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Molecular and Cellular Probes 40 (2018) 13–18

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Duplex real-time polymerase chain reaction assay for the detection of human KIPyV and WUPyV in nasopharyngeal aspirate pediatric samples

Marco Ligozzi[a,*], Liliana Galia[a], Maria Carelli[a], Pier Paolo Piccaluga[b,c,d], Erica Diani[a], Davide Gibellini[a]

[a] Microbiology and Virology Unit, Department of Diagnostics and Public Health, University of Verona, Strada delle Grazie 8, 37134 Verona, Italy
[b] Department of Experimental, Diagnostic, and Specialty Medicine, University of Bologna, Bologna, Italy
[c] Euro-Mediterranean Institute of Science and Technology (IEMEST), Palermo, Italy
[d] Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

ARTICLE INFO

Keywords:
WUPyV
KIPyV
PCR
Viral respiratory diseases

ABSTRACT

In this study, we describe a duplex real-time PCR assay for the simultaneous detection of KIPyV and WUPyV polyomaviruses based on TaqMan probes. This assay detected 500 copies/mL both for KIPyV and WUPyV in 100% of tested positive samples. We assessed this technique on 482 nasopharyngeal aspirate specimens from hospitalized pediatric patients with respiratory symptoms, previously analyzed with commercial multiplex assay for 16 major respiratory viruses. Our assay detected KIPyV genome in 15 out of 482 samples (3.1%) and WUPyV genome in 24 out of 482 samples (4.9%), respectively, and in three samples the coinfection of the two viruses was found. Interestingly, 29 out of 36 of samples with KIPyV and/or WUPyV infection exhibited a co-infection with one or more respiratory viruses confirming that KIPyV and WUPyV were often detected in association to other viral infections. Of note, KIPyV and WUPyV were detected singularly in 4 out of 15 cases and 3 out of 24 cases, respectively, suggesting a possible direct role of these viruses in the respiratory diseases. In conclusion, this method could be taken into account as an alternative technical approach to detect KIPyV and/or WUPyV in respiratory samples for epidemiological and diagnostic analyses.

1. Introduction

High throughput sequencing techniques found out the presence of two new polyomaviruses in respiratory secretions of children with acute respiratory symptoms [1,2]. These new viruses were called Karolinska Institut (KIPyV) and Washington University (WUPyV) polyomaviruses. KIPyV and WUPyV, as the BK and JC polyomaviruses, persist in a latent form in the human body but they could mainly reactivate in patients with immunosuppression. The initial identification of KIPyV and WUPyV in respiratory samples was progressively extended to other human fluids including blood, cerebrospinal fluid, lymphoid tissue, urine, stool and saliva [3–6]. Serological evaluations demonstrated that the primary infections by KIPyV and WUPyV occur early in life through the respiratory and fecal routes. It is noteworthy that the seroprevalence of antibodies to VP1 protein is detected in adult population of about 80% for WUPyV and 70% for KIPyV [7–10]. The genome detection of these two viruses in samples achieved from children is well documented whereas has been rarely revealed from immunocompetent adults. These viruses mainly infect young patients and their reactivation is related to immunosuppression cases including HIV positive patients and in stem cell transplanted patients [11–13]. The prevalence of KIPyV and WUPyV genome detection varied from 2 to 9%, in patients with respiratory symptoms [14] however, the detection of KIPyV and WUPyV genomes in respiratory samples is often related to high rate of co-infection with other major respiratory diseases such as Respiratory syncitial virus, Parainfluenza viruses, Adenovirus, Rhinovirus and Influenza virus [11,15–17]. Moreover, KIPyV and WUPyV were found at approximately similar percentages in healthy patients. Hence, the real pathogenicity of these viruses in the respiratory disease is still unclear even though KIPyV and WUPyV were detected in clinical cases without any viral co-infection in symptomatic patients with respiratory disorders including pneumonia, bronchiolitis and bronchitis [17,18]. These recent observations suggest that KIPyV and WUPyV may be directly involved in the onset of respiratory diseases thus determining the need of reliable molecular procedures for the infection diagnosis of these two viruses. In this study, we developed a duplex PCR procedure for a single tube detection of these two viruses in clinical samples. This method was also assessed on 482 nasopharyngeal aspirate

* Corresponding author. Microbiology and Virology Unit, Department of Diagnostics and Public Health, University of Verona, Strada delle Grazie 8, 37134 Verona, Italy.
E-mail address: marco.ligozzi@univr.it (M. Ligozzi).

https://doi.org/10.1016/j.mcp.2018.06.001
Received 4 May 2018; Received in revised form 25 May 2018; Accepted 4 June 2018
Available online 05 June 2018
0890-8508/

Table 1
Primers and probes for the duplex real-time PCR assay.

