Chapter 36
Co-Infections with Influenza and Other Respiratory Viruses

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Abstract Clinicians often do not consider the presence of more than one viral etiologic agent in respiratory infection, and in many cases they order diagnostics for influenza viruses or recently even only for A(H1N1)2009 virus. However, in a substantial number of patients with a respiratory tract disease, co-infection with various viral pathogens has been confirmed. Although the association between the occurrence of co-infection and substantially higher severity of disease is still unclear, a rapid and proper diagnostics of wide spectrum of viral respiratory pathogens reveals an accurate picture of the disease and is essential for appropriate therapeutic management and control of infection. In the present study we reported five cases of multiple respiratory infection in hospitalized immunosuppressed patients: two double infections with influenza virus (IV) type A/respiratory syncytial virus (RSV) type A and IV type A/coronavirus (CoV) OC43, one infection with four viruses – IV type A/RSV type A and B/CoV OC43, and two cases of mixed infections caused by five viral agents – IV type A and B/RSV type A and B/ parainfluenza type 3 or CoV OC43. Each patient had an underlying chronic disease and received immunosuppressive treatment. Despite a low number of tested specimens, our study shows that the inclusions of multiplex PCR methods for diagnostics of respiratory tract infections and the extension of diagnostic strategies by clinicians to detect viruses other than influenza are very important and make a contribution to identifying the true rate of co-infections and their correlation with the clinical symptoms and severity of disease.

Keywords Infection • Influenza • Diagnostics • Multiplex PCR • Respiratory viruses

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Abbreviations

AdV	adenovirus
ALRI	acute low respiratory tract infection
ARI	acute respiratory tract infection
CDC	Centers for Disease Control and Prevention
CMV	cytomegalovirus
C. pneumo	*Chlamydia pneumoniae*
CTX	cyclophosphamide
CVEV	coxsackie virus/echovirus family
EV	enteroviruses
hBoV	human bocavirus
hCoV	human coronavirus
hMPV	human metapneumovirus
hRV	human rhinovirus
IV	influenza virus
IV-A	influenza virus type A
IV-B	influenza virus type B
IV-C	influenza virus type C
M. pneumo	*Mycoplasma pneumoniae*
PCR	polymerase chain reaction
PIV	parainfluenza virus
PIV-1	parainfluenza virus type 1
PIV-2	parainfluenza virus type 2
PIV-3	parainfluenza virus type 3
PIV-4	parainfluenza virus type 4
RSV	respiratory syncytial virus
RSV-A	respiratory syncytial virus type A
RSV-B	respiratory syncytial virus type B
RT-PCR	reverse transcription-polymerase chain reaction

36.1   Introduction

Human respiratory tract infections represent a major public health problem because they are associated with a high rate of hospitalization, significant morbidity, and mortality. They thus cause a considerable financial burden on the health-care system throughout the world. The elderly and immunocompromised subjects with medical conditions are especially at high risk of developing severe course of infection and various complications. The most common respiratory viruses are influenza virus type A (IV-A) and B (IV-B), respiratory syncytial virus type A (RSV-A) and B (RSV-B), parainfluenza virus type 1, 2 and 3 (PIV-1, -2, -3), human rhinoviruses (hRV), human metapneumoviruses (hMPV), adenoviruses (AdV), human coronaviruses (hCoV), human bocavirus (hBoV) and enteroviruses (EV) (Do et al. 2011).

Infectious respiratory diseases caused by different viruses are characterized by a wide range of similar respiratory symptoms from mild cold to severe pneumonia. This makes the clinical distinction between different agents involved in infection very difficult. Although the high rates of infection with more than one respiratory virus have been reported, especially in children (Table 36.3), the real number of co-infections is undoubtedly underestimated, mainly due to sensitivity of diagnostic methods

used and limited number of tested viruses. In majority of cases the most likely or casual viral agents are tested first and the diagnostics stops with a detection of the primary relevant infectious agent. Meanwhile rapid, reliable, and accurate identification of every viral pathogen involved in infection is crucial for patient management, surveillance, and control of infection, including the prevention of nosocomial transmission.

