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DEVELOPMENT OF A NEW DUPLEX REAL-TIME POLYMERASE CHAIN REACTION ASSAY FOR DETECTION AND QUANTIFICATION OF KIHPYV AND WUHPYV IN PEDIATRIC SAMPLES

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Coordinatore: Prof. Giovanni Malerba

Firma _____

Tutor: Prof. Davide Gibellini

Firma _____

Dottorando: Dott.ssa Maria Carelli

Firma _____

Abstract

KI and WU are two recently-described human polyomaviruses worldwide distributed. An association between these two viruses and respiratory infections has been suggested, in particular in children and immunocompromised patients. Since no standardized detection methods are currently available, we developed a duplex real-time PCR assay for the simultaneous detection of KIHPyV and WUHPyV polyomaviruses based on TaqMan probes. We assessed this technique on 831 nasopharyngeal aspirate specimens from hospitalized paediatric patients with respiratory symptoms, retrospectively analysed with commercial multiplex assay for 16 other major respiratory viruses. Our assay detected 500 copies/mL for both KIHPyV and WUHPyV in all tested samples.

We detected KIHPyV and WUHPyV genome in 28 (3.36%) and 41 samples (4.93%) respectively. Moreover, in 3 samples, the co-infection of the two viruses was found. 55 out of all positive samples (n=69) for KIHPyV and/or WUHPyV infection exhibited a co-infection with one or more respiratory viruses, confirming that KIHPyV and WUHPyV were often detected in association with other viral infections. Interestingly, KIHPyV and WUHPyV were detected singularly in 8 out of 28 cases and 6 out of 41 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 KIHPyV and/or WUHPyV in respiratory samples helping in the definition of the pathogenic potential and of the epidemiological prevalence of these two viruses.

Sommario

KIHPyV e WUHPyV sono due polyomavirus umani recentemente isolati con una grande prevalenza nella popolazione mondiale. Fin dalla loro scoperta, KIHPyV e WUHPyV sono stati associati a patologie dell'apparato respiratorio soprattutto in bambini e soggetti immunocompromessi, ma non esistono ad oggi metodi commerciali e/o standardizzati per la diagnosi di KIHPyV e WUHPyV.

Abbiamo dunque messo a punto una originale Duplex Real-Time PCR in grado di identificare e discriminare KIHPyV e WUHPyV con una sensibilità di 500 copie/ml ed una specificità del 100%.

Questa metodica è stata valutata su 831 aspirati nasofaringei di bambini di età inferiore a 5 anni con sintomatologie respiratorie. Gli stessi campioni sono stati analizzati in maniera retrospettiva con il kit diagnostico standardizzato attualmente in uso per la diagnosi di virus respiratori.

La nostra metodica ha identificato la presenza di KIHPyV in 28 campioni (3.36%) e di WUHPyV in 41 campioni (4.93%) mentre in tre campioni è stato possibile rilevare la presenza di entrambi i virus.

Nonostante in 55 dei 69 campioni positivi per KIHPyV e/o WUHPyV siano stati anche identificati altri virus, confermando l'alto tasso di identificazione di co- infezione di KIHPyV e WUHPyV, è interessante notare che questi sono stati identificati singolarmente in 8 casi su 28 ed in 6 casi su 41, rispettivamente. L'identificazione singola di KIHPyV e WUHPyV in campioni di bambini con sintomi respiratori suggerisce che questi virus possano essere direttamente responsabili dell'insorgenza della sintomatologia.

In conclusione, abbiamo messo a punto un metodo in grado di identificare KIHPyV e WUHPyV con elevate sensibilità e specificità, che potrebbe contribuire alla definizione del potenziale patogeno e dell'epidemiologia dei due virus.

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1. INTRODUCTION

1.1 ACUTE RESPIRATORY TRACT INFECTION

Acute respiratory tract infections (ARI) represent an important global health issue and are responsible for significant morbidity and mortality worldwide. Word Health Organization (WHO) defines ARI as follows: Acute is defined as sudden onset of symptoms; Respiratory and Infection are defined as having at least one of the following symptoms:

- cough;
- sore throat;
- shortness of breath;
- coryza.

ARI includes a group of diseases that affect millions of people worldwide and could exert influence on the survival and the health of many of them, especially kids, elderly and immunocompromised patients. In fact, ARI is the most common cause of death in children after neonatal complications.

According with WHO's report, pneumonia, the most severe ARI form, accounts for 16% of all the deaths in children under 5 years old. Only in 2016, 880.000 children died from acute respiratory infections.

Although most of the mortality associated with ARI occurs in developing countries, considering that the true burden of disease is often underestimated in these areas, there are substantial economic issues associated to the spread of respiratory infections also in developed countries. In fact, respiratory diseases are accountable for more than 10% of all disability-adjusted life-years (DALYs), a metric that estimates the amount of active and productive life lost due to a condition (WHO, 2016).

For example, taking into account common cold, although it mainly causes mild and self-limiting symptoms, it led to loss of productivity and work absenteeism not only in patients, but also in care givers. Another important aspect that must be considered is the treatment cost, in fact respiratory infection is the most common reason for antibiotic prescription even though most of the case are of viral etiology, as will be extensively discuss in next chapters (Lindbaek, 2006).

1.1.1 Agents of respiratory infection

Pathogenic agents of acute respiratory infections are viruses, bacteria and fungi. Respiratory viruses are the most common causative agents of respiratory infections and their distribution differ by season, geographic region and age group (Fouchier et al. 2005; Brodzinski & Ruddy 2009; Cerone et al. 2017). Respiratory viruses can cause infections in either the upper or lower respiratory tract. Many of them cause common clinical syndromes including nasal congestion, cough, sore throat and fever; some of these pathogens may also lead to more serious clinical manifestations, such as bronchiolitis and pneumonia (Ruuskanen et al. 2011).

More than 300 different virus serotypes have been associated with human respiratory disease. Most common DNA Respiratory virus are human Adenovirus (ADV) and human Bocavirus (HBoV), while RNA Respiratory virus are Flu A (A-H1, A-H1N1 pdm09, A-H3) and B, human Parainfluenzavirus 1-4 (PIV1-4), Respiratory syncytial virus A and B (RSV-A, RSV-B), human Metapneumovirus (MPV), human Rhinovirus (HRV), human Enterovirus (hEV), human Coronavirus (CoV-229E, CoV- OC43, CoV NL63). In the pediatric population, respiratory syncytial virus, parainfluenza viruses, and Flu virus are known as the major causes of bronchiolitis and lower respiratory tract infections (Brodzinski & Ruddy 2009).

However, in approximately the 30% of all reported cases of respiratory diseases, none of the previous indicated viruses can be revealed, suggesting the existence of additional respiratory viruses (Wright et al. 1989; Nokso-Koivisto et al. 2002). Thanks to the advent of high throughput sequencing, new viruses have been recently described both in the respiratory tract and in other human district in people showing unknown etiology respiratory symptoms, such as Karolinska Institutet Polyomavirus (KIHPyV) (T. Allander et al. 2007) and Washington University Polyomavirus (WUHPyV) (Anne M Gaynor et al. 2007).

1.1.2 Diagnosis of viral respiratory infections

Typical respiratory illness diagnosis is clinical, based on symptoms and local epidemiology. Diagnosis of such pathogens is very challenging due to the lack of pathognomonic features, the co-circulation of respiratory pathogens and the wide range of non-specific symptoms of respiratory infections. Moreover, lack in appropriate differential diagnosis lead to difficulties in the administration of appropriate antiviral or antibacterial therapy, initiation of effective infection control measures and reduction of the length of hospital stay (Das et al. 2018).

Historically, conventional testing methods for viral diagnosis were: culture and virus isolation, which was considered the "gold standard" for diagnosis of respiratory viral pathogens (Olsen et al. 1993); viral detection by electron microscopy (Roingeard 2008); antigen detection assays like rapid immunoassays (Weinberg & Walker 2005); direct and indirect immunofluorescence assays (Weinberg & Walker 2005).

However, nowadays an increasing number of laboratories is adopting rapid molecular assays (Mahony et al. 2011). In particular, Multiplex Real Time PCR (RT-PCR) respiratory panel is the most popular diagnostic assay for improving infection control, timely treatment decisions and also because it is substantially less expensive than detecting individual pathogens. Nucleic acid-based FDA-approved diagnostic tests are listed in **Table 1** (modified from Das et al. 2018).

Test	Manufacturer	Technology	Targets
AllplexTM	Seegene	MuDT technology	Multiplex Panel (16 tagets)
Anyplex RV16	Seegene	TOCE technology	Multiplex Panel (16 targets)
NxTAG® respiratory pathogen panel	Luminex Corporation	Real-time RT-PCR	Multiplex Panel (20 targets)
eSensor® respiratory viral panel (RVP)	GenMark Diagnostics	Multiplex microarray, competitive DNA hybridization	Multiplex Panel (14 targets)
Verigene® RP flex	Luminex Corporation	RT-PCR & and microarray hybridization	Multiplex Panel (16 targets)
ePlex® respiratory pathogen (RP) panel	GenMark Diagnostics	RT-PCR	Multiplex Panel (17 targets)
FilmArray® respiratory panel (RP)	BioFire Diagnostics, Inc.,	Nested multiplex RT-PCR	Multiplex Panel (20 targets)
FilmArray® respiratory panel 2 (RP2)	BioFire Diagnostics, Inc.,	Nested multiplex RT-PCR	Multiplex Panel (21 targets)
FilmArray® respiratory panel® (RP) EZ	BioFire Diagnostics, Inc.,	Nested multiplex RT-PCR	Multiplex Panel (14 targets)
Lyra® parainfluenza virus assay	Quidel Corporation	Real-time RT-PCR	Parainfluenza virus types 1, 2, and 3
Lyra® RSV + MPV assay	Quidel Corporation	Real-time RT-PCR	RSV, MPV
Simplexa™ flu A/B & RSV Kit	Focus Diagnostics, Inc.,	Real-time RT-PCR	Flu A, Flu B, and RSV
Panther fusion flu A/B/RSV	Hologic, Inc.,	Real-time RT-PCR	Flu A, Flu B, and RSV
Panther fusion paraflu assay	Hologic, Inc.,	Real-time RT-PCR	Parainfluenza 1, 2, and 3
Panther fusion AdV/MPV/RV assay	Hologic, Inc.,	Real-time RT-PCR	Adenovirus, MPV, and Rhinovirus
ARIES® flu A/B & RSV assay	Luminex Corporation	Real-time PCR	Flu A, Flu B, and RSV
Simplexa™ flu A/B & RSV direct	Focus Diagnostics, Inc.,	Real-time RT-PCR	Flu A, Flu B, and RSV
Xpert® flu/RSV XC	Cepheid	Real-time RT-PCR	Flu A, Flu B, and RSV
Solana RSV + MPV assay	Quidel Corporation	Isothermal RT-helicase-dependent amplification (HDA)	RSV, MPV
Illumigene®mycoplasma direct DNA amplification assay	Meridian Bioscience, Inc.,	Loop-mediated isothermal DNA amplification (LAMP)	*Mycoplasma pneumoniae*
Xpert® xpress Flu/RSV	Cepheid	Real-time RT-PCR	Flu A, Flu B, and RSV
Xpert® xpress flu	Cepheid	Real-time RT-PCR	Flu A, Flu B
cobas® Lia tinfluenza A/B & RSV assay	Roche Molecular Diagnostics	Real-time RT-PCR	Flu A, Flu B, and RSV
cobas® Liat influenza A/B assay	Roche Molecular Diagnostics	Real-time RT-PCR	Flu A, Flu B
Alere i influenza A & B 2 test	Abbott Laboratories	Isothermal nucleic acid amplification	Flu A, Flu B
Alere i RSV	Abbott Laboratories	Isothermal nucleic acid amplification	RSV

Table 1. Commercially available nucleic acid amplification assays for respiratory pathogens

1.1.3 Seasonality of respiratory infection

In temperate regions, respiratory viruses mostly show distinct seasonal patterns:

- Typically, Flu viruses occur mainly during winter causing annual-recurrent epidemics in temperate areas, or more irregularly outbreaks in tropical regions. Aerosol transmission is facilitated in cold and dry climate, which is one reason for the seasonal epidemics during winter (Sundell et al. 2016). However, successive outbreaks are not comparable in magnitude and form with each other (Truscott et al. 2012).
- Comparably, RSV tends to peak during winters in temperate regions while exhibits a wide range of variability in the timing and duration of epidemics in the tropics, where viral circulation is seen primarily during the rainy season (Obando-Pacheco et al. 2018; Rose et al. 2018).
- Surveillance data from CDC's National Respiratory and Enteric Virus Surveillance System (NREVSS) shows that MPV is most active during late winter and spring in temperate climates; a biennial epidemic pattern of early and late MPV infection is suggested.
- PIV-1 and PIV-2 also shows fairly regular biennial seasonality with increased activity while PIV-3 infections have a regular annual seasonal peak between March and June and PIV-4 shows a peak in the last quarter of the year (Zhao et al. 2017).
- HEV typically shows late summer or early autumn seasonality (Fisman 2012).
- Higher incidence for HRV infection has been described from September to November and from April to May, but in some years (and in some geographical areas) spring was reported to be the more important time for HRV transmission (Rossi & Colin 2015). On the other hand, all HRV species have been identified in all months, independently on the climate regions (Jacobs et al. 2013).
- Also HBoV and ADV (Schildgen et al. 2008) infections have not a regular peak, whereas CoV display the marked seasonality typical of other respiratory viruses, with high detection frequencies in the winter months but few or no detections in the summer (Schildgen et al. 2008).

1.2 RESPIRATORY VIRUSES

In the following sections we will briefly summarize the main characteristics of the analyzed respiratory viruses.

1.2.1 Pneumovirus

The *Pneumoviridae* family comprises large enveloped negative-sense RNA viruses. This taxon was formerly a subfamily within the *Paramyxoviridae* but was reclassified in 2016 as a family with two genera, Orthopneumovirus and Metapneumovirus (Rima et al. 2017). As shown in **Table 2**, Orthopneumovirus genus is divided into tree species: Bovine-, Murine- and Human Orthopneumovirus, Metapneumovirus is divided into Avian- and Human Metapneumovirus (Amarasinghe et al. 2018).

Family *Pneumoviridae*		
Metapneumovirus	Avian metapneumovirus	Avian metapneumovirus (AMPV)
	Human metapneumovirus	Human metapneumovirus (MPV)
Orthopneumovirus	Bovine orthopneumovirus	Bovine respiratory syncytial virus (BRSV)
	Human orthopneumovirus	Human respiratory syncytial virus A2 (HRSV-A2)
		Human respiratory syncytial virus B1 (HRSV-B1)
	Murine orthopneumovirus	Murine pneumonia virus (MPV)

Table 2. Classification of *Pneumoviridae*.

Two important respiratory viruses belong to Orthopneumovirus family: Respiratory syncytial virus and Human Metapneumovirus (Collins & Karron 2013).

1.2.2 Respiratory syncytial virus (RSV)

Respiratory syncytial virus (RSV) belongs to the recently described Human Orthopneumovirus genus. RSV was first isolated from nasal secretions of young chimpanzees in 1955 and was initially named 'chimpanzee coryza agent' (Blount et al. 1956). A year later, it was also isolated from two child affected by pneumonia and bronchiolitis (Chanock et al. 1956).