Primer/probe name	Primer/probe sequence	Viral target	Gene position
KIF	5′-CAAGTGTTTAACAGACCCACAGAAAC-3′	KIPyV- VP1 gene	939–965
KIR	5′-GTGGGCCTATTTCTTCTACCATAGTC- 3′	KIPyV- VP1 gene	996–1021
FAM-KI MGB-probe	5′-TGATGCACAGGTTGGT-3′	KIPyV- VP1 gene	969–984
WUF	5′-GGTGTTTAATAAGCCAGCTGATGA-3′	WUPyV-VP1 gene	918–941
WUR	5′-GGGCCCTGTTTCTTCAGTCA-3′	WUPyV-VP1 gene	977–996
VIC-WU MGB-probe	5′-TAGTGGGCAACTGC-3′	WUPyV-VP1 gene	945–958

specimens collected from hospitalized pediatric patients previously analyzed for major respiratory viruses with a commercial multiplex PCR assay. The data obtained showed the analytical performances of the assay, the prevalence of KIPyV and WUPyV infection and the viral co-infection in this specific pediatric population.

2. Materials and methods

2.1. Clinical specimens

Nasopharyngeal aspirate (NPA) samples were collected from 482 hospitalized children (age < 5 years) with symptomatic respiratory tract infections. These specimens were collected between October 2015 and October 2017 at Verona Hospital. NPA swabs were transported to laboratory in sterile transport medium (Universal Transport Medium, Copan Diagnostics, Murrieta, CA, USA). All samples were stored at −80 °C prior to processing. These samples were analyzed for respiratory viruses with Allplex™ respiratory panel assay kit (Seegene, Seoul, South Korea) and, retrospectively, with TaqMan-based duplex real-time PCR for the detection of KIPyV and WUPyV genomes.

2.2. Nucleic acid extraction and purification

Nucleic acids extraction from samples was carried out with a Microlab Nimbus apparatus (Hamilton Robotics, Reno, NV, USA). Nucleic acids were extracted from 350 µL of NPA according to the manufacturer's instructions. Briefly, 350 µL of NPA sample was added to 340 µL of lysis buffer containing proteinase K and internal control provided in the extraction kit. This mixture was incubated at 56 °C for 5 min with 25 µL of silica followed by automatic magnetic separation. Nucleic acids were then recovered in 100 µL of elution buffer (10 mM Tris-EDTA pH 7.5). The purified nucleic acids were stored at −80 °C.

2.3. Allplex™ respiratory panel assay kit detection procedure

Allplex™ respiratory panel assay kit (Seegene, Seoul, South Korea) is able to detect two DNA viruses and fourteen RNA viruses including human Adenovirus (AdV) and human Bocavirus (HBoV 1/2/3/4) for DNA viruses, Influenza A and B viruses (FluA, FluB), human Parainfluenza viruses 1/2/3/4 (PIV1/2/3/4), human Rhinovirus A/B/C (HRV A/B/C), human Respiratory Syncytial viruses A and B (RSV A, RSV B), human Coronaviruses 229E, NL63 and OC43 (HCoV229E, HCoVNL63, HCoVOC43), human Metapneumovirus (MPV), and human Enterovirus (HEV) for RNA viruses. Allplex™ respiratory panel assay kit is composed by three detection panels: panel 1 includes the Flu A virus (subtypes H1, H1N1-pdm09, and H3), Flu B virus, RSV A and RSV B; panel 2 includes ADV, MPV, HEV and PIV1/2/3/4; panel 3 includes HBoV, HCoV229E, HCoVNL63, HCoVOC43, and HRV. This technique is based on one-step, real-time reverse transcription-PCR (rRT-PCR) assay based on Multiple Detection Temperature (MuDT) technology. A real-time PCR reaction mixture was prepared as follows: 8 µL eluted DNA, 5 µL 5× RV primer, 4 µL 8-methoxypsoralen solution, and 4 µL 5× Master Mix. Seegene unique real-time PCR and melting curve analysis technique called “Tagging Oligonucleotide Cleavage and Extension” was performed using CFX96 real-time PCR detection system (Bio-Rad Laboratories, Richmond, VA, USA) as follows: i) 1 cycle of initial denaturation at 95 °C for 15 min; ii) 50 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min, and extension at 72 °C for 30 s; iii) 1 cycle of cooling the reaction mixture at 55 °C for 30 s; iv) melting double-strand DNA into single strands by raising the temperature to 85 °C. The validation of DNA extraction and amplification was performed using an internal control, negative and positive controls as indicated by manufacturer.