Widespread use of PCR-based methods and their excellent sensitivity and specificity contributed to a great improvement of diagnostics of respiratory infections. While single PCR assay enables a detection of only one target, the multiplex molecular methods provide a simultaneous detection of multiple viruses in the single reaction and thus give a more accurate diagnosis of causative pathogens and provide a better understanding of the etiology of infection. The main advantages of using multiplex reactions are time saving and reducing the use of reagents and specimens, and consequently the cost of analysis. The Seeplex RV Detection Kit (Seegene Inc., Korea), used in our study, is a qualitative multiplex assay that detects 12 respiratory viruses including 11 types of RNA and one type of DNA virus: IV-A, IV-B, RSV-A, RSV-B, PIV-1, PIV-2, PIV-3, hRV, hMPV, Adv, hCoV OC43 and combined hCoV 229E/NL63. The assay uses dual priming oligonucleotide (DPO™) technology, which prevents non-specific amplification (it generates consistently high specificity by blocking the extension of non-specifically primed templates). The primers were designed for highly conserved regions in viral genome.

In the epidemic season 2010/2011, the National Influenza Centre in Poland received 140 specimens (paid diagnostics) from hospitalized patients with respiratory tract infection, including 49 specimens from severe cases (i.e., patients from intensive care units, with pneumonia, respiratory failure, immunocompromised patients). In the majority of these specimens, hospitals required only detection of influenza viruses (IV-A and IV-B (n=29) or IV-A and subtype A(H1N1)2009 (n=93)). Testing of specimens not only for influenza, but also other respiratory viruses was commissioned only for 18 patients. In these cases the multiplex Seeplex™ RV12 ACE Detection Kit was used to detect 12 respiratory viruses. In our paper, we present five cases of multiple infections caused by various respiratory viruses in immunocompromised patients.

36.2 Methods

All respiratory specimens (nasopharyngeal swabs) were suspended in 500 μl of physiological saline solution (within several hours after samples collection). Viral nucleic acid was extracted from 140 μl of sample using the QIAamp Viral RNA Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. The elution was performed with 60 μl of elution buffer. cDNA was synthesized by using First Strand cDNA Synthesis Kit with random hexamer primers (Fermentas, Lithuania). Reverse transcription reactions were performed in a final volume of 20 μl with 1 μl of random hexamers, 5 μl of water, 5 μl of total nucleic acids extracted from clinical specimen, 4 μl of 5× RT Buffer, 2 μl of dNTP Mix (10 mM each), 1 μl of RiboLock™ RNase Inhibitor and 2 μl of M-MuLV Reverse Transcriptase. After incubation for 5 min at 25°C, reverse transcription reactions were carried out for 60 min at 45°C, followed by inactivation of reverse transcriptase for 5 min at 70°C.

The next step was multiplex reaction with using the Seeplex™ RV12 ACE Detection Kit (Seegene, Inc., Korea) containing A and B sets of primer. Both panels were performed in 20 μl reaction volumes in accordance with the supplied protocols. The internal amplification control for detection of inhibitor of PCR, positive control (a mixture of 12 pathogens and internal control clones from the manufacturer as a template) and negative control (sterilized water instead of a template) were included in each reaction. After amplification, the obtained PCR products were analyzed by electrophoresis in 2% agarose gel stained with GelRed™ 10,000× solution. The respiratory viruses were identified by

comparison of amplicons with bands included in the positive control and molecular size marker for set A or B provided by the manufacturer and consisting of seven bands with the same length as PCR products for internal control and six appropriate viruses.

