, using these cells.

However, one of the limitations of this model is the low titer value. The titer value is influenced by several factors, such as the growth conditions of the host cells, the extraction, and purification of the virus, the storage of the viral particles, as well as the way in which the titer is determined.

So, in order to try to amplify the titer, we could repeat the process by optimizing the factors that influence this value, as well as increasing the initial production, i.e. using larger quantities of host cells.

The variation in outcomes observed for each cell type may be connected to the ability of each cell line to reproduce. MRC-5 cells require nearly a week to reach confluence, as opposed to Vero cells, which take 3 a 4 days. This difference may have been sufficient for the MRC-5 to produce new virions, unlike the Vero cells, since there was more time for the virus to replicate.

Overall, our results show that 216x2X strain can be adapted to an *in vitro* model using MRC-5 cells, and that when compared to the initial model using RPTEC cells, which is more difficult to manipulate and more expensive, this model is more effective because it is possible to produce a stock with the same concentration but in a larger final volume and at a lower cost.

6. Conclusion

The BKPyV infects around 80% of the world's population, subsequently establishing a latent infection, mainly in renal tubular epithelial cells. Although reactivation of the BKPyV has no consequences for the majority of the human population, for immunocompromised individuals, this reactivation can lead to serious consequences, such as BKVAN in kidney transplant recipients. The unavailability of effective antiviral drugs against the BK virus means that the decision taken to try to combat the progression and eventual loss of the organ is to reduce immunosuppressive therapy.

While on the one hand, this reduction allows the immune system to recover and fight the infection, on the other hand, too much immunosuppression can lead to episodes of acute rejection.

Over the last few years, several studies have been carried out in an attempt to find an effective drug against BKPyV. However, the lack of information on various processes related to the virus can hinder this research. The lack of *in vitro* and *in vivo* models capable of meeting the needs of these studies is also one of the major limitations to this phenomenon.

Because it is highly replicated, the strain most often used in studies on BKPyV is Dunlop. This is another problem added to the attempt to find an effective therapy against BKPyV, as it is a strain that is rarely present in the human population.

During the first part of this project, techniques were developed to isolate strains from the urine of patients in whom the BKPyV has reactivated, so that they can begin to replace the use of Dunlop in research into new drugs. Although this switch seems minor, it allows us to be more certain that if an effective drug is found, it will probably be more effective in the general population.

The aim of this project was to try to replicate one of the previously isolated strains in order to find an *in vitro* model that was more efficient in terms of time and cost. Quantifying the number of viral particles per mL was one of the ways used to assess whether this new model was more effective. This quantification is also important since the new viral particles will be used in future studies associated with research into antiviral drugs that are effective against BKPyV.

According to the results obtained, MRC-5 cells are a potential *in vitro* model to be used for this purpose. Although we were unable to increase the number of viral particles per mL, we were able to obtain the same number of viral particles per mL in a larger volume (45mL) when

compared to the initial volume of 3 mL. This model also has the advantages of being easier to handle and cheaper to use when compared to RPTE cells. Although this model has shown positive results, the search for an *in vitro* model that can obtain better results is extremely important.

One of the major limitations of *in vitro* models is their inability to produce large quantities of new viral particles. Finding a model that is capable of doing this would not only help in the discovery of new drugs, but also in studies on the virus, particularly on the replicative cycle of the BK virus, where there is a predominant lack of information.

It would also be interesting to test the ability of this model to produce results similar to or better than those obtained in this project with the other strains isolated previously.

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