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Development and evaluation of real-time PCR assays for the detection of the newly identified KI and WU polyomaviruses

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Abstract

Background: Recently, novel human polyomaviruses, KI (KIV) and WU (WUV) were described. Their role in human disease has not yet been determined.
Objectives: The aim of this study was to develop sensitive and specific assays for the detection of KIV and WUV.
Study: Two KIV (KI-A and KI-B) and three WUV (WU-A, WU-B and WU-C) real-time polymerase chain reaction (rtPCR) assays were developed and evaluated. Clinical sensitivities and specificities were determined by testing 200 respiratory specimens and the results compared to those for previously described conventional PCR assays. Limits of detection were determined, and the analytical specificities of the assays were investigated.
Results: No cross-reactivity was observed between the rtPCR methods and unrelated organisms. All five rtPCR assays could reliably detect 10 copies of genomic DNA equivalents per reaction, which was more sensitive than conventional methods. Compared to the conventional PCR assays, the sensitivity of the KI-A, KI-B, WU-A, WU-B and WU-C assays was 100%, 86.7% 95.5%, 100% and 100%, respectively. Specificity was 94.6%, 97.3%, 96.6%, 97.7% and 97.2%, respectively.
Conclusions: The KI-A, WU-B and WU-C assays provide the most sensitive detection of KIV and WUV in clinical specimens and may be used for further research into these viruses.

Keywords: Human polyomavirus; Real-time PCR assay; KI polyomavirus; WU polyomavirus

1. Introduction

The non-enveloped small double-stranded circular DNA viruses of the *Polyomaviridae* are known to infect a wide variety of birds and mammals. Until recently, only two, JC virus (JCV) and BK virus (BKV), were known to commonly infect humans. Both are ubiquitous in the human population (Knowles, 2006), establish persistent infections in the kidneys and in other tissue sites without overt disease (Randhawa et al., 2006), and may be detected in the urine of healthy adults and occasionally in the feces of children (Vanchiere et al., 2005; Knowles, 2006). Both JCV and BKV may cause human disease, particularly in immunocompromised patients. JCV is the causative agent of the neurological disease progressive multifocal leukoencephalopathy (PML), which occurs primarily in AIDS affected patients (Dubois et al., 1998). BKV-associated disease includes hemorrhagic cystitis and other urinary tract diseases, which most commonly manifest

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in transplant patients undergoing immunosuppressive therapy (Bogdanovic et al., 2006). In addition, both JCV and BKV exhibit oncogenic properties in experimental animals. Indeed, in the past few years JCV, BKV and a simian polyomavirus, SV-40, have come under greater scrutiny for their potential roles in several human cancers (Lee and Langhoff, 2006).

In 2007, two novel human polyomaviruses, KI polyomavirus (KIV) and WU polyomavirus (WUV), were described by independent groups in Sweden and the USA (Allander et al., 2007; Gaynor et al., 2007). The two viruses are genetically closely related and together form a new subfamily of polyomaviruses; they have comparable genomes in the early coding regions to other primate polyomaviruses, but are dissimilar in their late regions (Gaynor et al., 2007). So far, the role of these novel polyomaviruses in human disease has not been established. Initial data show that both KIV and WUV can be detected in up to 4.5% of respiratory samples obtained from patients with acute respiratory tract infection (Bialasiewicz et al., submitted for publication). However, a precise role in respiratory disease has been confounded by high co-detection rates with other respiratory viruses. Unlike JCV and BKV, KIV and WUV nucleic acids have not been detected in urine samples, raising further questions over the persistence of these viruses in the human body, as well as modes of transmission.

Clearly, further studies are needed to elucidate the epidemiology, pathogenesis and potential for oncogenesis of KIV and WUV. In order to facilitate these investigations, rapid and sensitive detection methods to specifically detect these viruses should be developed. Real-time PCR (rtPCR) assays offer enhanced sensitivity, specificity, rapid turnaround time, decreased contamination risk, and the ability to quantify pathogen targets within clinical samples (Mackay, 2004; Espy et al., 2006). In this study we describe the development and evaluation of rtPCR assays for the detection of KIV and WUV.