Based on serological differences, RSV can be divided in two subtypes (A and B) (Anderson et al. 1985), which can both circulate independently and co-circulate

(Kneyber et al. 1998). Despite the genomic differences between the two virus subtypes, the relationship with the clinical severity is still controversial (Martinello et al. 2002; Esposito et al. 2015; Liu et al. 2016; Vandini et al. 2017). The clinical severity of RSV infections can vary from mild upper respiratory tract infections to severe bronchiolitis. The most common symptoms of RSV-A are: bronchiolitis, dyspnoea, coryza and gastrointestinal symptoms, while systemic Flu-like symptoms such as chills, myalgia, rash, debility and headache are often associated with RSV-B (Liu et al. 2016).

RSV is worldwide recognized to be the leading cause of acute lower respiratory tract illness in infants and young individuals (Homaira et al. 2016) as well as among elderly and immunocompromised patients, usually not as primary infections (Falsey & Walsh 2005). RSV causes significant paediatric and adult morbidity and mortality, which have a significant economic impact on health care systems. In 2005, at least 66.000 children less than 5 years old died of RSV infection or of complications directly related to RSV infection, the 99% of these cases occurring in developed countries (Nair et al. 2010).

1.2.3 Human metapneumovirus (MPV)

This genus consists of viruses infecting human and avian hosts. Members of the Human metapneumovirus species are divided into four co-circulating subgroups A1, A2, B1 and B2; Subgroup A2 is again subdivided into A2a and A2b (Amarasinghe et al. 2018). Metapneumovirus was firstly discovered in 2001, in a 1-month-old child in the Netherlands with symptoms like those of RSV infection (van den Hoogen et al. 2001). This virus causes respiratory infections in children; with high susceptibility rates in children less than 2 years old, nearly all children have serologic evidence of infection by 5 years of age (Milder & Arnold 2009). Worldwide, MPV infections also occur in elderly and immunosuppressed patients. MPV infection in adults normally shows only mild flu-like symptoms but in some elders it can cause severe complications such as chronic obstructive pulmonary disease (COPD) (van den Hoogen et al. 2001). Studies conducted on solid organ and bone marrow transplant patients suggest that infection with MPV can cause significant morbidity and mortality in immunocompromised hosts (Milder & Arnold 2009).

1.2.4 Human Parainfluenza viruses 1–4 (PIV1 – 4)

Human Parainfluenza viruses are single-stranded, negative-sense RNA viruses in the *Paramyxoviridae* family and circulate globally as four species. These four species are classified into two genera based on their genome, PIV1 and PIV3 as genus Respirovirus, and PIV2, PIV4A and PIV4B as genus Rubulavirus (Amarasinghe et al. 2018). PIV were firstly discovered in the late 1950s from both human and animal species with respiratory symptoms (Fukumi Et Al. 1954; Abinanti & Huebner 1959; Andrewes et al. 1959).

PIV are common community-acquired respiratory pathogens, with a worldwide distribution leading to both acute upper and lower respiratory infections. PIV respiratory infections are generally self-limiting, but infants, children, and immunocompromised hosts could instead develop severe diseases (Linster et al. 2018). PIV has also been considered an important causative agent of virus-induced asthma exacerbations (Holtzman et al. 2004). The bulk of PIV-associated hospitalizations among children aged <5 years occurs from ages 0 to 2 years, with a marked increase among 1- to 2-year-olds (Abedi et al. 2016).

Individual PIV species have been related to different clinical outcomes: PIV1 and PIV2 infection with laryngo-tracheo-bronchitis (croup) and PIV3 with bronchiolitis (Henrickson 2003); PIV-4 cause mild clinical symptoms and is the only PIV that does not cause croup (Frost et al. 2014). Nevertheless, symptoms observation cannot be considered predictive of the PIV species (Schomacker et al. 2012).

1.2.5 Coronaviruses

Human Coronavirus are a group of large enveloped RNA viruses under the *Coronaviridae* family. *Coronaviridae* are divided into four genera (alpha-, beta-, delta-, gamma- Coronavirus) able to infect a wide range of hosts. Human Coronavirus are 229E, NL63 (alpha coronavirus); OC43, HKU1 (beta coronavirus); MERS-CoV (the beta coronavirus that causes Middle East Respiratory Syndrome, or MERS) and SARS-CoV (the beta coronavirus that causes severe acute respiratory syndrome, or SARS) as shown in **Table 3**.

Coronaviriniae Genera	Strains	Year of Discovery	Host
Alpha-coronavirus	CoV-229E	1966	Bats
	CoV-NL63	2004	Palm Civets, Bats
Beta-coronavirus	CoV-OC43	1967	Cattle
	CoV-HKU1	2005	Mice
	SARS-CoV	2003	Palm Civets, Bats
	MERS-CoV	2012	Bats, Camels

Table 3. Classification of human coronavirus. (Adapted from Lim et al. 2016)

CoVs are associated with respiratory diseases of various severity, from the common cold with benign outcomes to life-threatening pneumonia and bronchiolitis, especially in elderly, children and immunocompromised patients. SARS and MERS CoVs, that have clear zoonotic origins, are highly pathogenic causing atypical pneumonia. CoV-SARS was first identified in 2003 (Drosten et al. 2003) and, interestingly, clinical course and outcome of illness are milder in children younger than 12 years of age than in adults (Stockman et al. 2006).

CoV-MERS was first isolated in a patient from South Arabia (Zaki et al. 2012). Since September 2012 to October 2018, WHO notified 2.266 laboratory-confirmed cases of infection with MERS-CoV, leading to 804 MERS-CoV-associated deaths.

1.2.6 Enteroviruses

Enteroviruses, a genus within *Picornaviridae* family, are divided into 15 species, three RV species (RV-A to RV-C) and 13 EV (non-RV EV) species (EV-A to EV-L). Although they are closely related at a genetic level, these viruses have remarkably different phenotypic characteristics.

The tropism of RVs is restricted to upper respiratory airways, except in some rare cases of disseminated disease, whereas EVs can infect a wide range of different cells and cause a wide range of diseases with both mild and high pathogenicity (Royston & Tapparel 2016).

RVs are single-stranded positive RNA (ssRNA) viruses, firstly isolated in the 1950s (Price 1956). RVs are the leading cause of upper respiratory tract infections worldwide (Abedi et al. 2016). In fact, RVs are the primary etiological agents of "common cold" causing all the significant clinical and economic implications of this disease (Stobart et al. 2017). Nevertheless, RVs are frequently cause of relatively minor respiratory

symptoms, and have also been described in the context of severe respiratory tract infections (Lauinger et al. 2013). Moreover, the relationship between wheezing RV infections in early life and development of asthma were reported in high-risk children (Makris & Johnston 2018). Three species of human rhinovirus (HRV) are currently known: HRV-A, HRV-B, and HRV-C (Palmenberg et al. 2009), which comprise 83 HRV-A types, 32 HRV-B types, and 55 HRV-C. HRV-A and HRV-C are generally associated with more severe disease and more asthma exacerbations than HRV-B (Lee et al. 2012). Moreover, HRV-C infections are associated with severe infection in children (Makris & Johnston 2018).

EV can in fact lead to mild symptoms, such as a self-limiting fever, clinical syndromes like hand-foot-mouth syndrome but also encephalitis, myocarditis, poliomyelitis, acute heart failure and sepsis (Lugo & Krogstad 2016). Notably, EV-D68, have also been associated with outbreaks, occasionally resulting in significant morbidity and mortality. EV-68 was first identified in 1962 and has been associated with clusters of severe respiratory illness (Messacar et al. 2015) and with acute flaccid paralysis and cranial nerve dysfunction in children in 2014 (Messacar et al. 2015) and in 2016 (Knoester 2016).

1.2.7 Influenza viruses

Orthomyxoviridae is a family of segmented single stranded negative RNA viruses first recovered in 1933 (Smith et al. 1933). Influenza outbreaks have apparently occurred since at least the Middle Age, but probably also ancient times (Taubenberger & Kash 2010). *Orthomyxoviridae* family is composed by five genera: Influenzavirus A, Influenzavirus B, Influenzavirus C, Isavirus, Quaranjavirus and Thogotovirus as shown in **Table 4**.

Members of Influenza A viruses are pathogens of humans, horses, pigs, mink, seals, whales, and fowl causing high pathogenic zoonotic infections. Until 2018, six major pandemics had been described, typically named after the presumed location of origin: 1889: Russian influenza (H2N2); 1900: Old Hong Kong influenza (H3N8); 1918: Spanish influenza (H1N1); 1957: Asian influenza (H2N2); 1968: Hong Kong influenza (H3N2); 2009: Swine influenza (influenza A [H1N1] pdm09) (De Vlugt et al. 2018).

Influenza B only infects humans and seals and is associated with similar symptoms as type A, but differently from type A, does not cause pandemics. Influenza C viruses infect humans and pigs, rarely causing serious disease.

The member of the genus Thogotovirus and Quaranjavirus are tick-borne arboviruses infecting humans and livestock in Africa, Europe, and Asia. The genus Isavirus is named for its type species, infectious salmon anemia virus (Krammer & Palese 2015).

Genera	Virus	Segments	Lenght	Protein	Host
Alphainfluenzavirus	Influenza A virus (A/California/07/2009(H1N1))	8	13158 nt	11	vertebrates, human
	Influenza A virus (A/Hong Kong/1073/99(H9N2))	8	13498 nt	10	vertebrates, human
	Influenza A virus (A/Korea/426/1968(H2N2))	8	13460 nt	12	vertebrates, human
	Influenza A virus (A/New York/392/2004(H3N2))	8	13627 nt	12	vertebrates, human
	Influenza A virus (A/Puerto Rico/8/1934(H1N1))	8	13588 nt	12	vertebrates, human
	Influenza A virus (A/Shanghai/02/2013(H7N9))	8	13191 nt	12	vertebrates, human
	Influenza A virus (A/goose/Guangdong/1996(H5N1))	8	13590 nt	12	vertebrates, human
Betainfluenzavirus	Influenza B virus (B/Lee/1940)	8	14452 nt	10	vertebrates, human
Deltainfluenzavirus	Influenza D virus (D/swine/Oklahoma/1334/2011)	7	12800 nt	8	Vertebrates
Gammainfluenzavirus	Influenza C virus (C/Ann Arbor/1/50)	7	12906 nt	9	Vertebrates, human
Isavirus	Salmon isavirus	8	12686 nt	10	Vertebrates
Quaranjavirus	Quaranfil quaranjavirus	6	11452 nt	6	Vertebrates
	Wellfleet Bay virus	7	11958 nt	7	
Thogotovirus	Dhori thogotovirus	6	10616 nt	6	vertebrates, invertebrates, human
	Thogoto thogotovirus	6	10461 nt	7	vertebrates, invertebrates, human

Table 4. *Orthomyxoviridae* family

Influenza infections cause characteristic clinical symptoms, like rapid onset of fever, malaise, joint pain and cough, but in children atypical presentation are also common, such as febrile seizures or gastroenteritis. Other complications can include otitis media, myocarditis, dehydration and encephalitis (Esposito et al. 2011).

Influenza viruses are the most common causes of human respiratory infections and one of the major cause of morbidity and mortality. Basing on ECDC Annual

Epidemiological Report for 2017-2018 most influenza viruses detected were of type B, representing a higher level and a longer period of circulation of influenza B viruses compared to previous seasons.

1.2.8 Adenoviruses

The first human adenovirus was isolated in 1953 form adenoidal tissue. The *Adenoviridae* family is comprised of relatively large, icosahedral, non-enveloped viruses with linear, double-stranded DNA, classified into seven species (A to G), 51 serotypes, and over 70 genotypes (Robinson et al. 2013). Due to wing to their genetic heterogeneity, ADVs display broad tissue tropism (Ison 2006) and can infect several cell types causing different diseases (**Table 5**) (Ghebremedhin 2014).
ADV are most commonly associated with pediatric illnesses of the upper respiratory tract, including the common cold, but can also cause gastrointestinal, ophthalmologic, genitourinary, and neurologic symptoms (Lynch & Kajon 2016).

HAvD subgroup	Serotype	Type of infection
A	**12, 18, 31**	gastrointestinal, respiratory, urinary
B, type 1	**3, 7, 16, 21**	keratoconjunctivitis, gastrointestinal, respiratory, urinary
B, type 2	**11, 14, 34, 35**	gastrointestinal, respiratory, urinary
C	1, 2, 5, 6	respiratory, gastrointestinal including hepatitis, urinary
D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49	keratoconjunctivitis, gastrointestinal
E	**4**	keratoconjunctivitis, respiratory
F	40, 41	gastrointestinal
G	52	gastrointestinal

Table 5 *Adenoviridae* family

ADVs are estimated to cause 2 - 5% of the overall respiratory tract infections and 4 - 10% of all pneumonias. (Jobran et al. 2018). These viruses mostly cause chronic low-virulent infection with mild symptoms. In contrast, acute infection is mostly characterized by flu-like symptoms but could also lead to severe lower respiratory tract infection (Murtagh et al. 2009).

1.2.9 Bocaviruses

Human bocavirus (HBoV) is a small non-enveloped single-stranded DNA parvovirus of about 5300 nucleotides isolated in 2005 at Karolinska University (Allander et al. 2005) and found worldwide in both respiratory samples, mainly from children with acute respiratory infection, and in stool samples from patients with gastroenteritis (Bastien et al. 2006).

HBoV is prevalent worldwide in children with respiratory tract infections (Allander 2008) both singularly and in coinfection with other respiratory virus (Jartti et al. 2012) and it is often detected at low vial loads (Allander 2008). Since that high coinfection rate and since HBoV is also detected in respiratory secretions of asymptomatic individuals, there is a wide discussion about the real impact of HBoV in respiratory pathology (Ligozzi et al. 2017).

However, single HBoV infection observed several cases of severe pneumonia, asthma and/or bronchiolitis both in children and in adults (Kupfer et al. 2006; Vallet et al. 2009).

1.3 VIRAL CO-INFECTION

Respiratory viral co-infections, defined as the detection of more than one viral pathogen in the same sample, are detected in 15 to 60% of children with an acute respiratory tract infection (Liu et al. 2015; Martínez-Roig et al. 2015; Finianos et al. 2016).

The most frequently identified viral coinfections are dual RSV-HRV and RSV-HBoV infections (Garcia-Garcia et al. 2017) and subjects coinfected with RSV and at least another virus (especially RSV-HBoV) seems to had an higher rates of pneumonia compared to those with RSV infection alone (Midulla et al. 2010; Goka et al. 2013).

RSV and FLU-A have been mainly identified among children with single virus infection, other viruses, including HBoV, have been mainly reported in children with coinfection (Kuypers et al. 2004; Fairchok et al. 2010; Martin et al. 2010).

The clinical severity of virus coinfection compared to single virus infection remains uncertain, although an increased risk of mortality was observed when coinfection are detected amongst preschool children (Asner et al. 2014; Asner et al. 2015; Lim et al. 2016).

1.4 POLYOMAVIRUSES

1.4.1 Historic overview

The first polyomavirus infection was described in 1953 by Ludwik Gross, which discovered the Mouse polyomavirus (MPyV), a virus able to induce salivary tumours in experimentally exposed mice and, when inoculated into newborn mouse, various tumours in different site (Gross 1953; Stewart et al. 1957). Hence, the Greek name poly for "many" and oma for "tumor".