2.4. TaqMan-based duplex real-time PCR assay for KIPyV and WUPyV detection

Specific primers corresponding to the VP1 gene were selected from conserved regions of KIPyV (reference sequence FJ754236.1) and WUPyV (reference sequence FJ754242.1) genomes without any cross homology to other types of polyomaviruses including JC and BK polyomaviruses. This analysis was carried out comparing the selected sequences with the polyomavirus sequences in NCBI sequence database using the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). KIPyV and WUPyV primer and probes were described in Table 1. As positive control templates, synthetic double-strand oligonucleotides (BMR Genomics, Padua, Italy) from nucleotide 935 to 1024 (90 bases) for KIPyV and from nucleotide 913 to 1001 (89 bases) for WUPyV were designed [19]. The double-strand oligonucleotides were quantitated by spectrophotometry. TaqMan-based real-time quantitative PCR was carried out in a total reaction volume of 50 µL consisting of 25 µL of TaqMan Universal Master Mix (Applied Biosystems, CA, USA), 0.5 µM of each KIPyV and WUPyV specific primer, 0.25 µM of KIPyV and WUPyV specific probes, 10 µL of DNA extracted either from clinical samples or scalar dilutions of synthetic double strand oligonucleotide positive control (from 5×10^6 to 5×10^1 copies/mL) and 12.5 µL of double-distilled water. The amplification was carried out in a BioRad CFX96 real-time PCR system (BioRad Laboratories). The analysis of results was determined by real-time CFX Manager Software version 3.1 (BioRad Laboratories). All standard dilutions, controls and samples from patients were run in duplicate and the average value of the copy number was employed to quantify KIPyV or WUPyV genome contents. Exact values were employed for calculations excluding the decimal values. Quantitative analysis was determined by interpolating the Ct value of sample on external reference curve obtained with serial concentrations of KIPyV or WUPyV synthetic dsDNAs.

2.5. Statistical analysis

To determine the assay lower limit detection in at least the 95% of replicates, we used Probit regression analysis as indicated in Ref. [20] with IBM SPSS software (IBM, Armonk, NY, USA) using several target concentrations (5×10^2, 4×10^2, 3×10^2, 2×10^2, 1×10^2, 5×10^1copies/mL) either for KIPyV or WUPyV.

3. Results

3.1. Standardization of duplex real-time TaqMan-based PCR

We assessed a new duplex real-time TaqMan-based PCR to detect

Table 2
Analysis of scalar dilution replicates.

	Synthetic DNA target (copy number/mL)	Number of positive replicates/ Total replicates	%
KIPyV	5×10^5	10/10	100
	5×10^4	10/10	100
	5×10^3	10/10	100
	5×10^2	10/10	100
	5×10^1	1/10	10
	0	0/10	0
WUPyV	5×10^5	10/10	100
	5×10^4	10/10	100
	5×10^3	10/10	100
	5×10^2	10/10	100
	5×10^1	1/10	10
	0	0/10	0

KIPyV: Karolinska Institut Polyomavirus. WUPyV: Washington University Polyomavirus.