2. Materials and methods

2.1. Real-time PCR primer and TaqMan probe design

The primers and hydrolysis (TaqMan) probes used in the rtPCR methods were designed using Bioedit 7.0.5.3 (Hall, 1999) and Primer Express 2.0 software (Applied Biosystems Pty. Ltd., Australia). Briefly, Bioedit 7.0.5.3 was used to identify conserved sequence regions using alignments of full-length genomic KIV and WUV sequences (Genbank accession numbers EF127908, EF127907, EF127906, EF444554, EF444553, EF444552, EF444551, EF444550, EF444549). Predicted conserved regions were input into Primer Express 2.0 software to search for potential KIV and WUV primer and probe targets.

Two assays, KI-A and KI-B, targeting the regulatory region and the small T antigen, respectively, were identified as candidates for specific real-time detection of KIV (Table 1). Similarly, three WUV assays were designed, with both the WU-A and WU-C assays targeting the regulatory region, and WU-B targeting the terminal end of the large T antigen (Table 1).

2.2. Real-time PCR

Real-time PCR was performed on a RotorGene 3000 (Corbett Robotics, Australia). All five real-time assays utilized a

Table 1
Primer and probe sequences used in the development of real-time PCR assays for the detection of KI and WU polyomaviruses

Oligonucleotide	Sequence (5′-3′)	Amplicon size (bp)
KI-A-141-F	ACCTGATACCGGCGGAACT	95
KI-A-200-R	CGCAGGAAGCTGGCTCAC	
KI-A-182-TM	fam-CCACACAATAGCTTTCACTCTTGGCGTGA-tamra	
KI-B-4603-F	GAATGCATTGGCATTCGTGA	114
KI-B-4668-R	GCTGCAATAAGTTTAGATTAGTTGGTGC	
KI-B-4632-TM	fam-TGTAGCCATGAATGCATACATCCCACTGC-tamra	
WU-A-4933-F	GGCCTACAACAGGGCTTATTTG	97
WU-A-4986-R	GAACCCAAGGACGTCTCTGTTAA	
WU-A-4983-TM	fam-CTTTGTAGTCCAGCGGAAAGTGAAGGGT-tamra	
WU-B-2729-F:	CTACTGTAAATTGATCTATTGCAACTCCTA	136
WU-B-2808-R:	GGGCCTATAAACAGTGGTAAAACAACT	
WU-B-2797-TM	fam-CCTTTCCTCCACAAAGGTCAAGTAAA-tamra	
WU-C-4824-F:	GGCACGGCGCCAACT	115
WU-C-4898-R:	CCTGTTGTAGGCCTTACTTACCTGTA	
WU-C-4861-TM	fam-TGCCATACCAACACAGCTGCTGAGC-tamra	
WUp001-107-F	CCAATTAGCAGCCACAAGGT	570
WUp001-636-R	TCCAGATCCAGCAATTGATAGT	
KIp001-4313F	TGAACTGTGACCACCACTCAT	1149
KIp001-372R	CAGGTTCGAGACGATGGC	

common PCR mix and cycling conditions. Briefly, 12.5 μl of QIAGEN Quantitect Probe Master Mix (QIAGEN, Australia), 10 pmol of each primer, 4 pmol of the corresponding probe, and 2 μl of sample nucleic acid extract in a final reaction volume of 25 μl, were cycled under the following parameters: incubation of 15 min at 95 °C, followed by 55 cycles of 95 °C for 15 s, and 60 °C for 1 min. Signal acquisition was obtained at the latter end of extension on each cycle on the FAM channel.

2.3. Clinical sensitivity and specificity

Clinical sensitivity and specificity were determined by retrospectively testing 200 nasopharyngeal aspirate (NPA) specimens by the five rtPCR methods and comparing the results to those obtained using previously described WUV and KIV conventional PCR assays. The NPA specimens were collected between June and September 2003 from hospitalized patients or patients presenting at hospital emergency departments in Queensland, Australia with acute respiratory tract illness. Nucleic acid extraction was performed using the High Pure Nucleic Acid kit (Roche Diagnostics, Australia) according to manufacturer's instructions.

The previously described conventional KIV and WUV PCR assays utilized primers POLVP1-39F; POLVP1-363R (KIV; Allander et al., 2007) and AG0044 and AG0045 (WUV; Gaynor et al., 2007) and were performed with some modifications. Separate reactions were performed for each virus. Briefly, each reaction mix contained 2.5 pmol of each primer, 0.625 μl of 10 mM dNTPs, 0.5 μl of 25 mM $MgCl_2$, 2.5 μl of 10X QIAGEN PCR buffer (QIAGEN, Australia), 1.25U of QIAGEN HotStart Taq, and 2 μl of nucleic acid extract in a final volume of 25 μl. KIV PCR cycling was performed on an ABI GeneAmp 2700 instrument (Applied Biosystems Pty. Ltd., Australia) with the following parameters; a 15 min incubation at 95 °C, followed by 40 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, followed by a final extension of 72 °C for 10 min. The WUV screening assay used a similar cycling profile to the KIV method but with an annealing temperature of 56 °C. PCR products were visualised by electrophoresis on 2% agarose gel with ethidium bromide staining.