Many studies had followed this first observation, leading to the discovery the first primate polyomavirus: SV40 (Sweet & Hilleman 1960; Fenyves & Klein 1963). SV40 was identified in kidney cells of African green monkey, used to produce polio- and adenovirus- vaccines, and like MPyV showed oncogenic activities in mouse (Eddy et al. 1962) leading to a big public health concern for individuals receiving SV40 contaminated vaccines by early 1960's (Shah & Nathanson 1976).

The first two polyomaviruses with a natural human tropism, BKHPyV and JCHPyV, were independently identified in 1971 in specimens of immunocompromised patients (Gardner et al. 1971; Padgett et al. 1971). Early after their first isolation, was proposed a role for BKHPyV and JCHPyV in malignant transformation, but the apparent ubiquity of BKHPyV and JCHPyV make the association still controversial (Delbue et al. 2017; Levican et al. 2018).

Since then, many Polyomaviruses where isolated from a lot of different host, such as mammalian, birds, fish, avian, arthropods (Moens, Calvignac-Spencer, et al. 2017), Human Polyomaviruses that will be extensively described in the next paragraphs.

1.4.2 Polyomavirus classification

Polyomaviruses where taxonomically classified as a genus in *Papovaviridae* family until 2000 when, with the publication of the Seventh report of the international committee on taxonomy of virus (ICTV), *Papovaviridae* family was divided into two families: Polyomaviridae and Papillomaviridae.

Because of the growing quantity and variety of HPyVs the International Committee on Taxonomy of Viruses (ICTV) *Polyomaviridae* Study Group designed a rationale, based on the observed distance between large T antigen coding sequences, in order to update the taxonomy of the *Polyomaviridae* family.

Last ICTV update (July 2018) divides Polyomavirus into four genera: Alphapolyomavirus, Betapolyomavirus, Gammapolyomavirus and Deltapolyomavirus that, all together, include more than 80 species.

Human polyomavirus 5, Human polyomavirus 8, Human polyomavirus 12 and Human polyomavirus 13 belong to Alphapolyomavirus genus; Human polyomavirus 1, Human polyomavirus 2, Human polyomavirus 3 and Human polyomavirus 4 belong to Betapolyomavirus genus; Human polyoDNAJ

mavirus 6, Human polyomavirus 7, Human polyomavirus 10 and Human polyomavirus 11 belong to Deltapolyomavirus genus.

1.4.3 Genome organization

Polyomaviruses are small, non-enveloped icosahedral viruses of 40-45 nm of diameter with circular double-stranded DNA and genomes of approximately 5 kbp.

Despite the abundance of different HHPyVs, their genomic organizations are similar. Viral genome is usually divided into two oppositely oriented transcriptional units, the early and late region (EVGR and LVGR) referring to the stage of infection in which they are transcribed, separated by a non-coding control region (NNCR) (Gu et al. 2009).

EVGR encodes the regulatory large and small T antigens (LTAg and STAg), named for their corresponding protein size, that are involved in the coordination of viral replication and gene expression (Ajuh et al. 2018), and, for JCHPyV, BKHPyV and MCHPyV miRNAs (Seo et al. 2009; Bauman et al. 2011). LTAg is a multifunctional protein, with several domains common to all HHPyVs: a DNAJ domain, an origin-binding domain (OBD), a zinc (Zn)-binding domain, and a helicase/ATPase domain (Ahsan 2006). It also binds the tumour suppressor proteins Rb and p53, modulating a variety of cell cycle activities critical for tumour formation (De Caprio 2009).

LVGR encodes the structural VP capsid proteins. The capsid comprises 72 pentamers (capsomeres) of VP1, the major structural subunit of the polyomavirus capsid, associated with a single molecule of VP2 and, in some HHPyV also VP3, VP4 and a small non-structural cytoplasmatic protein called agnoprotein which may have transforming activities itself (Gerits & Moens 2012; Baez et al. 2017).

NNCR is the less conserved genomic region among HPyV which contain numerous transcription factor binding sites (TFBS) and harbours the origin of viral replication and bi-directional promoter/enhancer (Barth et al. 2016).

1.4.4 Human Polyomavirus and disease

Until 2007, the only two known Human Polyomavirus were BKHPyV and JCHPyV. JCHPyV was identified in a patient, John Cunningham (initials JC), with a history of Hodgkin's lymphoma and progressive multifocal leukoencephalopathy (PML) (Padgett et al. 1971).

JCHPyV infection is typically acquired in childhood (10-15 years of age) and is asymptomatic, resulting in a seroprevalance ranging between 50-90% worldwide by adulthood (Egli et al. 2009; Brew et al. 2010).

JCHPyV in immunosuppressed patients is mostly associated with a demyelinating disease of the brain, the progressive multifocal leukoencephalopathy (PML) (Tan & Koralnik 2010; Saribas et al. 2010; Brew et al. 2010).

JCHPyV reactivation is also implicated in pathogenesis of colorectal cancer (Goel et al. 2006; Jung et al. 2008; Selgrad et al. 2008) and with Multiple Sclerosis (Khalili et al. 2007).

During the same year, Brennan-Krohn virus (BKHPyV) was isolated from the urine of a Sudanese kidney transplanted patient with ureteric stenosis (Gardner et al. 1971). BKHPyV virus primary infection often occurs in childhood (3 - 4 years of age) and is usually first asymptomatic. Consequently, seropositivity across the adult population is as high as approximately 90% (Egli et al. 2009), with intermittent reactivation throughout life.

BKHPyV reactivation is associated with complication in immunocompromised host in particular with nephropathy (BKVAN), including haemorrhagic and non-haemorrhagic cystis, haematuria, urethral stenosis and interstitial nephritis. Less commonly, BKHPyV infection can lead to liver disease, pulmonary manifestation such as pneumonitis and ophthalmological and neurological complication wit retinitis and meningoenphalitis (Rajpoot et al. 2007; Dropulic & Jones 2008).

Since 1971, 11 new species of human polyomaviruses were identified. In 2007 two polyomaviruses have been firstly identified, by high-throughput sequencing of DNA from nasopharyngeal samples, KI polyomavirus (KIHPyV) (Tobias Allander et al.

2007) and WU polyomavirus (WUHPyV) (Anne M Gaynor et al. 2007), that received their name by the institutions of their first discovery (Karolinska Institute, Stockholm, Sweden and Washington University St. Louis, USA). Characteristics of KIHPyV and WUHPyV will be extensively discussed in next chapter.

In 2009, two new polyomaviruses were found in association with human diseases: Merkel cell polyomavirus (MCHPyV) and TS polyomavirus (TSHPyV).

MCHPyV was detected from the tissues of patients with Merkel cell carcinoma (MCC) (Feng et al. 2008) and then strongly associated to this a rare, aggressive, neuro-endocrine tumour mostly observed in elderly or immunocompromised patients (Arora et al. 2012; Richards et al. 2015).

It also has been suggested a common nature between MCC and chronic lymphocytic leukaemia (CLL) (J. Kaae et al. 2010; Koljonen et al. 2010; Jeanette Kaae et al. 2010), but this second association is still controversial (Pancaldi et al. 2011).

TS polyomavirus (TSHPyV) was identified in association with Trichodysplasia Spinulosa (van der Meijden et al. 2010). Trichodysplasia spinulosa is a rare skin disease exclusively found in severely immunocompromised patients, especially solid organ transplant recipients (Elaba et al. 2012; Bagasi et al. 2018).

Many other Polyomaviruses were then discovered, but with low or no association with disease or malignant transformation. Human polyomavirus 6 (HPyV6) and human polyomavirus 7 (HPyV7) where first identified in 2010 (Schowalter et al. 2010), and recently HPyV7, but not HPyV6, has been linked to a pruritic skin eruption in immunosuppressed patients (Nguyen et al. 2017).

Also, for human polyomavirus 9 (HPyV9) (Scuda et al. 2011), human polyomavirus 10 (HPyV10) (Yu et al. 2012), human polyomavirus 11 (HPyV11) (Lim et al. 2013), human polyomavirus 12 (HPyV12) (Mishra et al. 2014) and human polyomavirus 13 (HPyV13) (Mishra et al. 2014) pathogenic role is mostly still unclear, even if a correlation with diarrhea was hypothesized (Barth et al. 2016; Vanchiere et al. 2016).

HPyV9 was originally isolated from a kidney transplant recipient HHPyV9 as an emerging infection in immunosuppressed kidney transplanted patients, causing both systemic and skin infection. (Scuda et al. 2011) Whether HHPyV9 is pathogenic in immunocompromised patients, alone or in concert with the well-known pathogen BKHPyV, the association deserves further study (Nguyen et al. 2017).

In 2017 a new putative member of the family, the Human Polyomavirus 14 or Lyon

IARC PyV (LIPyV), has been described, awaiting further confirmation as a member of HPyV (Gheit et al. 2017).

1.5 KIHPyV AND WUHPyV

Since first identification of KIHPyV and WUHPyV, viral sequences where confirmed in respiratory specimens worldwide, leading a possible association between upper and/or lower respiratory tract infections and the presence of these two viruses (Anne M. Gaynor et al. 2007; Wattier et al. 2008).

In adult population these two viruses seem to be more frequently associated to upper tract infection, else in the youngest one to lower tract respiratory infection (Abedi Kiasari et al. 2008).

Various other biological specimens and tissue have been screened for the presence of KIHPyV and WUHPyV.

Recently, a Hungarian study described the presences of both KIHPyV and WUHPyV in tonsils and adenoids and evidence of detection of WUHPyV into the middle ear were (Csoma et al. 2018).

Same authors also reported the presence of KIHPyV in respiratory, blood and urine samples collected from renal transplant patients and presence of WUHPyV in in respiratory and blood samples (Csoma et al. 2015) but not in in kidney and urinary bladder tumor tissue samples (Csoma et al. 2016).

No WUHPyV was detected in either respiratory samples, blood or urine of pregnant and non-pregnant woman; the same study reported KIHPyV DNA detection in 2% of non-pregnant woman (Csoma et al. 2012).

In another study, involving fecal, urine, blood, cerebrospinal fluid and respiratory samples, the two viruses were primarily founded in NPAs, BALs, and feces of young and immunocompromised patients, but not in urine and in CSF (Bialasiewicz et al. 2009).

Presence of KIHPyV and WUHPyV in the CSF is still controversial. No WUHPyV or KIHPyV DNA has been reported analysing the CSF of patients with or without PML (Bialasiewicz et al. 2009; Giraud et al. 2009; Dang et al. 2011), but Barzon and colleagues reported presence of the two viruses in HIV-1 positive patients with and without progressive multifocal leukoencephalopathy (Barzon, et al. 2009). These

different findings are probably due to the differences in patients enrolled for the studies and to the lack of standardized methods for WUHPyV and KIHPyV detection. Also, other authors investigated presence of the two viruses in HIV-positive patients (Babakir-Mina, et al. 2009; Bialasiewicz et al. 2009; Tatiana F Robaina et al. 2013; Nunes et al. 2014), but no correlation between the presence of the two viruses and HIV-1 viral load or CD4+ count was found (Mourez et al. 2009).

Other studies reported the detection of the two polyomaviruses in gastrointestinal tract and stool of patients with clinical symptoms, often in coinfection with another pathogen virus (Babakir-Mina, Ciccozzi, Alteri, et al. 2009; Ren et al. 2009). These data, although are not enough for a causal association between these viruses and gastrointestinal disease, could suggest a trend of association with diarrhea and vomiting, proposing oro-fecal as transmission route of these viruses (Babakir-Mina et al. 2013).

WUHPyV and KIHPyV DNAs were also detected in saliva samples of healthy volunteers, posing the hypotheses that saliva may be a route for transmission, and that the oral cavity could be a site of virus replication as for BKHPyV (Tatiana F. Robaina et al. 2013).

Seroepidemiological studies showed that infection by these viruses is widespread in the human population: WUHPyV and KIHPyV positive samples were found in Australia (Anne M Gaynor et al. 2007; Bialasiewicz et al. 2009); France (Touinssi et al. 2016); South Corea (Han et al. 2007); Germany (Neske et al. 2010); Philippine (Rao et al. 2016); Brazil (Pena et al. 2018); Italy (Babakir-Mina et al. 2013) etcetera; with high detection rate in adults population (Nguyen et al. 2009) suggesting that infection occurs early in childhood (Moens, Krumbholz, et al. 2017).

As for other polyomaviruses, reactivation of the two viruses seems to be related to immunosuppression, including stem cell transplanted (Sharp et al. 2009; Kuypers et al. 2012) and, as previously mentioned, HIV-1 positive patients.

In patients with respiratory symptoms, the prevalence of KIHPyV varied from 0, 5 to 7%, else WUHPyV DNA genome is even detected more commonly, in fact the prevalence varied from 3 to 9% (Abedi Kiasari et al. 2008; van der Zalm et al. 2008; Essa et al. 2015; Gozalo-Margüello et al. 2015; Zhu et al. 2017a; Essa et al. 2017; Zhu et al. 2017b).

Detection of KIHPyV and WUHPyV genome in respiratory samples is often related to a high co-infection rate with other major respiratory diseases such as RSV, PIV, ADV, HRV and Flu Viruses (Debiaggi et al. 2012; Moens et al. 2013) making hard the correlation between viral DNA presence and disease.

Little is known about the oncogenicity of KIHPyV and WUHPyV (Prado et al. 2018). KIHPyV detection has been reported in lung cancer tissue, but the meaning of this finding remains unclear (Teramoto et al. 2011).

No KIHPyV or WUHPyV was detected in neuroendocrine tumor (Duncavage et al. 2009), urinary bladder tumors (Csoma et al. 2016) and melanoma (Giraud et al. 2008).

1.5.1 Genomic differences among WUHPyV, KIHPyV and other HHPyVs

Although the similar genomic structure of the entire human described human polyomaviruses, there are significant sequence differences between them that have affected at least on their life cycle and host cell tropism (DeCaprio & Garcea 2013).

KIHPyV (5040 bp) (T. Allander et al. 2007) and WUHPyV (5229 bp) (Anne M. Gaynor et al. 2007) genome sizes are within the range of other polyomaviruses. The two genomes, shown in Figure 1, have the highest sequence identity between each other, but results quite divergent from other polyomaviruses (Rima et al. 2017).

Interestingly, high divergence occurs in LTAg critical domains, in p53- and in DNA-binding sites of T-antigens: KIHPyV and WUHPyV show high aminoacidic identity both in T-antigen (70%) and P53/DNA-binding (68%), else, if compared with JCHPyV and BKHPyV, show high divergences (from 30 to 50%).

Hyper variability of LTag binding site affects viral transcription, replication and cytopathology in cell cultures and is also related to different ability to bind p53 and consequently to the different oncogenic value of HPyV.

In addition, KIHPyV and WUHPyV show a high homology rate between themselves (68%) and a higher difference with other HPyV (almost 30% of homology) (Johnson 2010) in VP1 protein sequences.

This VP1 homology comparison may reflect differences in tissue tropisms among the different classes of polyomaviruses (Moens, Krumbholz, et al. 2017).

Moreover, unlike the other HPyV, BKPyV and JCHPyV encode for the agnoprotein, which has a critical role in the regulation of viral gene expression and replication, in the modulation cell cycle progression and in DNA repair (Khalili et al. 2005).