KIPyV and WUPyV genomes in a single-tube reaction. The primers and probes (Table 1) were designed with the NCBI BLAST program selecting conserved regions for the KIPyV and WUPyV isolates deposited in NCBI database. To rule out possible cross-reactivity with human, microbial or unrelated viral sequences, we performed a homology analysis in NCBI database. No significant homologies were detected indicating that these oligonucleotides designed on VP1 region sequence of both viruses can be considered specific for KIPyV and WUPyV amplifications. The sensitivity of the duplex real-time PCR assay was determined by testing serial dilutions (from 5×10^7 to 5×10^1 copies/mL) of two different synthetic dsDNAs carrying specific sequences of VP1 region either of KIPyV (89 nt) or WUPyV (90 nt). The choice of specific fluorochrome conjugated to KIPyV or WUPyV TaqMan probes allows the detection of KIPyV and WUPyV in 510 nm or 555 nm channels, respectively. The analysis of 10 replicates of synthetic dsDNAs displayed positive detection in 100% samples with 5×10^2 copies/mL (approximately 17 copies/reaction) for either KIPyV or WUPyV (Table 2). In the next series of replicates, (5×10^2, 4×10^2, 3×10^2, 2×10^2, 1×10^2, 5×10^1 copies/mL), linear regression analysis with Probit model analysis has been calculated: the limit of detection in at least the 95% of replicates were determined at 328 copies/mL (12 copies/reaction) with a 95% confidence interval from 256 to 444) for KIPyV and 345 copies/mL (15 copies/reaction) with a 95% confidence interval from 284 to 472) for WUPyV, respectively (Table 3). Our assay showed an efficiency > 90% with a correlation coefficient $R^2 > 0.99$ for both the viral targets. The linearity of this assay is detectable in 10-fold scalar dilutions (n = 5 for each dilution) from 5×10^6 copies/mL to 5×10^2 copies/mL. The specificity of the duplex real-time PCR assay was verified by examining negative controls and positive samples for different respiratory viruses

Table 3
Probit analysis.

	Synthetic DNA target (copy number/mL)	Number of positive replicates/ Total replicates	%
KIPyV	5×10^2	10/10	100
	4×10^2	10/10	100
	3×10^2	10/10	100
	2×10^2	4/10	40
	1×10^2	3/10	30
	5×10^1	1/10	10
WUPyV	5×10^2	10/10	100
	4×10^2	10/10	100
	3×10^2	9/10	90
	2×10^2	4/10	40
	1×10^2	2/10	20
	5×10^1	1/10	10

KIPyV: Karolinska Institut Polyomavirus. WUPyV: Washington University Polyomavirus.

Table 4
Intra-assay and inter-assay analysis.

	Number of copies/mL	Ct (mean)	Standard Deviation (SD)	Coefficient of Variation (%)
Intra-assay analysis				
KIPyV	5×10^6	23.00	0.21	0.9
	5×10^5	25.52	0.27	1.1
	5×10^4	28.92	0.41	1.4
	5×10^3	33.12	0.32	1
	5×10^2	36.81	0.47	1.2
WUPyV	5×10^6	22.12	0.37	1.7
	5×10^5	25.26	0.22	0.9
	5×10^4	28.56	0.26	0.9
	5×10^3	32.41	0.22	0.7
	5×10^2	36.1	0.48	1.3
Inter-assay analysis				
KIPyV	5×10^6	22.46	0.35	1.57
	5×10^5	25.87	0.54	2.08
	5×10^4	29.13	0.44	1.53
	5×10^3	32.96	0.51	1.55
	5×10^2	36.74	0.58	1.57
WUPyV	5×10^6	22.26	0.37	1.72
	5×10^5	25.61	0.36	1.42
	5×10^4	28.78	0.39	1.36
	5×10^3	32.55	0.36	1.12
	5×10^2	36.32	0.43	1.19

KIPyV: Karolinska Institut Polyomavirus. WUPyV: Washington University Polyomavirus.

including Flu A, Flu B, PIV, AdV, HRV, RSV A, RSV B, HBoV, HCoV, hMPV and HEVs (n = 3 for each virus). All these samples did not show any positive signal either for KIPyV or WUPyV determination.