The results positive for influenza type B were confirmed by further analysis by one-step real-time RT-PCR assay according to CDC rRT-PCR Protocol (version 2007) and those positive for influenza A were tested by RealTime ready Influenza A/H1N1 Detection Set (Roche Diagnostics, Switzerland) with using primer set only for detection of type A (M2). Specimens with laboratory results indicative RSV- A or/and RSV-B were confirmed by one-step RT-PCR using Transcriptor One-Step RT-PCR Kit (Roche Diagnostics, Switzerland) and primers: 5' – TGTTATAGGCATATCATTGA – 3' and 5' – TTAACCAGCAAAGTGTTAGA – 3' described by Gröndahl et al. (1999). Amplification was carried out in a 25 μl reaction volume, including 5 μl of 5 × Transcriptor buffer, 0.4 μM of each primer, 0.5 μl of enzyme, 5 μl of extracted template and water to a final volume. Cycling reaction was performed in a Veriti™ 96-Well Thermal Cycler (Applied Biosystems Inc., USA) as follows: a single cycle of reverse transcription for 30 min. at 50°C and reverse transcriptase inactivation and initial denaturation for 7 min at 94°C, than 45 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 30 s and extension at 68°C for 55 s. After the last cycle, the reaction was completed by a final extension at 68°C for 7 min. Amplification products (20 μl) were separated in 1.5% agarose gel stained with GelRed™ 10,000× solution in 1 × TAE buffer. GeneRuler™ 100 bp DNA Ladders was used for estimating the molecular size weight of the obtained bands (Fermentas, Lithuania). Clinical specimens, isolated nucleic acids, and cDNA were stored at −70°C.

36.3 Results

Ten out of the 18 respiratory specimens were positive for at least one respiratory virus. Single infections were found in five cases and were caused by IV-A (three cases) and RSV-B (two cases). Co-infections (infections with two or more viruses) were found in five patients: double infections were identified in two cases, one patient was co-infected with four different viral pathogens, and in other two patients multiple infections caused by five viral agents was detected. Table 36.1 shows the pathogens involved in multiple infections.

Four co-infected patients (P1, P2, P3, and P4) were hospitalized in the same hospital and Department. The swabs were collected on the 4th of March 2011 (from patients P1 and P2) and the 11th of March 2011 (from patients P3 and P4). Patient 5 was hospitalized in another hospital and the swab was collected on the 3rd of February 2011. All five patients were immunosuppressed, including one child (4-year-old boy) and have different underlying chronic diseases (Table 36.2). Only two patients (P1 and P3 with co-infections caused by IV-A, IV-B and three other viruses) developed symptoms of acute respiratory infection with signs of bronchial obturation and they required longer antiviral therapy. One of them (P1) was mechanically ventilated for 3 days. The condition of the remaining patients was medium or good. Three patients received oseltamivir, one oseltamivir and then zanamivir, one patient received antibiotics and no antiviral drugs. The condition of two patients after therapy was medium, although one of them received oseltamivir. In this patient mixed infection with five viral agents: IV-A, IV-B, RSV-A, RSV-B, and PIV-3 were confirmed.

Monoplex PCR with different primer sets for detection of IV-A, IV-B and RSV-A/B (without identification of subtype) showed the results consistent with multiplex Seeplex PCR. In case of Patient 2, a second specimen (bronchoalveolar lavage) was collected on the 23rd of March 2011 and the result was positive for RSV-B and negative for the other pathogens, including IV-A and RSV-A, which were detected in the previous specimen.

Table 36.1 Type of detected co-infection

Virus detected		
2 viral agents:		
1.	Influenza type A	Patient 2 (P2)
	Respiratory syncytial virus type A	
2.	Influenza type A	Patient 5 (P5)
	Coronavirus OC43	
4 viral agents:		
1.	Influenza type A	Patient 4 (P4)
	Respiratory syncytial virus type A	
	Respiratory syncytial virus type B	
	Coronavirus OC43	
5 viral agents:		
1.	Influenza type A	Patient 1 (P1)
	Influenza type B	
	Respiratory syncytial virus type A	
	Respiratory syncytial virus type B	
	Parainfluenza type 3	
2.	Influenza type A	Patient 3 (P3)
	Influenza type B	
	Respiratory syncytial virus type A	
	Respiratory syncytial virus type B	
	Coronavirus OC43	