In addition, viral encoded miRNAs, which have the ability to negatively regulate the expression of viral gene expression, have been found to be encoded by BKHPyV, JCHPyV and MCHPyV (Chen et al. 2015) but not by KIHPyV and WUHPyV.

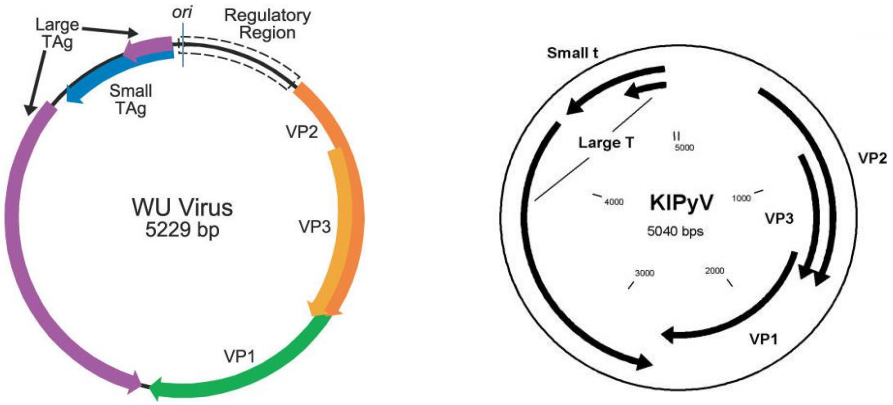

Figure1. KIHPyV and WUHPyV, from (T. Allander et al. 2007) and (Anne M Gaynor et al. 2007).

1.5.2 KIHPyV and WUHPyV diagnosis and treatment

KIHPyV and WUHPyV were discovered by Random PCR amplification, large-scale sequencing, library construction and bioinformatics analysis (Allander et al. 2005).

Since then, various detection methods for these two viruses have been developed, mostly molecular ones. In fact, almost all the previously described KIHPyV and WUHPyV-DNA detection methods are based on PCR amplification. Differences in sensibility and specificity of the assays can be ascribed to the choice of target region and to the choice of primer and probe sequences (Bialasiewicz et al., 2007a, Bergallo et al., 2009): primers/probes designed in the VP1 region give a higher detection rate than the primers/probes designed in the regulatory or sTAG (Bergallo et al., 2009).

In addition, several enzyme-linked immunosorbent assays (ELISA) tests had been developed for human VP1 antibodies, reporting the high serological prevalence of the two viruses (Nguyen et al. 2009).

Anyway, based on the currently available data, KIHPyV and WUHPyV routine testing of respiratory samples from immunocompromised have been suggested (Kuypers et al. 2012).

As the hypothesis that these two viruses do play an etiological role in childhood respiratory-tract disease is still controversial, no treatments regimen for KIHPyV and WUHPyV have still been implemented.

2. AIMS OF THE STUDY

Respiratory infections are one of the major causes of morbidity and mortality. These diseases affect the respiratory tree and cause a spectrum of diseases ranging from rhinitis to pneumonia. In particular, the groups of patients more susceptible to these diseases are children, patients with immunodeficiency and the elderly.

If we consider the number of cases per year and the pathogenic viruses that cause these infections, we can observe how high the clinical and social impact of viral respiratory infections is.

Cell cultures were the first diagnostic methods employed for the detection of respiratory viruses. This approach was overcome by the advent of molecular methods. Several molecular formats are currently in use for the identification of many respiratory viruses, in particular Multiplex PCR systems that lead to the simultaneous amplification of selected viral panels.

Despite their undoubted usefulness, these methods do not completely satisfy the need for diagnosis of newly characterized viruses, such as the recently discovered KIHPyV and WUHPyV. These two viruses have been associated with respiratory diseases, even though their real clinical significance is still debated.

In order to better investigate the role of these viruses, their epidemiology and diagnosis, we set up a molecular method for the detection of KIHPyV and WUHPyV infections that could be added to the multiplex PCR methods used in the screening and diagnosis of viral respiratory infections. To do so, we developed a specific, TaqMan–based real-time PCR diagnostic method for simultaneous detection of KIHPyV and WUHPyV in respiratory samples. To validate this method, we applied it on the NPA collected between October 2016 and October 2018 in the hospital of Verona from a cohort of pediatric patients with respiratory symptoms, previously analyzed by Multiplex PCR for the presence of 16 major respiratory viruses.

3. MATERIAL AND METHODS

3.1. CLINICAL SAMPLES

Nasopharyngeal aspirate (NPA) samples were collected from 831 hospitalized children (age <5 years) with symptomatic respiratory tract infections. These specimens were collected between October 2016 and October 2018 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 -70°C prior to processing.

These samples were analyzed for respiratory virus with Allplex™ respiratory panel assay kit (Seegene, Seoul, South Korea) and retrospectively with home-made TaqMan-based duplex real-time PCR for the detection of KIHPyV and WUHPyV genomes.

3.2 NUCLEIC ACID EXTRACTION AND PURIFICATION

Nucleic acids extraction from samples was carried out with a Microlab Nimbus apparatus (Hamilton Robotics, Reno, NV, USA). The procedure is based on reversible adsorption of nucleic acid to paramagnetic beads under appropriate buffer conditions. Lysis of NPA samples is performed with SDS/Proteinase K solution (LB) and Binding Buffer (BB) is added to adjust binding condition under which nucleic acids can bind the paramagnetic beads. After magnetic separation, the paramagnetic beads are washed with four Washing Buffer (WB, WB1, WB2, and WB3) to remove salt, contaminants and PCR inhibitors. Finally, purified nucleic acid can be eluted with low salt Elution Buffer EB (10mM Tris-EDTA pH 7.5).

Briefly, nucleic acids were extracted adding 350 µL of NPA to 340 µL of LB, 25 µL of proteinase K and 10 µL of internal control provided in the extraction kit. This mixture was incubated at 56°C for 5 min with 25 µL of silica and 1160 µL of BB. When all the magnetic beads have been attracted to the magnets of magnetic separator supernatant is removed. Magnetic beads are than washed with 600 µL of WB1, separated with magnetic separator, supernatant is discarding, and the same operation is performed with 600 µL of WB2. Two more washing steps are performed with 600 µL of WB (82% Ethanol) and 900 µL of WB3 (water-based). Nucleic acid was then incubated for 3 minutes at 56 C with 100 µL of EB recovered in microtubes. The purified nucleic acids were stored at -70°C until use.

3.3 Allplex™ respiratory panel assay kit detection procedure

The samples were analyzed for respiratory virus with Allplex™ respiratory panel assay kit (Seegene, Seoul, South Korea). Allplex™ technology is based on MuDT™ (Multiple Detection Temperatures) Technology, which enables the detection of multiple target in a single fluorescence channel. MuDT™ algorithm comprises of DPO™ and TOCE™ enable assay for the detection of fluorescence intensity and multiple temperature.

DPO™ technology provide specific multiplex amplification because of a priming structure that is composed of two priming regions: a 5' conserved (longer, 18–25 nt) region and a 3' specific (shorter, 6–12 nt) region connected by a poly-deoxyinosine stretch that bridges the two segment pairs. The poly-deoxyinosine linker would form a bubble- like structure and separate a single DPO™ primer into two functional regions that, because of the different size, leads to different annealing temperature. As 5' portion is approximately two times longer than 3' has a higher melting temperature and will preferentially bind the target DNA first during PCR acting as a "stabilizer" and initiating the annealing to the target sequence. In contrast, the short 3'-segment cannot bind target DNA by itself at standard PCR annealing temperatures so act as a "Determiner" specificity, because his binding is required for the elongation of DPO™ primer.

DPO™ primer are, together with Pitcher and Catcher, a key component of TOCE™ technology, for establishing arbitrary melting temperature to identify different target. Pitcher is a tagging oligonucleotide composed by a targeting portion, which hybridize specifically the target region, and a tagging portion, designed not to bind target region. Catcher is a fluorescently a dual-labeled and single-stranded artificial template with a portion complementary to the Pitcher's tagging region. During PCR extension, the unbound 5' portion (tagging portion) of the Pitcher oligonucleotide is cleaved off by Taq DNA polymerase exonuclease activity. Tagging portion is than released and anneals to the complementary portion of the Catcher. Extension of the Tagging portion on the Catcher template results in a Duplex Catcher causing an increasing of the physical distance of the fluorescent reporter from the quencher, thereby generating signal that is directly correlated to the quantity of the target DNA.

Since the Catcher is constructed to have a designated Tm, this expected specific Tm value can be controlled by adjusting length and composition of the Catcher and will not be affected from variations in the target sequence.

In addition, Catchers with unique Tm profiles can be detected in the same reaction and in the same color channel, significantly increasing the number of targets that can be simultaneously detected in the conventional four-channel Real-Time thermocycler.

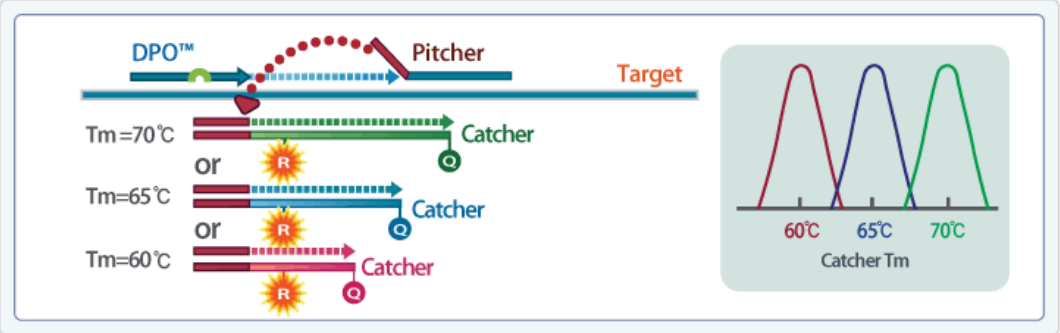

Figure 2. A representative example of Pitcher-Catcher designs to differentiate between three unique melting curves for three unique targets in a single channel, with Tm values ranging from 60°C, 65°C, and 70°C.
Modified from http://seegene.com/neo/en/introduction/core_toce.php

Implementing DPO™ and TOCE™, MuDT™ technology can detect multiple targets in a single fluorescence channel using Detection Temperature and without requiring the melting curve analysis. Detection temperatures are defined as the temperature where unquenched fluorescence signal is measured at each cycle during Real-time PCR reaction. As with TOCE technology different target can be designed with different Tm (as shown in **Figure 2**) relatively different Detecting Temperature can be selected for the collection of fluorescent signals. At the lower Detection Temperature signal of all the targets will overlap, whereas at higher Detection Temperature only signal of the target with the higher temperature will be detected. To discriminate between overlapping signal and find the real Ct value of all the single target MuDT™ algorithm exactly subtract fluorescence signal from the target designed with the lower Detection Temperature from other florescence signal collected at the same temperature. MuDT™ algorithm than converts subtracted fluorescence signal Δ RFU (Relative fluorescence Units) into a Ct enabling the measurement of target Ct values by analyzing fluorescence signals collected at different detection temperatures.

Allplex™ respiratory panel assay kit is able to detect two DNA viruses and fourteen RNA viruses including human adenovirus (ADV) and human Bocavirus (HBoV) 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) without subtype it, human respiratory syncytial viruses A and B (RSV A, RSV B), human bocaviruses 1/2/3/4 (HBoV1/2/3/4) without subtype it , human coronaviruses 229E, NL63 and OC43 (CoV229E, CoVNL63, CoVOC43), human metapneumovirus (MPV), and human enterovirus (HEV) for RNA viruses. Three detection panels compose AllplexTM respiratory panel assay kit: 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, CoVOC43, CoV229E, CoVNL63, and HRV.

Real-time PCR reaction mixture was prepared as follows: the extracted nucleic acid (8 µl), 17 µl of one -step RT -PCR premix.

TOCE™ assay was performed using CFX96 real-time PCR detection system (Bio-Rad Laboratories, Richmond, VA, USA) as follows:

- initial reverse transcription step at 50°C for 20 min
- 1 cycle of initial denaturation at 95°C for 15 min
- 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 1 min, and extension at 72°C for 10 s (fluorescence detection at 60 °C and 72 °C)

The validation of DNA extraction and amplification was performed using an internal control (IC) and negative and positive controls.

Bacteriophage MS2 was added as an IC to clinical specimens before nucleic acid extraction and was incorporated into the product as an exogenous whole process control, in order to monitor all the analyses, from nucleic acid isolation to result interpretation. The IC was co-amplified with the target nucleic acids within the clinical specimens a positive test result was defined as a well-defined exponential fluorescence curve that crossed the CT at a value of <42 for individual targets.

3.4 DUPLEX REAL-TIME PCR

The amplification was carried out in a Bio-Rad CFX96 real-time PCR system.

To perform simultaneous amplification and quantification of two target sequences in a single qPCR assay two reporters are required, in order to distinguish each reaction. Reporter fluorophores had to be chosen with minimally overlapping emission spectra, compatible with the excitation and emission filters of your real-time PCR detection system. We decide to use FAM™ (emission spectra 517 nm) and VIC® (emission spectra 551 nm) dye-labeled TaqMan® probes.

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 KIHPyV or WUHPyV genome contents.

Exact values were employed for calculations, excluding the decimal values. The efficiency of the assay should be 90–105%, the R2 of the standard curve should be >0.980 (or r > –10.990).

3.4.1 Bioinformatics analysis

BLAST (http://www.ncbi.nlm.nih.gov/BLAST) searches were performed to check for the presence of known sequence variations and to avoid any cross homology both to other types of polyomaviruses and to human sequences.

Multiple Sequence alignment (MSA) was performed using Clustal Omega https://www.ebi.ac.uk/Tools/msa/clustalo, an offline multiple sequence alignment program. Phylogenetic tree based on the Neighbour Joining method was constructed using the alignment scores with ClustalW2 Simple Phylogeny program https://www.ebi.ac.uk/Tools/phylogeny/simple_phylogeny/ and with the graphical editor TreeDyn http://www.treedyn.org/.

Mfold (http://unafold.rna.albany.edu/?q=mfold) was used to predict whether the amplicon would form any secondary structure at the annealing temperature.

Primers applied were designed by using:

Primer3 (http://primer3.ut.ee/)

and analyzed using:

OligoAnalyzer by IDT (http://eu.idtdna.com/calc/Analyzer/application/oligoanalyzer).

3.4.2 Target sequence selection

Phylogenetic comparison of sequences for each viral protein clearly groups KIHPyV and WUHPyV together in a new branch (Dalianis, 2009); the two viruses show a high similarity in the high conserved VP1 major capsid protein region. (Delianis, 2013).