3.2. Repeatibility of assay and interference test

The intra-assay and inter-assay analyses were performed in serial dilutions of synthetic dsDNA targets (Table 4). We assayed intra-assay analysis with 10-fold serial dilutions (from 5×10^6 copies/mL to 5×10^2 copies/mL) of dsDNA specific for KIPyV or WUPyV in triplicate. The intra-assay reproducibility data were indicated in Table 4. The analysis of coefficient of variation (CV) calculated on Ct average was less than 2% for all dilutions of either KIPyV or WUPyV. Similarly, we have compared the data obtained in three separate experiments performed in triplicate for the inter-assay evaluation. The results showed a coefficient of variation calculated on Ct average always less than 3% for all scalar dilutions either for KIPyV or WUPyV. It is well known that the presence of very different concentration of viral targets in duplex PCR can interfere on sensitivity reaction [21,22]. We assayed a combination of a high concentration (10^6 copies/mL) and a low concentration (10^3 copies/mL) of synthetic target of KIPyV and WUPyV in an interference test [23]. The results (Table 5) did not show systematic difference in the

Table 5
Analysis of duplex PCR amplification in simultaneous presence of different target concentrations.

KIPyV (number of copies/mL)	WUPyV (number of copies/mL)	KIPyV Ct mean	WUPyV Ct mean
10^3	–	32.31	–
10^6	–	21.57	–
–	10^3	–	35.03
–	10^6	–	21.22
10^3	10^6	33.07	21.34
10^6	10^3	21.7	35.89

KIPyV: Karolinska Institut Polyomavirus. WUPyV: Washington University Polyomavirus.

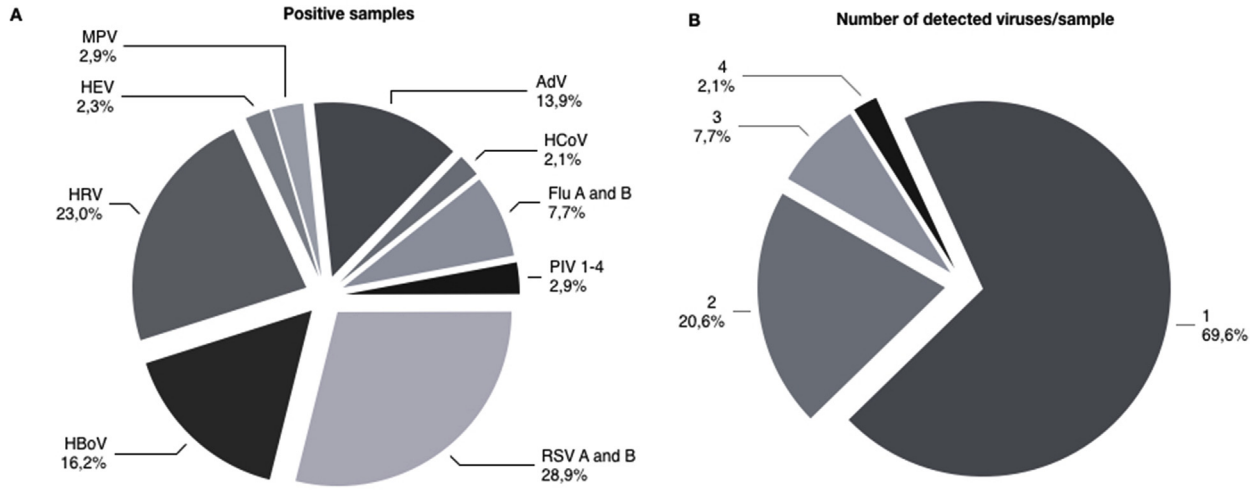

Fig. 1. Respiratory virus detection by Allplex™ respiratory assay kit in pediatric patients. Panel A: the percentage of viruses detected in the clinical samples. Panel B: analysis of co-infection in the same clinical samples. It is shown the number of detected virus in the same sample.

amplification curves of the mixed targets with respect to the run performed with a single target. The CV mean value showed a difference less than 2% suggesting a low interference when different template concentrations of targets were simultaneously amplified.