36.4   Discussion

Conventional methods of diagnostics of viral respiratory infections base mainly on virus isolation by cell culture, antigen detection, and serological assays. Cell culture has been considered as the gold standard for virus diagnosis but is not adapted to all medically important respiratory viruses. In contrast to this method, PCR permits rapid detection of viruses that are difficult to culture or have not been well replicated *in vitro*, such as hMPV, hCoV NL63 and hRV, with excellent sensitivity and specify (Calvo et al. 2008). Additionally, viruses present in specimens may considerably differ with viral loads and rate of replication, and this would make the detection of slowly replicating pathogens difficult. Therefore, numerous studies have been developed over recent years to evaluate the PCR-based methods for detection and typing/subtyping of respiratory viruses (Templeton et al. 2004; Bellau-Pujol et al. 2005; Gunson et al. 2005; Wu et al. 2008). When a viral infection is taken into consideration, because of very similar and nonspecific clinical symptoms caused by various respiratory viruses, the list of possible etiologic agents is very long. In contrast to monoplex PCR reaction, the use of multiplex PCR allows detecting various respiratory pathogens in a single reaction, including the identification of the type and the detection of co-infections with multiple agents, which could be missed by the monoplex reaction. The introduction of multiplex PCR to routine diagnostics revealed a higher prevalence of co-infections than has previously been reported by conventional techniques (Freymuth et al. 1997). A review of the literature indicates that the rate of co-infections may range from approximately 15 to as high as 50% of all positive specimens (Table 36.3). The majority of papers presenting the results of molecular assays for simultaneous detection of respiratory viruses and mixed infections focus on pediatric patients, infants, and young children in whom viral shedding in the respiratory tract is usually much higher. In these patients, the respiratory syncytial viruses were the most common pathogen occurred in respiratory viral co-infection, the next most often identified viruses in mixed infection were hRV, PIV, hMPV, and others. There also are observed co-infections with different subtypes of influenza A virus (Pajak et al. 2011).

Table 36.2 Characteristics of patients with diagnosed co-infections

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age/sex	58/female	33/female	70/female	40/male	4/male
Medical conditions	Wegener's granuloma with affecting of central nervous system, deep lymphocytope-nia, and 3 months earlier the pulmonary embolism	Wegener's granuloma and Takayasu disease, lymphocytopenia	Dermatomyositis with interstitial pulmonary disease	Allergic pulmonary alveolitis	Hemosiderosis
Therapy	Immunosuppressive therapy (CTX)	Immunosuppressive therapy (CTX, prednisone)	Glucocorticoid therapy	Glucocorticoid therapy	Immunosuppressive therapy (encorton)
Symptoms	Severe course of infections with pneumonia, respiratory failure and pancytopenia	No symptoms of acute respiratory infection	Typical influenza-like illness with malaise, fever and cough	No symptoms of acute respiratory infection	No symptoms of acute respiratory infection; intensified cough, tachypnea and decrease of carbonation
Received antiviral treatment	Oseltamivir (2×75 mg) for 5 days, than oseltamivir (2×150 mg) for 11 days	Oseltamivir (2×75 mg) for 5 days	Oseltamivir (2×75 mg) for 6 days, than zanamivir (2×5 mg) for 6 days	Oseltamivir (2×75 mg) for 11 days	No treatment (only antibiotics)
Condition of patients before/ after therapy	Severe, mechanical ventilation for 3 days/ medium	Good/good	Medium/good	Medium/good	Medium/medium
Other laboratory tests and results	Culture of bronchial secretion– *Acinetobacter baumanii*; stool culture– *Clostridium difficile*	PCR positive for CMV (bronchial secretion); the culture of bronchial secretion – negative results	Culture of sputum- negative results	Culture of bronchial secretion – negative results	Culture of throat swab– negative result; the culture of bronchial secretion– *Streptococcus pneumoniae*