To analyse VP1 target region of KIHPyV and WUHPyV we perform Multiple Sequence Alignment and we generate phylogenetic tree based on the Neighbour Joining method using the ClustalW2 Simple Phylogeny program and TreeDyn, a graphical editor for binary trees (shown in **Figure 3**)

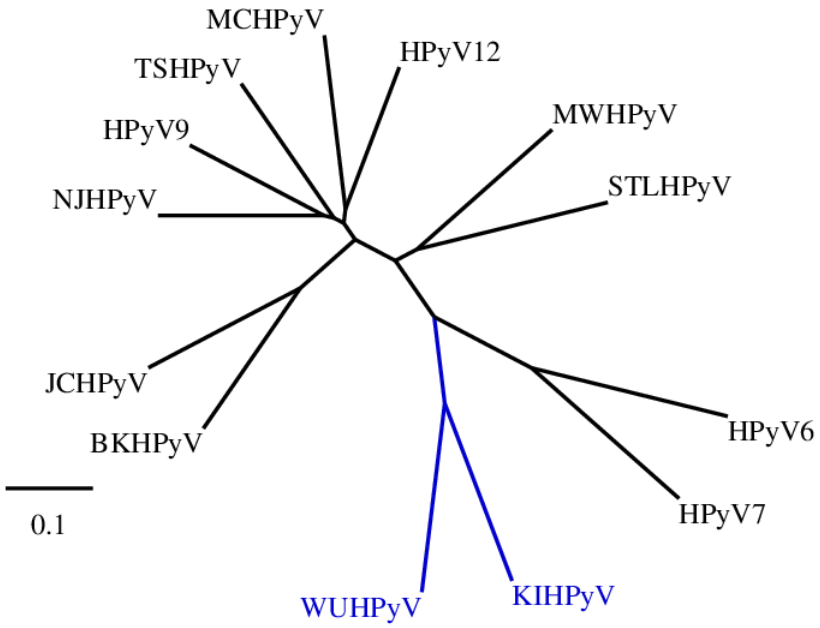

Figure 3. Phylogenetic tree of known Human Polyomavirus.

We perform BLAST searches to check for the presence of known sequence variations and to avoid any cross homology to both other types of polyomaviruses and human sequences. In order to confirm the high degree of similarity of KIHPyV and WUHPyV candidate target region VP1 against another human polyomavirus (Table 6) we performed an MSA using Clustal Omega.

Name	VP1 genome position	Reference Sequence
KIHPyV	1498 to 2634	NC_009238.1
WUHPyV	1670 to 2279	NC_009539.1
BK polyomavirus	1564 to 2652	NC_001538.1
JC polyomavirus	1469 to 2533	NC_001699.1
Merkel cell polyomavirus	1156 to 2427	NC_010277.2
Human polyomavirus 6	1287 to 2450	NC_014406.1
Human polyomavirus 7	1305 to 2447	NC_014407.1
Trichodysplasia spinulosa-associated polyomavirus	1311 to 2438	NC_014361.1
Human polyomavirus 9	1443 to 2558	NC_015150.1
MW polyomavirus	1353 to 2564	NC_018102.1
STL polyomavirus	1242 to 2447	NC_020106.1
Human polyomavirus 12	1405 to 2499	NC_020890.1
New Jersey polyomavirus	1062 to 2531	NC_024118.1

Table 6. Polyomavirus reference strains.

After MSA, we generate phylogenetic tree based on the Neighbour Joining method using the ClustalW2 Simple Phylogeny program and TreeDyn, a graphical editor for binary trees.

MSA was also performed to confirm the high degree of similarity between all KIHPyV and WUHPyV deposited strains.

Target regions to amplify were chosen following these criteria:

- Select a short region (in order to obtain a 75–150 bp product) Efficiency of amplification is higher for short PCR products than longer ones, but the PCR product should be at least 75 bp long to easily distinguish it from primer-dimer
- Avoid regions that have secondary structures
- Avoid regions with long (>4) repeats of single bases
- Choose a region that has a GC content of 50–60%

3.4.3 Primers and probe design

Specific primers were selected from conserved regions corresponding to the major capsid protein VP1 major capsid protein of KIHPyV (from nucleotide 1498 to 2634, 378 aa) NCBI Reference Sequence: NC_009238.1 and WUHPyV (from nucleotide 1670 to 2779, 369 aa) NCBI Reference Sequence: NC_009539.1 genome.

Primers were selected using the following parameters:

- Melting temperature (Tm) between 58◦C and 62◦C, Tm values is calculated by using the nearest-neighbor method
- Less than 2◦C difference in Tm between the two primers
- (G+C) content between 40-60%, avoiding repeats of Gs or Cs longer than 3 bases
- Length between 18 - 22 bp
- Avoid the formation of secondary structure of the primers and prevent primer dimer formation, especially at 3'

Probes were designed using Primer3 and selected within the target, possibly to anneal to the strand that has more Gs than Cs and using the following parameters:

- Tm 5–10°C higher than that of the primers
- Length <30 nucleotides
- No G at its 5' end because this could quench the fluorescence signal even after hydrolysis

In order to perform a duplex Real-Time PCR, all primers and probes will be present in one reaction, so we design it following next parameters:

- All primers have approximately the same Tm
- All probes to have approximately the same Tm (~5–10°C higher than that of the primers)
- The different primer and probe sets do not exhibit complementarity to one another

BLAST searches were performed to test the specificity of the probes and primer pairs.

3.4.4 Absolute quantification

Absolute quantification was performed with standard curve method. Standard curve method measures expression through comparison with serial dilutions of known copy number of a selected sequence in a separate PCR assay. Synthetic oligonucleotides (BMR Genomics, Padua, Italy) were designed from nucleotide 2437 to 2518 of KIHPyV NCBI Reference Sequence: NC_009238.1 (82 bp) and from nucleotide 2587 to 2665 of WUHPyV NCBI Reference Sequence: NC_009539.1 (78 bp) (Yang et al. 2012). The oligonucleotides were quantified by spectrophotometry. We used scalar dilutions of synthetic positive control, from $5x10^6$ to $5x10^1$ copies/mL as reference curves.

3.4.5 TaqMan-based duplex real-time PCR assay for KIHPyV and WUHPyV detection

TaqMan-based real-time quantitative PCR was carried out in a total reaction volume of 50µL consisting of 25µL of TaqMan Universal Master Mix (Applied Biosystems, CA, USA), 0.5 µM of each KIHPyV and WUHPyV specific primers, 0.25 µM of KIHPyV and WUHPyV specific probes, 10µL of DNA extracted either from clinical samples or scalar dilutions of synthetic positive control (from 5x106 to 5x101 copies/mL) and 12.5µL of double-distilled water.

Quantitative analysis was determined by interpolating the Ct value of sample on external reference curve obtained with serial concentrations of KIHPyV or WUHPyV synthetic DNA.

3.4.6 Validation of TaqMan-based duplex real-time PCR Assay for KIHPyV and WUHPyV detection

The development and validation of lab-developed assays (LDTs) are strictly regulated by the Clinical Laboratory Improvement Amendments (CLIA). Performance specifications of our LDT test are settled for the following characteristics: analytical sensitivity (limit of detection), precision (replication study) and analytical specificity (cross-reactivity and interference studies) as reviewed from Eileen M. Burd (Burd 2010).

3.4.6.1 Probit analysis

Probit analysis is a type of regression analysis widely used to empirically determining the LOD of an assay when the number of replicated tested at each concentration is low. We used 10 replicates at each concentration, even if it is not necessary to have the same number of replicates for each concentration. Probit analysis transforms the concentration-response curve to a straight line that can then be analyzed by regression using, in our case, maximum likelihood. Endpoint used to compare different concentration is C95, which represents the concentration at which 95% of the samples containing that target concentration are detected as positive.

Data needed for the construction of a graph of the Probit values versus the log of the concentration's analysis are:

- list of the concentrations tested, that will be transformed in log10
- the number of positive samples per each concentration, that will be than converted to Probit values
- the total number of samples tested per concentration

The graph is than constructed and line of regression is fit. The C95 is determined as the inverse log of the concentration associated to Probit value of 9.50.

3.4.6.2 Precision of the assay

In order to assess the repeatability (or within run imprecision) defined as the "the smallest measure of precision and involving measurements carried out under the same conditions" (International Organization for Standardization. 2006. Statistics—vocabulary and symbols, part 1. Probability and general statistical terms. ISO 3534-1. International Organization for Standardization, Geneva, Switzerland. International Organization for Standardization. 1993. International vocabulary of basic and general terms in metrology. International Organization for Standardization, Geneva, Switzerland) of the assay, we performed our assay on serial dilutions of known concentration of KIHPyV or WUHPyV synthetic DNA in triplicate. We use % Coefficient of variation (CV %) as indicator of precision, determined as the ratio of the standard deviation to the mean multiplied by 100 and represents the extent of variability of an assay.

3.4.6.3 Repeatability of the assay

The repeatability of the Duplex real-time RT-PCR assays was evaluated by determining the intra- and inter-assay coefficient of variation (CV). A 10-fold serial dilution of synthetic positive control, from $5x10^6$ to $5x10^1$ copies/mL was tested in triplicate in two independent sessions.

Intra-assay CV% was determined for identical sample triplicates as the ratio of average of each replicate's Ct standard deviation (SD) to the average of each replicate's Ct average x 100.

Inter-assay CV% was determined for identical sample triplicates analyzed in three-separated experiment, performed in three different days as the ratio of SD of the Ct averages for each replicate to the average of the averages for each replicate x100. (Duplex Real-Time RT-PCR Assays for the Detection and Typing of Epizootic Hemorrhagic Disease Virus)

3.4.6.4 Analytical sensitivity

"Analytical sensitivity" is an inherent characteristic of an assay, defined as the capability of an assay to detect the lowest concentration of a given substance in a biological specimen (Saah and Hoover, 1997) and is often referred to as the "limit of detection" (LOD). LOD of our assay is expressed as a concentration (copies/ml) considering that the lower is the value the greater is the analytical sensitivity of the assay. As our assay is a Duplex assay LOD had been calculated both individually for each target (KIHPyV and WUHPyV) tested and using combinations of KIHPyV and WUHPyV synthetic DNA at high (10^6 copies/ml) and low (10^3 copies/ml) concentrations. Combination of high and low concentrations of target show that a high concentration target will not outcompete a low-concentration target present in the specimen and to demonstrate that there are no cross-reactivity or interference between the pathogen and the different primers/probes.

LOD can be empirically determined by testing serial dilutions of known concentration of the target (CLSI/NCCLS, 2004) using Probit, defined as "unit of measurement of statistical probability based on deviations from the mean of a normal distribution".

3.4.6.5 Analytical specificity

"Analytical specificity" refers to the capability of an assay to detect the target of interest, avoiding "false-positive" results and any cross-reactivity that can be caused, in our case, by potentially interfering nucleic acids. As in our assay two target are quantified by the same method, each one can also be considered as a possible interference for the others. Assay specificity was investigated by TaqMan-based duplex real-time PCR amplification of sample negative for both KIHPyV and WUHPyV. In addition, different both positive and negative samples for different respiratory viruses including Flu A virus, Flu B virus, RSV A and RSV B, ADV, MPV, HEV, PIV, HBoV, CoV and HRV and negative for KIHPyV and WUHPyV were targeted for TaqMan-based duplex real-time PCR.

4. RESULTS

4.1 RETROSPECTIVE ANALYSIS OF 831 RESPIRATORY SAMPLES

We have collected 831 nasopharyngeal aspirate specimens from paediatric patients (age <5 years) with respiratory symptoms followed at the Hospital of Verona between October 2016 and October 2018.

These samples were analysed with a multiplex PCR represented by Allplex™ respiratory panel assay kit. This assay can determine the 16 major viruses associated to respiratory infection through three specific panels.

This multiplex amplification technique detected specific genome sequences of one or more respiratory viruses in 646 out of the 831 analysed specimens (77,73%). Interestingly, the most common viruses we found were: RSV (A and B) in 28.8% of positive samples, followed by HRV (26.7%), HBoV (12.23%), AdV (11%), and Flu A/B viruses (6.97%). In **Figure 4** we show the percentage of detected viruses in 831 analysed samples.

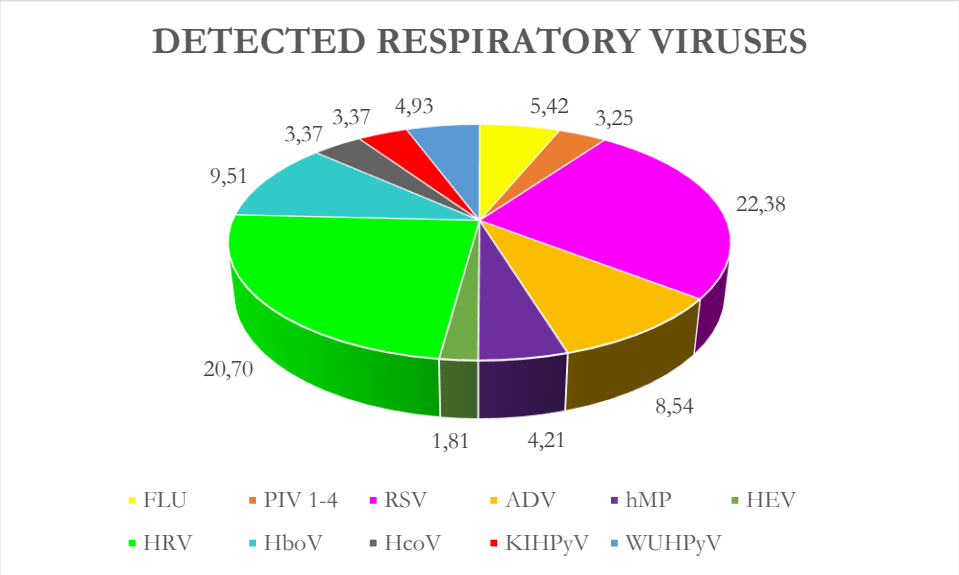

Figure 4. Epidemiology of detected respiratory viruses in 831 analysed paediatric respiratory samples (data are expressed as percentage of the positive samples)

4.2. SEASONALITY OF DETECTED RESPIRATORY VIRUSES

The viral infections of respiratory tract were also analysed as seasonal distribution. The detection rate of respiratory viruses was highest in March (138/831, 21.4% of positive

samples) followed by January (130/831, 20.1% of positive samples) and February (127/831, 19.65% of positive samples), and lowest in October and November (37 and 35/831, respectively 5.7 and 5.4% of positive samples). In **Figure 5** we show the percentage of total detected viruses and their monthly distribution. Moreover, the rates for respiratory viruses in autumn, spring and winter were 9,9%, 49,5% and 40,6%, respectively. These data confirm previous findings that indicated a similar epidemiological distribution of respiratory infections (Cabello et al. 2006; Dey et al. 2013; Leotte et al. 2017; Ge et al. 2018).