2.4. Analytical specificity

A panel of commensal and pathogenic organisms was used to investigate assay specificity (Table 2). Also, human nucleic acid was extracted from whole blood and used to test for non-specific cross-reactions with human genomic DNA.

2.5. Detection limit

The detection limits of the assays were determined using dilutions of viral genomic DNA. Initially, plasmids were created using the pGEM-T-Easy vector system (Promega, Madison, USA). The plasmid KIp001 contained an 1149 bp KIV genome fragment, which was synthesized using primers KIp001-4313F and KIp001-372-R (Table 1), and encompassed the targets for the KI-A real-time assay. Similarly, plasmid WUp001 incorporating a 570bp fragment of WUV was synthesized using primers WUp001-107-F and WUp001-636-R (Table 1), and contained the target sequences for the WU-A real-time PCR assay. Plasmid copy numbers were calculated based on molecular weight and optical density measurements. Ten-fold dilutions of the plasmids were tested in their respective rtPCR assays and used to generate standard curves using the RotorGene 3000 software (Corbett Robotics, Australia). These standard curves were then used to quantify secondary control samples containing the viral genomic DNA previously extracted from clinical specimens collected from a KIV- and WUV-positive patient, respectively. Detection limits were determined by testing 10-fold dilutions of the quantified genomic KIV and WUV DNA in triplicate in all assays, including the conventional PCR methods. The limit of detection was defined as the final dilution in which all three replicates tested positive.

Table 2
Panel of organisms tested to determine the specificity of the KIV and WUV real-time PCR assays

Viruses:
Adenovirus 14 ATCC VR15
CMV wild-type
Coronavirus HKU-1 wild-type
Coronavirus NL63 wild-type
Coronavirus OC43 wild-type
Coronavirus 229E wild-type
EBV wild-type
HSV 1 wild-type
HSV 2 wild-type
Influenza A ATCC VR544
Mumps vaccine virus Jeryl Lynn Strain
hMPV wild-type
Parainfluenza 1 ATCC VR94
Parainfluenza 2 ATCC VR92
Parainfluenza 3 ATCC VR93
Polyomavirus JCV ATCC 45027
Polyomavirus BKV ATCC 45024
RSV ATCC VR1400
Rubella virus wild-type
VZV wild-type
Measles attenuated vaccine virus Ender's line
Bacteria:
Bartonella henselae wild-type
Bordetella parapertussis ATCC 15237
Bordatella pertussis ATCC 12742
Campylobacter coli NCTC 11366
Haemophilus influenzae ATCC 10211
Klebsiella pneumoniae wild-type
Mycoplasma pneumoniae ATCC 15293
Neisseria meningitidis wild-type
Pneumocystis carinii ATCC 50385
Legionella pneumophillia ATCC 43111
Listeria monocytogenes wild-type
Streptococcus pneumoniae wild-type

Table 3
Clinical sensitivity and specificity of the KIV and WUV real-time PCR assays calculated after testing 200 clinical samples collected from patients with acute respiratory tract infections

Real-time assay		Conventional PCR		Sensitivity (%)	Specificity (%)
		Positive	Negative		
KI-A	Positive	15	10	100	94.6
	Negative	0	175		
KI-B	Positive	13	5	86.7	97.3
	Negative	2	180		
WU-A	Positive	21	6	95.5	96.6
	Negative	1	172		
WU-B	Positive	22	4	100	97.7
	Negative	0	174		
WU-C	Positive	22	5	100	97.2
	Negative	0	173		

3. Results

3.1. Clinical sensitivity and specificity

A total of 200 clinical respiratory sample extracts were tested to determine clinical sensitivity and specificity of the real-time assays (Table 3). KIV DNA was detected in 13 samples by the KIV conventional PCR and both KIV rtPCR assays. Two specimens were positive by the KIV conventional and KI-A real-time methods, but were negative by the KI-B assay (Samples 1 and 2; Table 4). These were considered false-negative results by the KI-B assay. Five samples gave positive results in both KIV rtPCR assays and were negative by conventional PCR, and a further five samples were positive by KI-A alone. All of these samples provided high cycle threshold (Ct) values in the rtPCR methods (Samples 3–11, 21; Table 4).