Table 36.3 Review of literature regarding co-infections

Reference	Tested pathogens	The number of co-infections (percentage of all positive specimens)	The number of pathogens detected per sample	Co-infections rates by respiratory viral pathogens	Study group
Freymuth et al. (1997)	IV-A; RSV; PIV-1, -2; PIV-3; hRV; Adv	50 (28.2%)	Double infection, n=50	RSV=90.0%; PIV-1/2/3=40.0% (PIV-3=34.0%); AdV=28.0%; IV-A=24.0%; hRV=18.0%	277 hospitalized infants
Bellau-Pujol et al. (2005)	IV-A; IV-B; IV-C; RSV; PIV-1; PIV-2; PIV-3; PIV-4; hMPV; hRV; hCoV OC43/229E	33 (20.6%)	Double infection, n=30 Three pathogens, n=3	hRV=81.8%; RSV=42.4%; hMPV=39.4%; PIV-1/2/3=27.3% (PIV-3=21.2%); hCoV OC43, IV-A/B=6.1%	203 from hospitalized children
Yoo et al. (2007)	IV-A; IV-B; RSV-A; RSV-B; PIV-1; PIV-2; PIV-3; hMPV; hRV; AdV; hCoV OC43; hCoV 229E/NL63	16 (14.3%)	Double infection, n=15 Three pathogens, n=1	RSV-A/B=81.3%; PIV=62.5%; hMPV=18.8%[a]	200 children ≤5 years old
Brunstein et al. (2008)	IV-A; IV-B; RSV-A; RSV-B; PIV-1; PIV-2; PIV-3; PIV-4; hMPV; hRV; AdV; CVEV; bacterial pathogens[b]	475 (40.4%)	Double infection, n=371 Three pathogens, n=89 Four pathogens, n=14 Five pathogens, n=1	No data of this type available	1,742 patients with ARI (80% pediatric and 20% adult)
Calvo et al. (2008)	IV-A; IV-B; IV-C; RSV; PIV-1; PIV-2; PIV-3; PIV-4; hMPV; hRV; AdV; hCoV 229E/OC43; EV	86 (17.4%)	Double infection, n=70 Three pathogens, n=12 Four pathogens, n=4	RSV-A/B=79.1%; AdV=43.0%; hRV=30.2%; hMPV=22.1%; IV-A=18.6%; EV=10.5%; IV-C, PIV-1/2/3/4=9.3%; hCoV=1.2%	749 hospitalized children under 24 months of age with ALRI
Roh et al. (2008)	IV-A; IV-B; RSV-A; RSV-B; PIV-1; PIV-2; PIV-3; hMPV; hRV; AdV; hCoV OC43; hCoV 229E/NL63	7 (21.2%)	Double infection, n=7	AdV=57.1%; IV-B, hRV=28.6%; RSV-A, RSV-B, PIV-1, PIV-3, hCoV=each 14.3%	50 patients with ARI, (45 children, 5 adults)
Kim et al. (2009)	IV-A; IV-B; RSV-A; RSV-B; PIV-1; PIV-2; PIV-3; hMPV; hRV; AdV; hCoV OC43; hCoV 229E/NL63	13 (17.1%)	Double infection, n=12 Three pathogens, n=1	RSV-A/B=69.3%; PIV-1/2/3=84.7%; hRV, AdV=23.1%; IV-B=7.7%	92 patients (86 children, 6 adults)
Lee et al. (2010)	IV-A; IV-B; RSV-A; RSV-B; PIV-1; PIV-2; PIV-3; hMPV; hRV; AdV; hCoV OC43; hCoV 229E/NL63; hBoV; EV	30 (29.1%)	Double infection, n=25 Three pathogens, n=4 Four pathogens, n=1	RSV-A/B=90.0%; hMPV=33.3%; hRV=20%; IV-A/B=16.7%; PIV-3=16.7%; AdV=16.7%; hCoV 229E/NL63=13.3%; hCoV OC43=10.0%; PIV-1=3.3%	220 pediatric patients with ALRI

Table 36.3 (continued)