3.3. Analysis of nasopharyngeal aspirate from pediatric patients by duplex real-time PCR

A total of 482 nasopharyngeal aspirate specimens from pediatric patients (age < 5 years) with respiratory symptoms were tested with Allplex™ respiratory panel assay kit. This commercial multiplex PCR assay detects the major human respiratory viruses. The multiplex assay has determined the presence of viral pathogens in 339 out of 482 specimens (70.3%). The analysis of the 339 positive samples has shown that 30% of positive samples were co-infected with two or more viruses (Fig. 1). Based on frequency of detection (Fig. 1), RSV (A and B) was detected in 28.9% of positive samples, followed by HRV (23%), HBoV (16.2%), AdV (13.9%) and Flu A/B viruses (7.7%). Hence, we have assessed our duplex real-time PCR in the same samples and our data indicated that 15 out of 482 samples (3.1%) were positive for KIPyV whereas WUPyV was detected in 24 out of 482 samples (4.9%). The mean value of KIPyV viral load of positive samples was determined between 5.71×10^2 and 8.64×10^6 copies/mL with a median value of 1946 copies/mL whereas the WUPyV viral load in positive specimens was calculated between 4.09×10^2 and 1.22×10^7 copies/mL with a median value of 53444 copies/mL (Table 6). Since these two Polyomaviruses are often associated to other viral infection, we determined whether the KIPyV and/or WUPyV positive samples showed a viral co-infection analyzing the data obtained with commercial Allplex™ respiratory panel assay kit. This analysis displayed that 11 out of 15 KIPyV positive samples showed one or more viral co-infections, whereas WUPyV was associated to other viruses in 21 out of 24 cases. Interestingly, in this patient group, KIPyV is preferentially associated with HRV (9 out of 15 samples) followed by RSV A/B and AdV (3 out of 15 samples). On other hand, WUPyV exhibited a major association with HRV (9 out of 24 samples) followed by RSV A/B viruses (7 out of 24 samples) and AdV (6 out of 24 samples). It is noteworthy that KIPyV and WUPyV were simultaneously detected in three samples. All these three KIPyV/WUPyV double positive cases showed that these two viruses were associated with at least another respiratory virus (Table 6).

4. Discussion

In this paper, in the first set of experiments, we set up a TaqMan-based real-time duplex PCR for the simultaneous determination of KIPyV and WUPyV. This method was applied on respiratory samples to study the presence of these viruses and co-infection with other respiratory viruses in hospitalized pediatric patients with respiratory symptoms. Our technique detects the 100% of KIPyV and WUPyV positive samples with 5×10^2 copies/mL. In addition, Probit analysis indicated that our assay is able to detect until 328 copies/mL for KIPyV and 345 copies/mL WUPyV in the 95% of replicates. The sensitivity of this assay is similar to previous real-time PCRs [24–26] and its analytical performances demonstrated that this approach might be considered a useful tool for KIPyV and WUPyV genome detections. The choice of a TaqMan format with a combined detection show clearer advantages than separate analysis as this format may be developed in multiplex assays with additional respiratory viruses. This aspect may be interesting because commercial and laboratory developed multiplex assays for respiratory viruses have not KIPyV and WUPyV among the targets.

We have applied this duplex real-time PCR technique on 482 nasopharyngeal aspirate samples from pediatric cohort previously analyzed for viral infection detection by Allplex™ respiratory panel assay kit. This commercial test detects 16 human viruses causing respiratory diseases but it is not able to reveal the presence of KIPyV and WUPyV genomes. We found a prevalence of 3.1% and 4.9% for KIPyV and WUPyV positive samples, respectively during the screening of these 482 pediatric samples. These data are in according to other studies [27–32] on KIPyV and WUPyV detection. In fact, the prevalence of KIPyV and WUPyV varies from 0.5 to 5% for KIPyV and 0.4–9% for WUPyV in immunocompetent pediatric and adult patients, whereas in immunodeficiency patients, the prevalence of KIPyV and WUPyV viruses significantly increased in respiratory samples. Our data also confirmed that the prevalence of WUPyV is higher than KIPyV as previously indicated [14]. The difference of prevalence described in past studies may be related either to regional prevalence variations or age population selection. In particular, a higher detection of KIPyV and WUPyV in respiratory samples of pediatric patients than in adult patients was noticed. It is noteworthy that several observations proved a correlation between age and KIPyV and/or WUPyV DNA detection: KIPyV showed a prevalence peak of genome detection at 1–2 years of age whereas WUPyV displayed the peak later, between 5 and 24 years of age [11,33–35].