WUV DNA was detected in 21 of the 200 clinical samples by the WUV conventional PCR and the three WUV real-time assays. Assay WU-A failed to detect one specimen that was

Table 4
Discordant results obtained from 200 respiratory samples tested by the real-time PCR and conventional reference PCR assays

Sample	KI-A	KI-B	KI Conventional	WU-A	WU-B	WU-C	WU Conventional
KIV discordant results							
1	37.5	ND	Pos	ND	ND	ND	ND
2	34.0	ND	Pos	21.4	21.0	19.3	Pos
3	34.2	33.3	ND	ND	ND	ND	ND
4	35.2	34.4	ND	ND	ND	ND	ND
5	35.2	34.6	ND	32.9	31.7	32.7	Pos
6	35.7	35.9	ND	ND	ND	ND	ND
7	37.8	36.7	ND	ND	ND	ND	ND
8	34.4	ND	ND	ND	ND	ND	ND
9	37.9	ND	ND	ND	ND	ND	ND
10	38.3	ND	ND	30.0	29.7	29.8	Pos
11	34.5	ND	ND	ND	ND	ND	ND
WUV discordant results							
12	ND	ND	ND	ND	37.0	36.0	Pos
13	ND	ND	ND	36.5	36.4	37.3	ND
14	ND	ND	ND	ND	36.5	37. 5	ND
15	ND	ND	ND	ND	ND	36.7	ND
16	ND	ND	ND	ND	ND	37.0	ND
17	ND	ND	ND	37.7	37.5	ND	ND
18	ND	ND	ND	37.4	ND	ND	ND
19	ND	ND	ND	38.3	ND	ND	ND
20	32.8	31.4	Pos	38.5	ND	ND	ND
KIV and WUV discordant result							
21	35.7	ND	ND	35.8	38.6	37.1	ND
KIV and WUV result for one specimen co-infected with KIV and WUV							
22	21.8	21.1	Pos	19.8	18.8	17.7	Pos

Cycle threshold (Ct) values are shown for real-time PCR assays. ND = not detected.

detected by the conventional PCR, WU-B and WU-C assays and so was considered to be a false-negative result (Sample 12; Table 4). Two samples gave positive results in the three WUV rtPCR assays and were negative by the WUV conventional PCR (Samples 13 and 21; Table 4). A further seven samples provided positive results in one or more of the real-time methods but were negative in the WUV conventional PCR assay (Samples 14–20; Table 4). Again, all of the additional detections made by the WUV real-time assays yielded high Ct values.

One specimen provided positive results for both KIV and WUV in all real-time and conventional PCR assays and was considered a co-infection (Sample 22; Table 4). A further five specimens provided positive results for both KIV and WUV using one or more of the real-time and conventional PCR assays (Samples 2, 5 10, 20 and 21; Table 4).

3.2. Assay specificity

Twenty-one different viruses and 12 different bacterial species were used to test assay specificity (Table 2). No cross-reactions were observed with these organisms or with human genomic DNA. In addition, the two KIV real-time assays did not cross-react with wild-type WUV genomic DNA, and the three WUV real-time assays did not cross-react with wild-type KIV genomic DNA.

3.3. Detection limit

Dilutions of genomic KIV DNA tested in the KI-A and KI-B assays demonstrated a reliable detection limit of approximately 10 copies per reaction for both assays. The KIV conventional reference assay (39F; 363R) had a detection limit of approximately 100 copies per reaction, and so was considered to be one log concentration less sensitive than the real-time KIV methods (Table 5). Similarly, the WUV conventional assay was considered to be comparatively one log less sensitive than the WUV real-time assays, with the three WUV real-time assays consistently detecting 10 copies per reaction and the WUV conventional reference assay (AG44; 45) detecting 100 copies per reaction (Table 5).

4. Discussion

The clinical significance of the newly described human polyomaviruses KI and WU is currently unknown. What is clear, however, is the need for sensitive and specific detection methods to help facilitate further investigations. The successful development of any PCR assay for microbial detection is dependant on the availability of sufficient sequence information to ensure the assays' primer and probe targets are both conserved and specific. However, for many organisms, both novel and characterized, the necessary spectrum of sequence data is lacking. This is particularly so for newly described organisms. Therefore, in our opinion, a comprehensive validation of potential PCR assays for the detection of novel micro-organisms in samples from different population groups requires the simultaneous evaluation of multiple targets, in case unknown genetic variants exist.