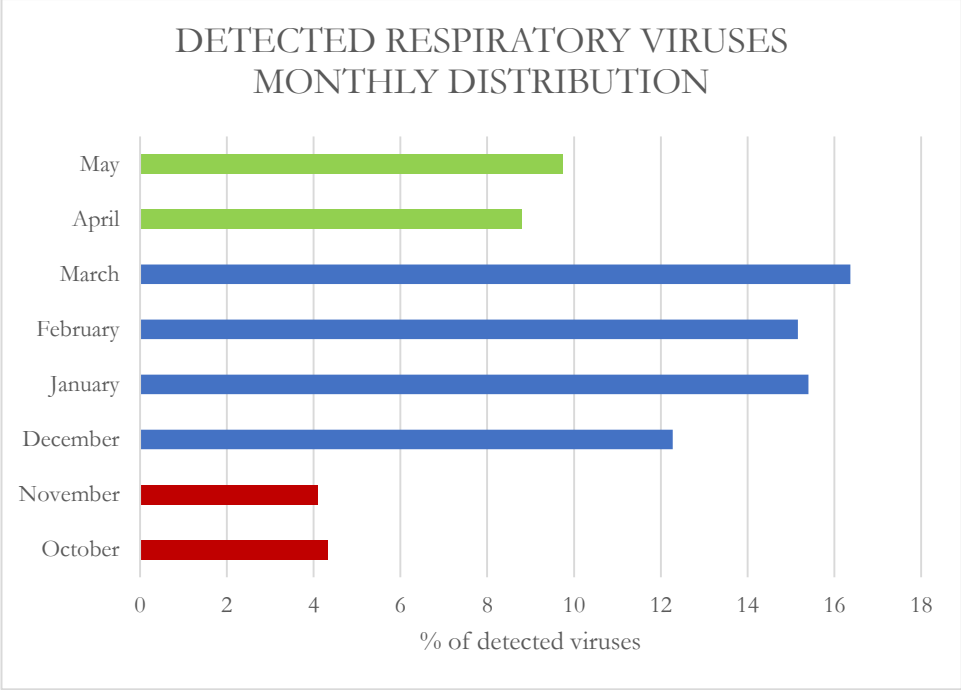

Figure 5. Monthly distribution of detected respiratory viruses. Brick red is used for autumn months, blue for winter months and green for spring months.

The detection of RSV as the most common respiratory virus in infants is also in line with previously reported data, as its winter seasonality (**Figure 6**) (Asner et al. 2014; Weinberg et al. 2009 Kuhdari et al. 2018; Botti et al. 2018).

As shown in **Figure 6**, the pattern of prevalence of HBoV infection (found in 12% of positive samples) resembled the RSV infection seasonality, as previously reported (Manning et al. 2006; Allander et al. 2007).

FLU viruses were found in almost 7% of the 646 positive children's samples, with a strong late fall/early spring seasonality, in line with previously reported findings (Wolf et al. 2006; Committee on infectious diseases 2013; Centers for Disease Control and Prevention (CDC) 2013).

In contrast, HRV (found in the 27% of positive samples) and ADV (11%) are most commonly detected in spring. In addition, HRV is the most frequently detected virus in autumnal season (October/November) (Monto 2002). In **Figure 6** we show seasonal distribution of most commonly detected respiratory viruses (RSV, ADV, FLU, HRV, HBoV)

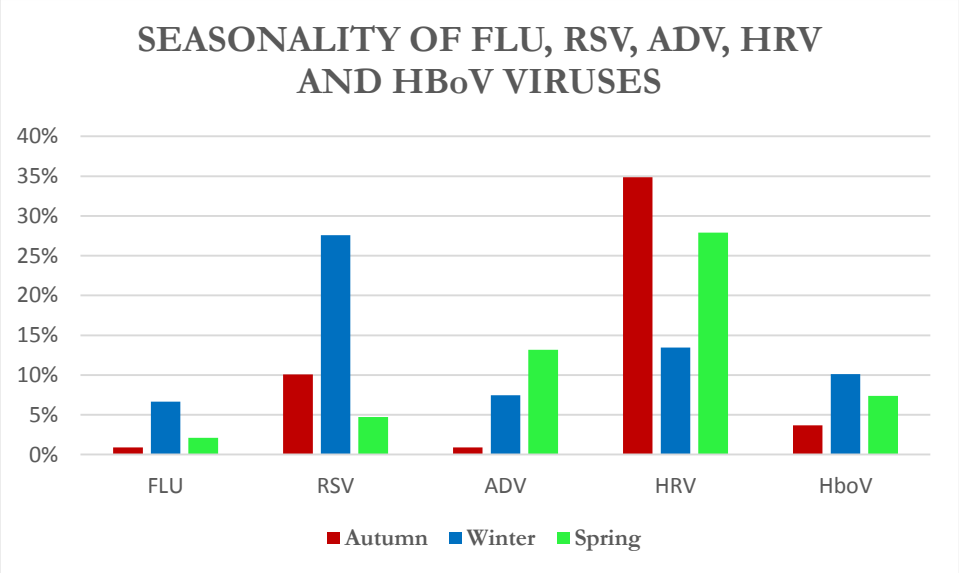

Figure 6. Seasonality of FLU, RSV, ADV, HRV, HBoV viruses

4.3. CO-INFECTION OF DETECTED RESPIRATORY VIRUSES

As reported by several studies, co-infection with respiratory viruses is often detected by molecular methods (Debiaggi et al. 2012 Martínez-Roig et al. 2015).

Indeed, analysis with Allplex™ respiratory panel assay demonstrated that 39% of the positive samples were co-infected with two or more viruses (**Figure 7**)

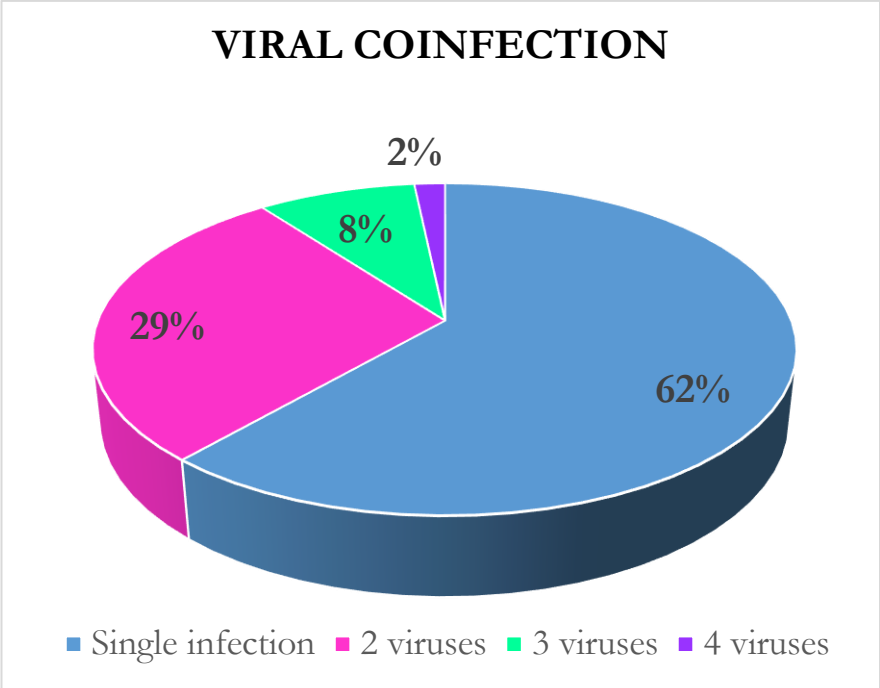

Figure 7. Percentage of viral coinfection in positive respiratory samples

We have also evaluated the seasonality of respiratory viral co-infection. The data we obteined are also in accord with another italian study (Pierangeli et al. 2007), where the viral co-infections occured mainly, but not only, in the coldest months. In **Figure 8** we show the percentage of coinfection in every month.

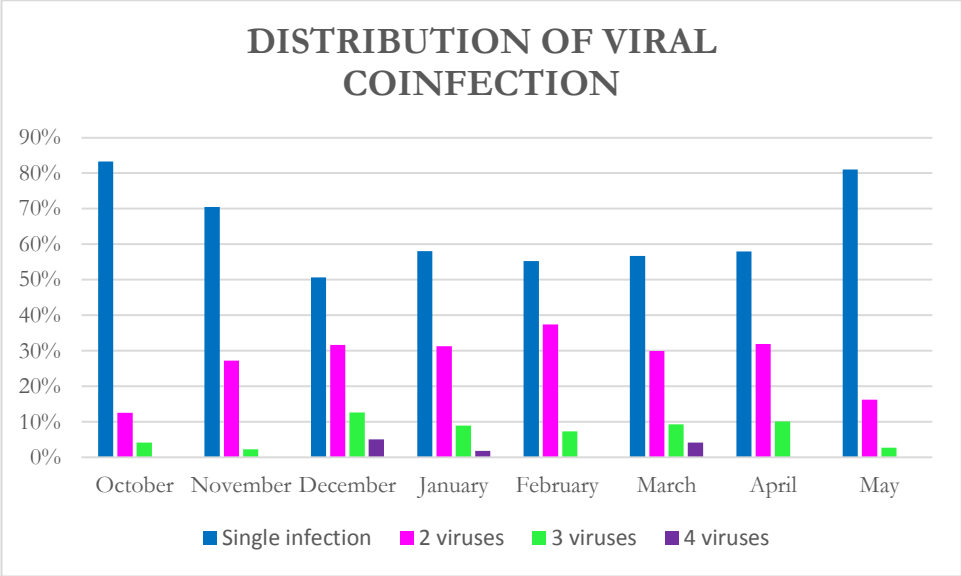

Figure 8. Monthly distribution of viral coinfection

4.4. TaqMan-based duplex real-time PCR assay for KIHPyV and WUHPyV detection set-up

4.4.1. Primer and probe design

Allplex™ respiratory panel assay is a valuable method for the analysis of viral infection in respiratory samples. This assay can detect 16 viruses involved in the respiratory infections, but it is not designed to account for some new respiratory viruses including KIHPyV and WUHPyV polyomaviruses.

Our main aim was to set up a duplex PCR for the KIHPyV and WUHPyV detection in respiratory viruses.

To do so, we evaluated the genomes of KIHPyV and WUHPyV isolates deposited in NCBI database to design specific primers and probes (**Table 6**) for a duplex PCR using NCBI BLAST program.

We have selected conserved sequences of KIHPyV and WUHPyV VP1 regions and performed BLAST (http://www.ncbi.nlm.nih.gov/BLAST) searches to check for the presence of known sequence variations and to rule out any cross homology to both other types of polyomaviruses and human sequences.

In addition, we have compared the selected sequences of primers and probes against the sequences of all KIHPyV and WUHPyV deposited strains and of other human polyomavirus (**Table 6**).

We also performed a multiple sequence alignment (MSA) using Clustal Omega as shown in **Figures 9, 10, 11**.