Reference	Tested pathogens	The number of co-infections (percentage of all positive specimens)	The number of pathogens detected per sample	Co-infections rates by respiratory viral pathogens	Study group
Meerhoff et al. (2010)	IV-A; IV-B; RSV-A; RSV-B; PIV-1, PIV-2; PIV-3; PIV-4; hMPV; hRV; AdV; hCoV; BoV; M. pneumo; C. pneumo	45 (47.9%)	Double infection, n=25 Three pathogens, n=17 Four pathogens, n=2 Five pathogens, n=1	BoV=91%; RSV=72%; hCoV=71%; hMPV=60%; hRV=57%[a]	98 infants
Richard et al. (2008)	IV-A/B; RSV-A/B; PIV-1/2/3/4; hCoV NL63; hRV; EV	44 (25.4%)	Double infection, n=44	RSV-A/B=81.8%; hRV=50.0%; hMPV=18.2%; EV=13.6%; IV-A/B, hCoV NL63=11.4%	180 hospitalized infants with bronchiolitis
Nascimento et al. (2010)	IV-A; IV-B; RSV; PIV-1; PIV-2; PIV-3; hMPV; hRV; AdV; hCoV NL63/HKU1; hBoV; EV	34 (47.2%)	Double infection, n=22 Three pathogens, n=8 Four pathogens, n=4	RSV=67.6%; hRV=61.8%; EV=44.1%; hMPV, BoV=23.5%; PIV-3=17.6%; hCoV=5.1%; IV-A, PIV-1=2.9%	77 infants with acute bronchiolitis
Do et al. (2011)	IV-A; IV-B; RSV-A; RSV-B; PIV-1; PIV-2; PIV-3; hMPV; hRV; AdV; hCoV OC43; hCoV 229E, NL63; hBoV; EV	62 (27.9%)	Double infection, n=55 Three pathogens, n=6 Four pathogens, n=1	BoV=43.5%; IV-A/B=33.9%; RSV-A/B=33.9%; hCoV NL63=25.8%; EV=24.2%; AdV=19.4%; PIV-1/2/3=12.9%; hRV=9.7%; hMPV=8.1%	309 children (2–13 year old) with ARI

Abbreviations: *ARI* acute respiratory tract infection, *ALRI* acute low respiratory tract infection, *IV* influenza virus: A (IV-A), B (IV-B) and C (IV-C), *RSV* respiratory syncytial virus: type A (RSV-A) and type B (RSV-B), *PIV* parainfluenza virus: type 1 (PIV-1), type 2 (PIV-2), typa 3 (PIV-3) and type 4 (PIV-4), *hMPV* human metapneumovirus, *hRV* human rhinovirus, *AdV* adenovirus, *hCoV* human coronavirus, *hBoV* human bocavirus, *EV* enteroviruses, *CVEV* coxsackie virus/echovirus family, *M. pneumo Mycoplasma pneumoniae*, *C. pneumo Chlamydia pneumoniae*

[a] Co-infections rates by the most common viral pathogen

[b] *Haemophilus influenza* (all types); *Haemophilus influenzae* (strains a, b, c and d); *Haemophilus influenzae* (strains e and f); *Mycoplasma pneumoniae*; *Chlamydia pneumoniae*; *Legionella pneumophila*; *Streptococcus pneumoniae*; *Neiseria meningitidis*

The Seeplex™ RV Detection Kit has recently been found as a good tool for the detection of multiple infections caused by common respiratory viruses (Yoo et al. 2007; Do et al. 2011), with sensitivity higher than viral culture assays (Drews et al. 2008; Roh et al. 2008; Kim et al. 2009; Lee et al. 2010). Our findings confirmed that co-infections are not a rare occurrence, particularly in immunocompromised patients with medical conditions receiving immunosuppressive therapy. Nevertheless, a limitation of this study is a low number of tested specimens (18 specimens) that does not allow making more significant observations, especially the comparison of clinical and epidemiological features between patients with single infection and patients with multiple infections.

A rapid and accurate identification of etiologic agents involved in a respiratory infection is of essential clinical value. This may support the physician's decision regarding the selection of appropriate treatment and a prompt use of antiviral drugs, such as neuraminidase inhibitors in case of influenza infections, for a patient, the exposed family members, or contact persons. Clinicians are inclined to use antibiotics in cases when the etiological agent of infection is not identified. Therefore, in spite of lack of specific antiviral drugs and the symptomatic and supportive treatment in majority of respiratory viral infections, a rapid detection of viral pathogen may help avoid an inappropriate use of antibiotics. Moreover, the multiplex methods that enable detection of a broad spectrum of common viral pathogens are clinically desirable in case of specific groups of patients: children, the elderly, and immunocompromised individuals who tend to have prolonged virus shedding and who are at increased risk of serious complications and mortality. Such tests allow detecting agents not primarily expected in a patient.