In this study two KIV and three WUV real-time PCR assays were evaluated in parallel and compared to conventional PCR for the respective viruses. Although all of the real-time methods proved to have lower detection limits than the conventional PCR methods, two real-time assays, KI-B and WU-A, demonstrated a reduced clinical sensitivity in specimens (86.7% and 95.5%, respectively) when using the respective conventional assays as the reference. The false-negative results obtained with these assays may have been due to sequence variation in the oligonucleotide targets. In our experience, very few mismatches between primers and/or probes and their respective targets are needed to affect the sen-

Table 5
KIV and WUV real-time PCR cycle threshold (Ct) values and conventional reference PCR results for serial dilutions of quantified KIV or WUV reference genomic DNA

	Estimated KIV genome copy number						
	$1.0E^6$	$1.0E^5$	$1.0E^4$	$1.0E^3$	$1.0E^2$	$1.0E^1$	$1.0E^0$
KI-A	17.8[a]	21.8[a]	24.4[a]	28.4[a]	31.7[a]	35.1[a]	ND[b]
KI-B	15.9[a]	19.4[a]	22.7[a]	26.7[a]	29.9[a]	33.8[a]	ND[c]
Conventional	Pos	Pos	Pos	Pos	Pos	ND[b]	ND
	Estimated WUV genome copy number						
	$1.0E^5$	$1.0E^4$	$1.0E^3$	$1.0E^2$	$1.0E^1$	$1.0E^0$	
WU-A	19.9[a]	23.9[a]	27.9[a]	31.9[a]	35.8[a]	ND	
WU-B	20.0[a]	23.4[a]	26.8[a]	31.6[a]	35.7[a]	ND	
WU-C	17.9[a]	22.2[a]	26.1[a]	30.0[a]	33.7[a]	ND	
Conventional	Pos	Pos	Pos	Pos	ND	ND	

Pos = positive. ND = not detected.
[a] Mean Ct values of triplicate reactions.
[b] Template detected in 2 of 3 replicates.
[c] Template detected in 1 of 3 replicates.

sitivity of a PCR assay. It is for these reasons that multiple genetic targets should be examined in initial evaluations, and then the chosen targets should be re-examined at later time periods, particularly when dealing with new viral agents that may exhibit considerable genomic variation. On this basis, we deemed the KI-B and WU-A assays to be unsuitable for use in further research of these viruses.

Compared to the conventional assay, none of the rtPCR methods achieved a clinical specificity of 100% (Table 3). This was because all assays made additional detections of either KIV or WUV. These additional positive results were assumed to be false-positive reactions for the purpose of specificity calculations. Nevertheless, it is unlikely that all of these represent false-positive results by the rtPCR methods, as five of the nine additional KIV positive specimens were positive by both the KI-A and KI-B methods (Table 4). Similarly, four of the nine additional WUV-positive specimens were positive by more than one WUV rtPCR assay. Further, all of the real-time assays target different sequences on the respective KIV and WUV genomes, making simultaneous cross-reactions much less likely. Significantly, no cross-reactions were observed when the assays were used to test a panel of other unrelated viruses and bacteria.

In contrast, it is likely that these additional positive results represent true false-negative results by the conventional PCR assays. This is supported by our demonstration that the conventional assays were approximately one log of concentration less sensitive than the real-time PCR methods. Standards containing KIV or WUV DNA at a concentration returning a Ct value of 33 or greater in the real-time methods could not be detected by the conventional PCR assay (Table 5). Sensitivity issues are commonly encountered when evaluating rtPCR methods, as the assay under evaluation is often more sensitive than the reference method. Approaches such as discrepant analysis are often used to circumvent these problems. However, we refrained from using discrepant analysis on these specimens, given this approach has previously been criticised for introducing bias when used to recalculate sensitivity and specificity values (Hadgu, 2000).

In this study, we describe the development and validation of sensitive rtPCR assays for the detection of the recently described KI and WU polyomaviruses. Overall, the results show that the KI-A, WU-B and WU-C assays provide sensitive detection of KIV and WUV in clinical specimens. We will use these assays to conduct further research into the epidemiology and pathogenesis of these viruses.

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