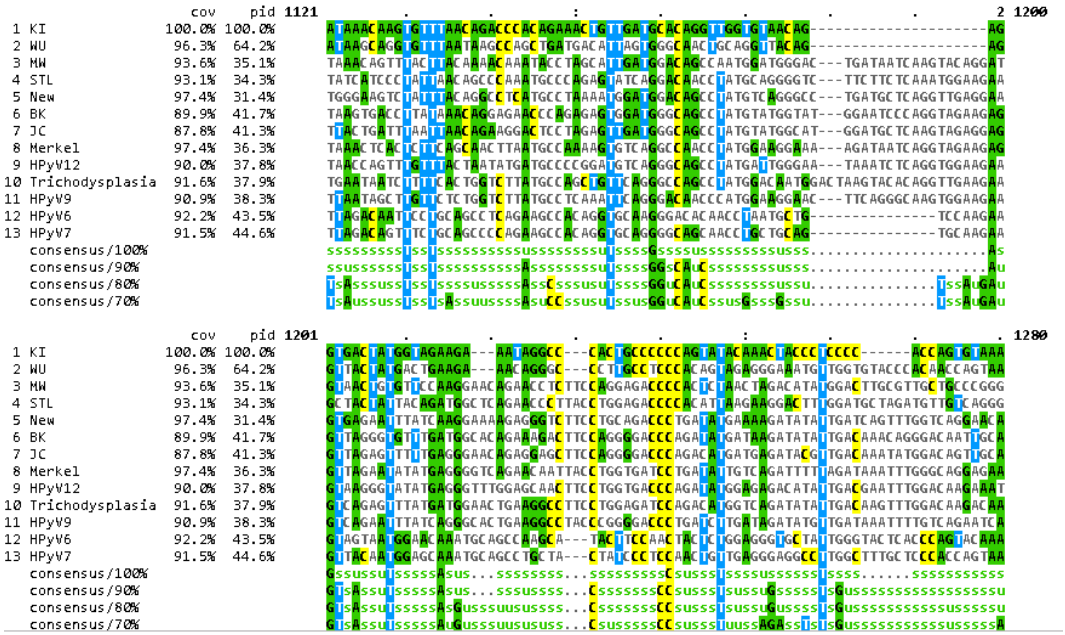

Figure 9. MSA of VP1 against another human polyomavirus

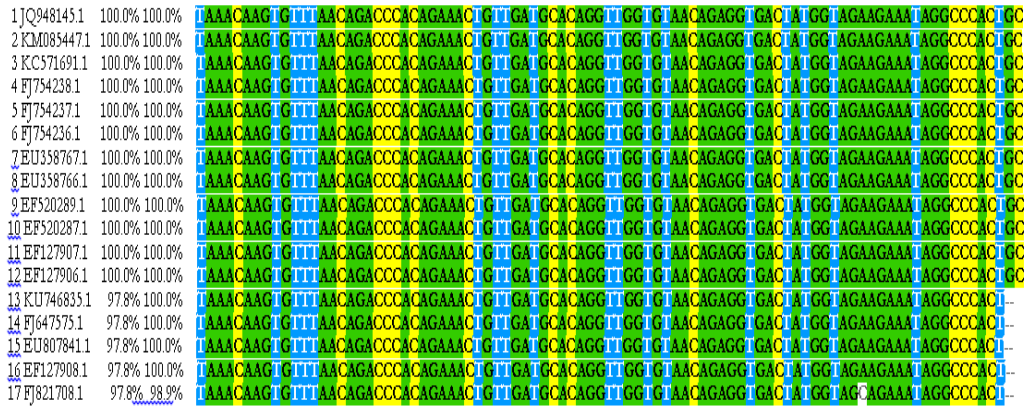

Figure 10. MSA of VP1 target region against deposited KIHPV strain

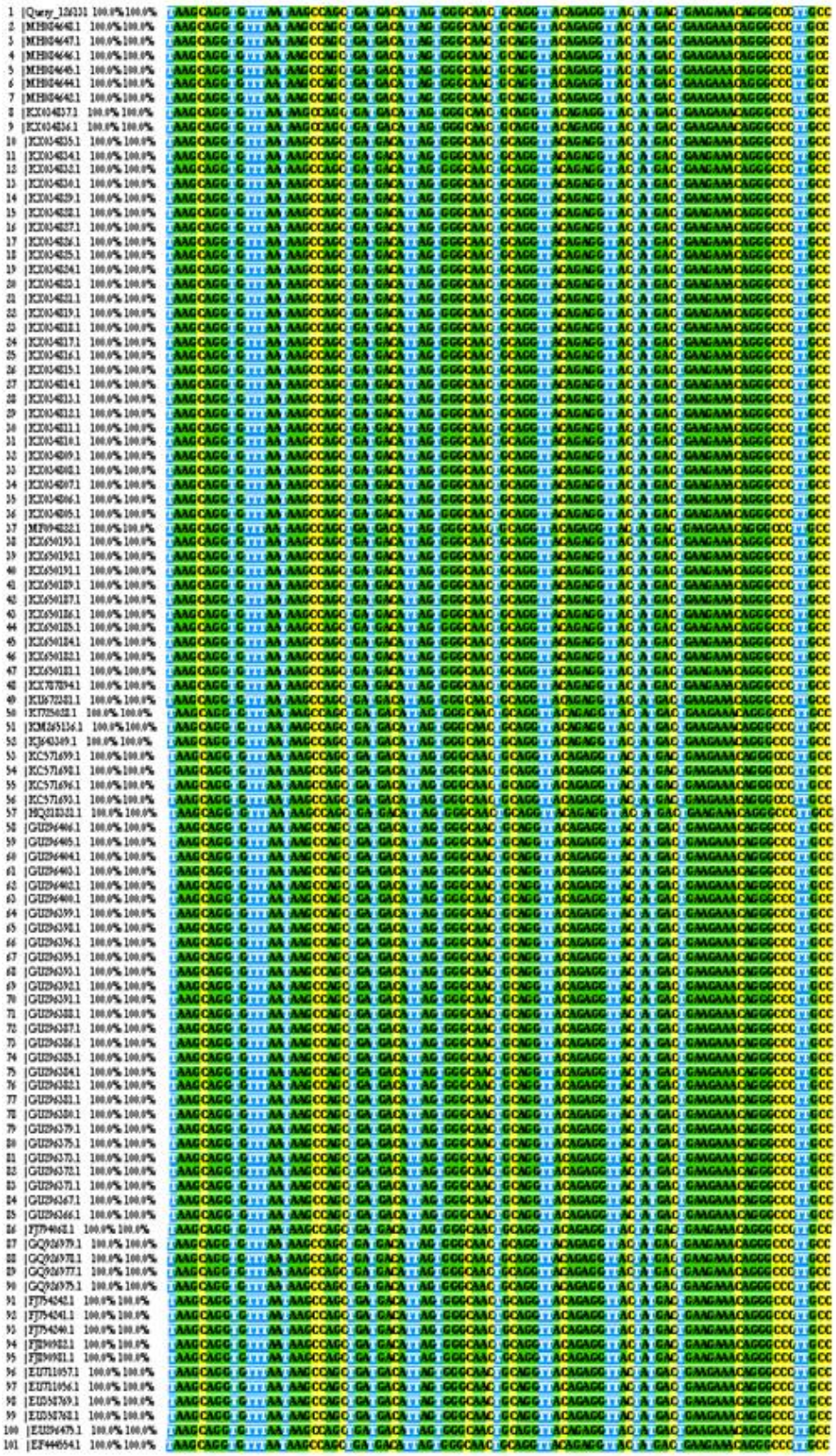

Figure 11. MSA of VP1 target region against deposited WUHPV strain

As shown in **Figure 9**, no significant homologies were detected, indicating that the oligonucleotides designed on VP1 region sequence of both viruses can be considered specific for KIHPyV and WUHPyV amplification (**Figures 10, 11**).

Specific primers were selected from conserved regions corresponding to VP1 major capsid protein of KIHPyV (from nucleotide 1498 to 2634, 378 aa) NCBI Reference Sequence: NC_009238.1 and WUHPyV (from nucleotide 1670 to 2779, 369 aa) NCBI Reference Sequence: NC_009539.1 genome (**Table 7**).

The choice of specific fluorochromes conjugated to KIHPyV or WUHPyV TaqMan probes allows the detection of KIHPyV and WUHPyV in 510 nm or 555 nm channels, respectively.

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

Table 7 Primers and probes used for the duplex real-time PCR assay

4.4.2. Duplex real time PCR set-up

Absolute quantification was performed with standard curve method. Synthetic oligonucleotides were designed from nucleotide 2437 to 2518 of KIHPyV NCBI Reference Sequence: NC_009238.1 (82 bp) and from nucleotide 2587 to 2665 of WUHPyV NCBI Reference Sequence: NC_009539.1 (78 bp). We used scalar dilutions of synthetic positive control from $5x10^6$ to $5x10^1$ copies/mL as reference curves.

The sensitivity of the duplex real-time PCR assay was determined by testing serial dilutions (from 5×10^5 to 5×10^1copies/mL) of two different synthetic dsDNAs carrying specific sequences of VP1 region of either KIHPyV (89 nt) or WUHPyV (90 nt). 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 KIHPyV or WUHPyV (**Table 8**).

	Synthetic DNA target (copy number/mL)	Number of positive replicates/ Total replicates	%
KIHPyV	$5x10^5$	10/10	100
	$5x10^4$	10/10	100
	$5x10^3$	10/10	100
	$5x10^2$	10/10	100
	$5x10^1$	1/10	10
	0	0/10	0
WUHPyV	$5x10^5$	10/10	100
	$5x10^4$	10/10	100
	$5x10^3$	10/10	100
	$5x10^2$	10/10	100
	$5x10^1$	1/10	10
	0	0/10	0

Table 8. Analysis of scalar dilution replicates

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 was determined at 328 copies/mL (12 copies/reaction) with a 95% confidence interval from 256 to 444 for KIHPyV and 345 copies/mL (15 copies/reaction) with a 95% confidence interval from 284 to 472 for WUHPyV(**Table 9**).

	Synthetic DNA target (copy number/mL)	Number of positive replicates Total replicates	%
KIHPyV	$5x10^2$	10/10	100
	$4x10^2$	10/10	100
	$3x10^2$	10/10	100
	$2x10^2$	4/10	40
	$1x10^2$	3/10	30
	$5x10^1$	1/10	10
WUHPyV	$5x10^2$	10/10	100
	$4x10^2$	10/10	100
	$3x10^2$	9/10	90
	$2x10^2$	4/10	40
	$1x10^2$	2/10	20
	$5x10^1$	1/10	10

Table 9. Probit analysis

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 5×10^2copies/mL. The specificity of the duplex real-time PCR assay was verified by examining negative controls and positive samples for different respiratory viruses including Flu A, Flu B, PIV, AdV, HRV, RSV

A, RSV B, HBoV, CoV, MPV and HEVs (n = 3 for each virus). Not all these samples showed positive signals for either KIHPyV or WUHPyV determination.

The intra-assay and inter-assay analyses were performed in serial dilutions of synthetic dsDNA targets. 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 KIHPyV or WUHPyV in triplicate. The intra-assay and inter-assay reproducibility data are indicated in **table 10** and **11**.

The coefficient of variation (CV) calculated on average Ct was lower than 2% for all dilutions of either KIHPyV or WUHPyV. 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 lower than 3% for all scalar dilutions for either KIHPyV or WUHPyV.

KIHPyV (copies/ml)	Ct (mean)	Standard Deviation (SD)	Coefficient of Variation (%)
$5x10^6$	22.31	0.28	1.2
$5x10^5$	25.52	0.27	1.1
$5x10^4$	28.92	0.41	1.4
$5x10^3$	33.12	0.32	1
$5x10^2$	36.81	0.47	1.2
WUHPyV (copies/ml)	Ct (mean)	Standard Deviation (SD)	Coefficient of Variation (%)
$5x10^6$	22.12	0.37	1.7
$5x10^5$	25.26	0.22	0.9
$5x10^4$	28.56	0.26	0.9
$5x10^3$	32.41	0.22	0.7
$5x10^2$	36.1	0.48	1.3

Table 10. Intra-assay analysis

KIHPyV (copies/ml)	Ct (mean)	Standard Deviation (SD)	Coefficient of Variation (%)
$5x10^6$	22.46	0.35	1.57
$5x10^5$	25.87	0.54	2.08
$5x10^4$	29.13	0.44	1.53
$5x10^3$	32.96	0.51	1.55
$5x10^2$	36.74	0.58	1.57
WUHPyV (copies/ml)	Ct (mean)	Standard Deviation (SD)	Coefficient of Variation (%)
$5x10^6$	22.26	0.37	1.72
$5x10^5$	25.61	0.36	1.42
$5x10^4$	28.78	0.39	1.36
$5x10^3$	32.55	0.36	1.12
$5x10^2$	36.32	0.43	1.19

Table 11 Inter-assay analysis

As the presence of very different concentrations of viral targets in duplex PCR can interfere on sensitivity reaction, we combined a high concentration (10^6 copies/mL) and a low concentration (10^3 copies/mL) of synthetic target of KIHPyV and WUHPyV in an interference test.

The results obtained did not show systematic difference in the amplification curves of the mixed targets with respect to the run performed with a single target (**Table 12**). The CV mean value showed a difference lower than 2%, suggesting a low interference when different template concentrations of targets were simultaneously amplified.

Synthetic oligo samples	KIHPyV Ct mean	WUHPyV Ct mean
KIHPyV (10^3 copies/ml)	32.31	-
KIHPyV (10^6 copies/ml)	21.57	-
WUHPyV (10^3 copies/ml)	-	35.03
WUHPyV (10^6 copies/ml)	-	21.22
KIHPyV (10^3 copies/ml) + WUHPyV (10^6 copies/ml)	33.07	21.34
KIHPyV (10^6 copies/ml) + WUHPyV (10^3 copies/ml)	21.7	35.89

Table 12. Analysis of duplex PCR amplification in simultaneous presence of different target concentrations

Several conventional PCR methods have been previously assessed for separate identification of WUHPyV and KIHPyV

Although TaqMan-based PCR assay is the most rapid, specific and sensitive approach for the detection/discrimination of KIHPyV and WUHPyV. Our original method has a higher sensitivity and a specificity comparable to other published Single-Tube Real-Time PCR methods (Lindau et al. 2009).

4.4.3. KIHPyV and WUHPyV analysis in 831 clinical samples

To validate our assay, the same 831 clinical samples collected from paediatric patients followed at the Hospital of Verona between 2016 and 2018 were analysed for detection of KI and WU Human Polyomaviruses.

Our data indicated that 28 out of 831 samples (3.36%) were positive for KIHPyV whereas WUHPyV was detected in 41 out of 831 samples (4.93%) (**Figure 12**).

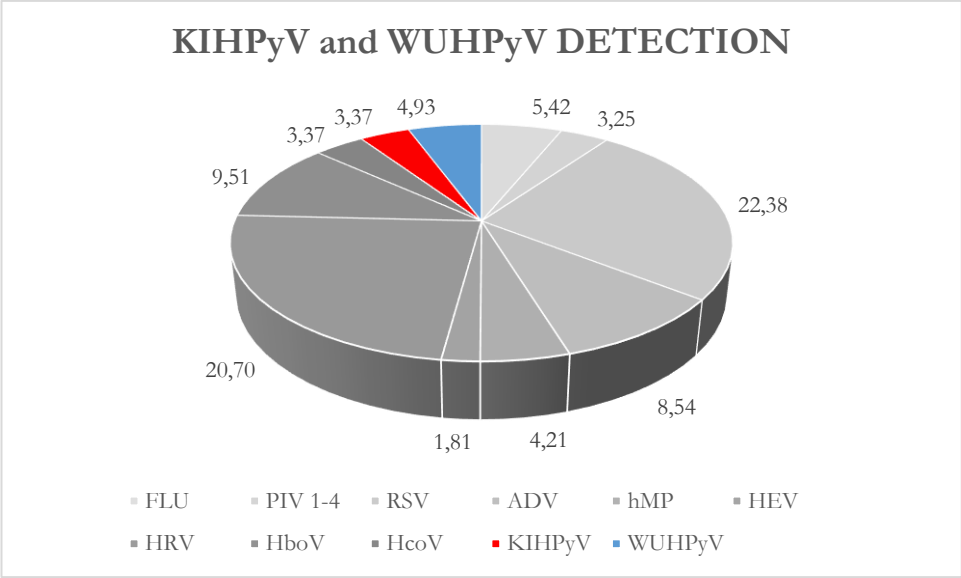

Figure 12. Percentage of KIHPyV /WUHPyV positive samples in analysed pediatric samples

Our results are in line with previously published epidemiological studies, and also confirms that the prevalence of WUHPyV is higher than KIHPyV as previously indicated (Ramqvist et al. 2009).

The quantitative data (**Figure 13**) of viral load on respiratory samples displayed different amounts of KIHPyV and WUHPyV genomes in respiratory samples. The KIHPyV viral load in positive samples was detected between 5.71×10^2 and more than 1×10^8 copies/mL with a median value of 16017 copies/mL, whereas the WUHPyV viral load in positive specimens was calculated between 4.09×10^2 and more than 1×10^8 copies/mL with a median value of 214786 copies/mL.

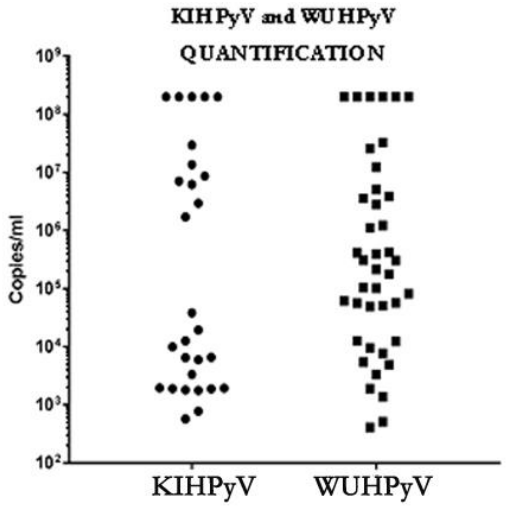

Figure 13. KIPHyV and WUHPyV quantification

4.4.3.1 KIHPyV and WUHPyV coinfection

The association between KIHPyV and/or WUHPyV 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 WUHPyV roles in the respiratory pathogenesis.

Since that, we determined whether the KIHPyV and/or WUHPyV positive samples showed viral co-infections by analysing the data obtained with commercial Allplex™ respiratory panel assay kit. This analysis displayed that 20 out of 28 KIHPyV positive samples showed one or more viral co-infections, whereas WUHPyV was associated to other viruses in 35 out of 41 cases. Thus, in 8 cases KIHPyV was the only virus identified, while in 6 cases this was true for WUHPyV. Interestingly, KIHPyV is preferentially associated with HRV (17 out of 20 co-infected samples) followed by HBoV and AdV (6 out of 20 samples). In **Figure 14,** we show the percentage of KIHPyV coinfection. On other hand, WUHPyV exhibited a major association with HRV (16 out of 35 co-infected samples) followed by RSV A/B viruses (10 out of 35 samples) and AdV (8 out of 35 samples). In **Figure 15,** we show the percentage of WUHPyV coinfection. It is noteworthy that KIHPyV and WUHPyV were simultaneously detected in three samples (**Figure 16**), in association with at least another respiratory virus.