Another important aspect of the identification of mixed infections is that the presence of more than one pathogen may influence the course of infection; although the exact clinical significance of mixed infection remains unclear (Paranhos-Baccalà et al. 2008). In numerous studies that have described co-infections, no clinical differences among patients with single and multiple infections are reported, including the severity of disease, clinical signs of infection, their intensity, or duration of hospital stays (Brouard et al. 2000; Lee et al. 2010; Nascimento et al. 2010). In contrast, Calvo et al. (2008) reported that infants with co-infections of RSV and one or more other respiratory pathogens have a significantly higher incidence of fever and the duration of hospitalization was longer in comparison to infants infected only with RSV. In the study of Richard et al. (2008), a significant correlation between dual viral infection and increased severity in bronchiolitis was found and infants with co-infections were at 2.7 times higher risk for admission to the pediatric intensive care unit than those with single infections. The other significant factors that bore on the severity of infection were: age (<42 days), premature birth, and the underlying chronic disease. There were no statistically significant differences between host conditions, duration of assisted ventilation or of supplemental oxygen administration, and duration of hospitalization (Richard et al. 2008). Certainly, further studies for a better understanding of these various interactions and the clinical differences between single and multiple respiratory viral infections need to be carried out.

Brunstein et al. (2008) suggested the occurrence of pathogen co-suppression, when infection with one pathogen reduces a risk for infection with others. Such interactions have been observed for the following viruses: RSV and IV; RSV-A and RSV-B; RSV and PIV; hRV and RSV/IV. However, in the present study we identified four cases of simultaneous infections with RSV and IV. Two of these cases were caused by RSV-A and RSV-B and one by both types of RSV and PIV. These four co-infected patients (P1, P2, P3 and P4) were hospitalized in the same unit, multiple infections were caused by four different combinations of only six viruses (IV-A in four patients, IV-B in two patients, RSV-A in four patients, RSV-B in three patients, hCoV OC43 in two patients, and PIV-3 in one patient) and all specimens were collected during 1 week, which all suggests a high probability of nosocomial transmissions. Additionally, in the case of Patient 2, the second specimen collected after an interval of 19 days was negative for IV-A and RSV-A but positive for RSV-B.

The present study has some limitation. In electrophoretic gel patterns of PCR products obtained for patients with co-infections, the intensity of bands was variable, with some faint bands. Due to excellent sensitivity of PCR and its very low detection limit (even several copies per reaction, including

non-replicated viruses), weak positive results may be related not with active but passing infection (Calvo et al. 2008). Especially in children and immunosuppressed patients, shedding of a low concentration of viruses from a previous infection would be expected. This may be relevant for the interpretation of multiple infections and for the role of particular pathogens in the clinical picture of disease. The identified presence of viral agents does not exclude the possibility of concomitant infection caused by bacterial pathogens. In our study, in Patients 2, 3, and 4 cultures of bronchial secretion or sputum gave all negative results. In Patient 1, culture of bronchial secretion was positive for *Acinetobacter baumanii* and in Patient 5 it was positive for *Streptococcus pneumoniae* but that from a throat swab was negative.

36.5   Conclusions

In conclusion, our findings show that in case of hospitalized patients with respiratory tract infection, especially with severe infections observed in children, elderly, and immunocompromised patients who are susceptible to serious complications, the simultaneous detection of multiple viral agents is advisable and more reliable than detection of only influenza viruses. This enables to reveal an accurate picture of the disease and thus is essential for the control of infection, including the nosocomial transmission, prioritizing diagnostic and improvement of both preventive and therapeutic managements. Identifying the accurate etiology of infection also allows the physician to determine better prognosis and to inform patients about the measures that should be implemented to limit the spread of infections to other persons.

Conflicts of interest: The authors declare no conflicts of interest in relation to this article.

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