The detection of KIPyV and/or WUPyV genome sequences in respiratory samples suggested that these viruses establish persistent replication in immunocompetent hosts and may elicit respiratory disorders. However, the association between KIPyV and/or WUPyV

Table 6
KIPyV and/or WUPyV positive samples in pediatric patients.

KIPyV and/or WUPyV positive samples	KIPyV (number of copies/mL)	WUPyV (number of copies/mL)	Viral co-infections
1	1780	–	–
2	1798	–	HRV
3	1885	–	–
4	1946	–	HRV, HBoV,HEV, RSV A
5	1938	56113	HEV, PIV3, HBoV, HRV
6	1904	–	–
7	7068800	–	RSV B
8	5970	50775	HRV, AdV, RSV B
9	8635969	–	HRV, AdV, HBoV
10	6566	–	HRV
11	12564	7676	HBoV
12	3330	–	HRV
13	774	–	–
14	571	–	AdV, PIV3, HRV
15	2949000	–	HRV
16	–	1908	RSV A
17	–	2801236	HCoV
18	–	390127	–
19	–	512	RSV B
20	–	9546	Flu A, RSV B
21	–	214786	RSV A
22	–	1222628	HBoV
23	–	3859264	Flu B, HBoV, HRV
24	–	5426	Flu B
25	–	3558464	Flu A, RSV A, HBoV, HRV
26	–	3320	RSV B
27	–	12242560	AdV
28	–	103415	–
29	–	409	MPV, HRV
30	–	12300	AdV
31	–	102362	HRV
32	–	1367	AdV, PIV3, HRV
33	–	419315	HRV
34	–	12633	AdV, PIV3
35	–	56821	AdV, HRV
36	–	4905	–

KIPyV: Karolinska Institut Polyomavirus, WUPyV: Washington University Polyomavirus, AdV: human Adenovirus, HBoV 1/2/3/4: human Bocaviruses. Flu A: Influenza A virus; FluB: Influenza A virus; PIV1/2/3/4: human Parainfluenza viruses 1/2/3/4; HRV: human Rhinovirus A/B/C; RSV A: human Respiratory syncytial virus A, RSV B: human Respiratory syncytial virus B; HCoV: human Coronaviruses 229E, NL63 and OC43; MPV: human Metapneumovirus; HEV: human Enterovirus.

infections and respiratory diseases is controversial because these two viruses showed very high co-infection rates with other respiratory viruses increasing the complexity of KIPyV and/or WUPyV roles in the respiratory pathogenesis. In particular, previous studies have displayed KIPyV co-detection rate with other respiratory viruses around 74% whereas WUPyV showed variable rates between 68% and 97% [27,28,33,36,37]. In our paper, we also investigated these viral co-infections using the data previously generated on these samples with Allplex™ respiratory panel assay kit that can show the presence of 16 respiratory viruses by multiplex approach. Multiple infections (Table 6) were detected in 11 out of 15 KIPyV infected specimens whereas WUPyV was detected with other respiratory viruses in 21 out of 24 cases. Interestingly, we have described, in our group of patients, the contemporary presence of KIPyV and WUPyV genomes in three cases. In all three cases, however, at least one additional respiratory was detected. The co-infection detection have suggested that KIPyV and WUPyV may be not directly involved in the disease induction and these viruses may be considered as either opportunistic agents, or viruses that other infections could reactivate in respiratory epithelia. In spite of these observations, we have detected seven cases where KIPyV or WUPyV were detected in apparent absence of concomitant respiratory viral infection. The detection of single infection in our patients with respiratory disease may suggest the direct role of these viruses as causative agents of respiratory disorders even though we cannot exclude the possibility that these patients may be infected with other pathogens (i.e. bacteria) not revealed with commercial multiplex analysis.

5. Conclusion

In conclusion, this study showed a new duplex real-time PCR for the contemporary detection of KIPyV and WUPyV. This method might be considered as a valuable diagnostic alternative for these viruses. We applied this method on NPAs achieved from a cohort of pediatric patients with respiratory symptoms to validate the test. This analysis determined that these viruses were detected in this group of patients with a prevalence of 3.1% and 4.9% for KIPyV and WUPyV positive samples, respectively.

Funding

This work was supported by Verona University Dipartimental Research Funds 2016.

Acknowledgments

We thank Dr.ssa Erika Carboni for editing the manuscript.

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