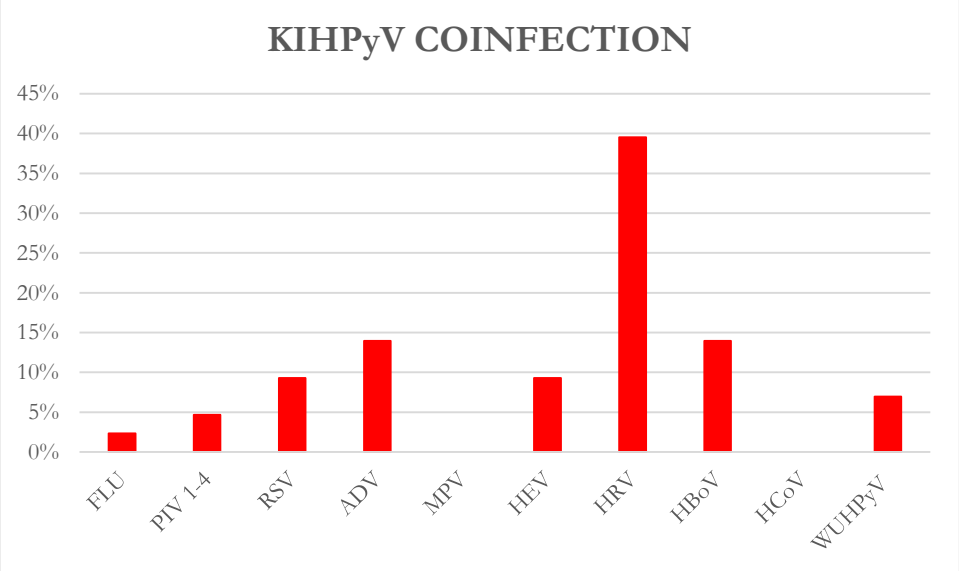

Figure 14. KIHPyV coinfection

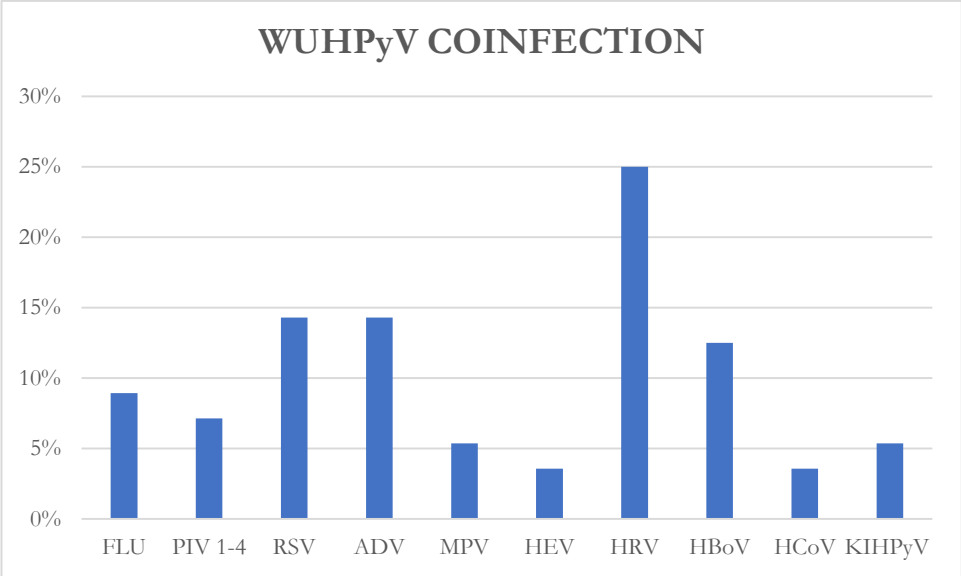

Figure 15. WUHPyV coinfection

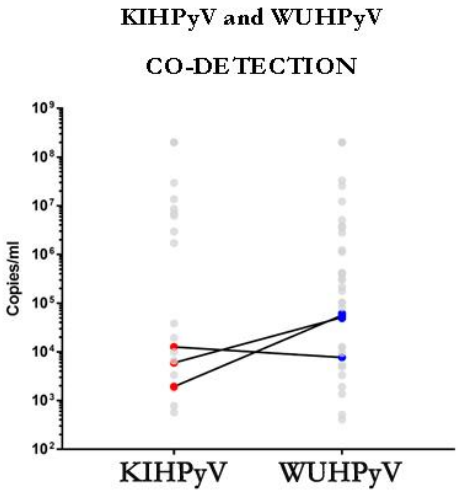

Figure 16. KIHPyV and WUHPyV double positive samples

4.4.3.2 KIPHyV and WUHPyV seasonal distribution

We also analysed the seasonal distribution of KIHPyV and WUHPyV in our patients. As shown in **Figure 17** and **18**, the two viruses were detected more frequently in spring. This is probably due to the high co-infection rate of these two polyomaviruses with HRV and HBoV, that were the more frequently detected viruses in the same season. This seasonal pattern is similar to the distribution observed by Han and co-workers (Han et al. 2007b) whereas Ramqvist and colleagues showed a wider distribution of these two viruses (Ramqvist et al. 2009).

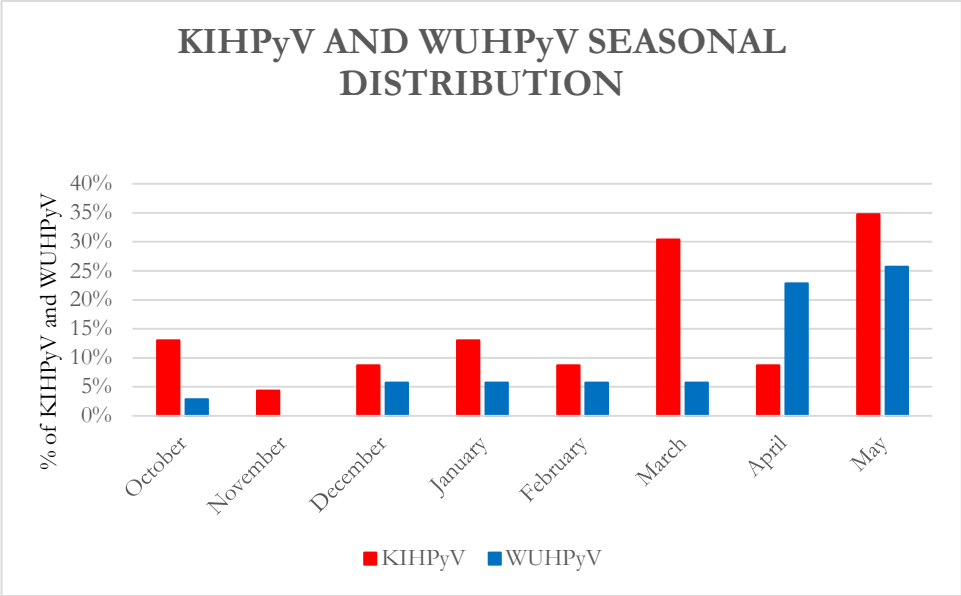

Figure 17 Monthly distribution of KIHPyV and WUHPyV

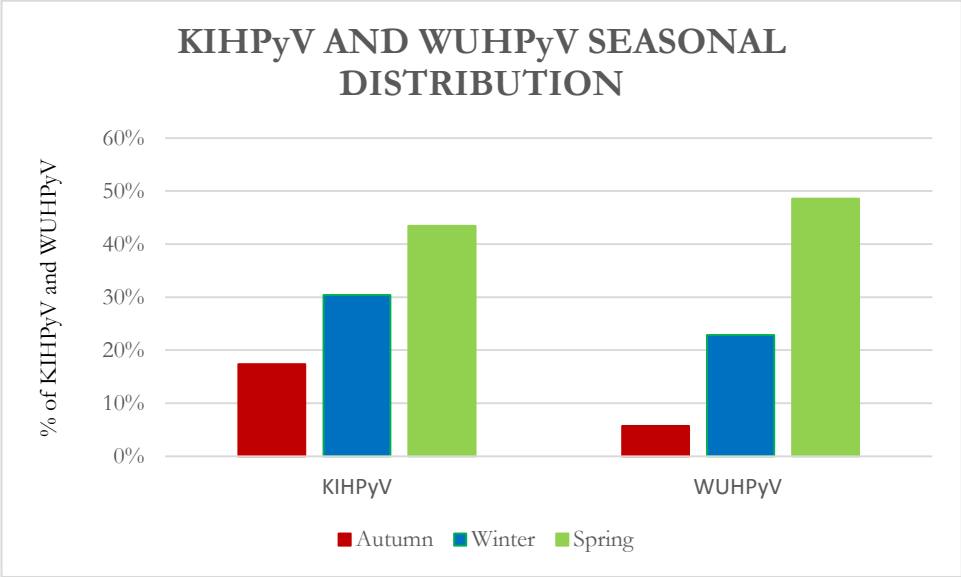

Figure 18. Seasonal distribution of KIHPyV and WUHPyV

5 DISCUSSION

Viral respiratory tract infection can lead to a series of respiratory tract diseases ranging from simple rhinitis to pneumonia.

These respiratory viral diseases are very important at both pathogenic and clinical point of view, because the cellular and tissue damage induced by the viral infection itself can determine the conditions for a bacterial co-infection leading to more serious clinical conditions.

The morbidity and mortality of viral respiratory infections are very important, especially in children, elderly, immunosuppressed and transplant patients where viruses such as FLU Virus and RSV may result in severe symptomatology leading, in most severe cases, to patient's death.

In this study, our objective was the identification and epidemiologic characterization of two recently isolated viruses, the *Polyomaviridae* KIHPyV and WUHPyV, using a new molecular method.

In particular, this study allowed defining:

I. A new Duplex real-time quantitative PCR method for the direct molecular identification of KIHPyV and WUHPyV;
II. Validation of this method retrospectively on a cohort of paediatric patients with respiratory disorders. The nasopharyngeal aspirate samples were also analysed by a commercial Multiplex PCR method identifying 16 respiratory viruses placed in three distinct panels;
III. Local epidemiological study of both these two viruses and other 16 respiratory viruses;
IV. Epidemiological study of the co-infection with the 16 respiratory viruses, KIHPyV and WUHPyV.

We set-up a Duplex real time PCR method based on a TaqMan system. Our original method is based on the amplification of the most conserved region of HHPyV genome, VP1, that in the past was also considered as an ideal region for the single amplification of these two viruses.

As previously described (Bialasiewicz et al 2007; Bergallo et al 2009), the location of primer and probe sequences can affect KIHPyV and WUHPyV detection rates. Indeed, in the KIHPyV VP1 region (Bergallo et al., 2009), primers and probes located

in the sTAG or regulatory regions have been investigated. Hence, we selected VP1 domain of the two viruses as target region for our original Duplex real-time PCR in order to optimize both sensitivity and specificity. In fact, our method allows the detection of up to 500 copies/mL equal to 17 copies/reaction in all the replicates of the two viruses, with a sensitivity comparable to described single amplification assays. Several conventional PCR methods have been previously assessed for separate identification of WUHPyV and KIHPyV (Abed et al. 2007; Abedi Kiasari et al. 2008; Foulongne et al. 2008; Neske et al. 2008), but sensitivity hasn't been often evaluated. Other authors describe conventional PCR methods followed by sequencing (Norja et al. 2007) but besides the longer time required and the higher risk of contamination, conventional end-point PCR also shows a lower sensitivity than Real-Time PCR. Data acquisition in Real-time PCR is made during linear phase or in early log-phase where conditions are optimal, instead of the end-point acquisition that could be affected by poorly optimized conditions or saturation of inhibitory PCR products or amplicons (Bergallo et al. 2009). Real-Time PCR for the separate identification of KIHPyV and WUHPyV (Bialasiewicz et al. 2007; Bergallo et al. 2009; Rao et al. 2011) and Real-Time PCR methods based on melting curve analysis have been published (Payungporn, et al. 2008), but these assays are not able to co-detect KIHPyV and WUHPyV in the same samples, because the melting curves would not be distinguished.

TaqMan-based PCR assay is a specific and sensitive approach for the detection/discrimination of KIHPyV and WUHPyV, and our original method has a higher sensitivity and comparable specificity to other published Single-Tube Real-Time PCR methods (Lindau et al. 2009). For these reasons, our method could be strongly taken into account as an alternative technical approach to detect KIHPyV and/or WUHPyV in respiratory samples for epidemiological and diagnostic analyses.

To validate our assay, we retrospectively analysed 831 clinical samples collected from paediatric patients admitted to Verona Hospital, Italy during 2016-2018 for detection of KI and WU Human Polyomaviruses. These samples had also been analysed for the detection of other conventional respiratory viruses with the Allplex™ respiratory panel assay kit able to determine the 16 major viruses associated to respiratory infection through three specific panels.

Our data indicated that 28 out of 831 samples (3.37%) were positive for KIHPyV whereas WUHPyV was detected in 41 out of 831 samples (4.93%). These results are

in line with previously published epidemiological studies. In fact, in patients with respiratory symptoms the prevalence of KIHPyV varied from 0.5 to 7%, whereas the prevalence of WUHPyV varied from 0.4 to 9% (Abedi Kiasari et al. 2008; van der Zalm et al. 2008; Essa et al. 2015; Gozalo-Margüello et al. 2015; Essa et al. 2017; Zhu et al. 2017) in immunocompetent paediatric/adult population. Our data also confirmed that the prevalence of WUHPyV is higher than KIHPyV as previously indicated (Ramqvist et al. 2009). Differences in prevalence described in past studies may be related to either regional prevalence variations or age population selection. In particular, a higher detection of KIHPyV and WUHPyV in respiratory samples of pediatric patients than in adult patients was noticed (Csoma et al. 2015; Abedi Kiasari et al. 2008).

Our results suggest that infection with these two viruses should be carefully considered as a leading cause of respiratory disease. The importance of KIHPyV and WUHPyV as etiological agents of respiratory pathology is currently controversial because previous studies showed that co-infection with other respiratory viruses is common in both KIHPyV- and WUHPyV-positive samples. The high rates of co-infection may support the notion that KIPHyV and WUHPyV do not have a truly significant pathogenic action (Wattier et al., 2008). Our data displayed that 20 out of 28 KIHPyV positive samples showed one or more viral co-infections, whereas WUHPyV was associated to other viruses in 35 out of 41 cases. In our cohort, KIHPyV is preferentially associated with HRV (17 out of 20 co-infected samples) followed by HBoV and ADV (6 out of 20 samples) (Figure 13A). On the other hand, WUHPyV exhibited a major association with HRV (16 out of 35 co-infected samples) followed by RSV A/B viruses (10 out of 35 samples) and ADV (8 out of 35 samples). Moreover, in 3 cases KIPHyV and WUHPyV are both present, in association also with other viruses. It is very important to note that there are 14 cases in which KIPHyV and WUHPyV are present individually (8 and 6 cases, respectively).

This observation confirms some previous studies (Han et al., 2007, Kleines et al., 2009) in which KIHPyV and WUHPyV could be detected as a single infection in patients with severe acute respiratory illnesses.

Although there are no clear evidences, the possibility that these viruses may establish persistent replication in immunocompetent hosts and may elicit respiratory disorders is suggested. Furthermore, we have to consider how the complex interaction between

the biology of these viruses and host clinical status can act in development of respiratory tract infection, as there may be several acute, persistent and chronic conditions of infection that contribute to a very hard-to-interpret scenario.

For example, not only viral coinfections but also the immunological status of the patients themselves can play an important role in the evolution of the infection and on its clinical outcome. It is worth mentioning that immunocompromised patients show increased viral load values and a tendency to infection persistence (Mourez et al. 2009; Rao et al. 2011).

In conclusion, this study allowed the investigation of some methodological aspects in the diagnosis of the two newly identified KIHPyV and WUHPyV. Our new method of Duplex real-time PCR based on TaqMan technology allows a simultaneous quantitative evaluation of the presence of these two viruses. The inter- and intra-assay tests confirmed the high sensitivity, specificity and repeatability of the system that was appropriately validated on clinical samples. This indicates that this original methodological approach can be considered as an alternative method to classical PCR methods, showing some advantages such as the simultaneous evaluation of the two viruses and a high sensitivity comparable to conventional PCR molecular methods.

This study also provides important observations on the epidemiological side: to our knowledge, this is the first study that describes at a local level the prevalence of these two viruses in paediatric patients with respiratory symptoms, suggesting how these two viruses can be considered as part of the basic virological screening when viral aetiology of a respiratory pathology is suspected.

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