

Screening of respiratory pathogens by Respiratory Multi Well System (MWS) r-gene™ assay in hospitalized patients

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SUMMARY

Novel respiratory viruses have been identified as possible agents of upper and lower respiratory tract infections. Multiplex real-time PCRs have been developed to identify clinically relevant respiratory pathogens. In this study, 178 respiratory samples already screened for influenza virus types A and B by Flu A/B ASR real-time PCR kit were retrospectively analyzed with the Respiratory Multi Well System (MWS) r-gene™ real-time PCR kit which detects a wide spectrum of respiratory pathogens. The goal was to demonstrate the importance of a wide spectrum screening compared to a single diagnostic request. The Flu A/B ASR kit detected influenza B virus in 1.7% of the samples (3/178) and no influenza A virus. The MWS r-gene™ kit detected influenza virus in 6.7% (12/178) of samples (0.6% influenza A, and 6.2% influenza B), while the overall detection rate for respiratory pathogens was 54% (96/178). Co-infections were detected in 8/178 (4.5%) samples. Adenovirus was the infectious agent detected most frequently, followed by respiratory syncytial virus. The risk of being infected by respiratory syncytial virus is almost threefold higher in patients older than 65 years compared to the younger age group (OR:2.7, 95% CI: 1.2-6.2). Wide spectrum screening of respiratory pathogens by real-time PCR is an effective means of detecting clinically relevant viral pathogens.

KEY WORDS: Respiratory viruses, Real-time PCR, Wide spectrum screening.

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Acute respiratory infections are responsible for a large proportion of acute morbidities in developed countries with a major economic and social burden. Indeed, they are an important cause of hospitalization among children and adults with underlying chronic diseases. In developing countries, acute respiratory infections are an important cause of death. The majority of these infections have a viral origin (Mahony *et al.*, 2011), and transmission occurs through

air droplets, aerosol and fomites that may come into contact with nasal and conjunctival epithelium.

The etiological viral agents involved include: influenza virus types A and B, parainfluenza viruses types 1, 2, 3, and 4 (Nichols *et al.*, 2008), respiratory syncytial virus, adenovirus, and rhinoviruses/enteroviruses (Pillet *et al.*, 2013). In the last few years, other respiratory viruses have been discovered and linked to the upper and lower respiratory tract infections: human metapneumovirus (van de Hoogen *et al.*, 2001), SARS coronavirus (Ksiazek *et al.*, 2003), HKU1 coronavirus (Woo *et al.*, 2005), NL63 coronavirus (van der Hoek *et al.*, 2006), MERS coronavirus (Zaki *et al.*, 2012) and bocavirus (Allander *et al.*, 2005). In 2007, two novel human poly-

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omaviruses named KIPyV and WUPyV were discovered in the respiratory secretions of patients with acute respiratory symptoms (Allander *et al.*, 2007a; Gaynor *et al.*, 2007). However, the association of these latter viruses with the respiratory disease remains to be proven (Babakir-Mina *et al.*, 2008; Babakir-Mina *et al.*, 2011).

There are no clinical symptoms that can be specifically linked to a viral agent, and it is often difficult to differentiate between viral and bacterial pneumonia. An accurate and rapid laboratory diagnosis can have a positive impact on patient management reducing the length of hospital stay, avoiding the unnecessary use of antibiotics and suggesting the use of appropriate antiviral agents.

With the introduction of the real-time PCR assay, a sensitive and specific amplification method, the diagnosis of respiratory infections improved greatly. It is possible to search for up to 21 different respiratory pathogens including viruses and bacteria using a multiplex PCR format (Poritz *et al.*, 2011; Babady *et al.*, 2012; Brittain-Long *et al.*, 2010). Unlike conventional viral cell cultures, these new technologies allow the detection of viruses that are not detected at all by conventional methods. In addition, it is possible to detect co-infections that may have implications on disease severity or therapeutic strategies (Paranhos-Baccala' *et al.*, 2008).

It is noteworthy that the use of this technology gives the best results when the respiratory samples are collected in a timely fashion (Brittain-Long *et al.*, 2010).

In this work, respiratory samples screened routinely for influenza virus types A and B were retrospectively reanalyzed by Respiratory Multi Well System (MWS) r-gene™ real-time PCR assay which detects up to ten respiratory pathogens. The goal of the study was to assess the importance of performing a wide range PCR screening of respiratory pathogens, and to determine the prevalence of this panel of pathogens among hospitalized patients.

The present study covers an observation period from February 2009 to May 2011. During the study period, 178 patients with influenza like symptoms as defined by the presence of fever >37.5°C and at least another symptom (i.e., headache, malaise, myalgia, thrill or sweats,

retrosternal pain, asthenia) and one respiratory symptom (cough, sore throat, dyspnea), were eligible for the study. The median age of the patients was 61.5 years (range 16-96 years old); 74 (41.6%) were females and 104 (58.4%) males.

Nasopharyngeal swabs and broncoalveolar lavages were from different inpatient departments. The majority of the specimens were collected in the Hematology Department, Intensive Care Units and Emergency Room of the University Hospital Tor Vergata. Upon arrival in the Molecular Virology Laboratory, the samples were split into two aliquots and stored at -80°C until analysis.

For diagnostic purposes, the influenza viruses (including the H1N1 pandemic strain 2009) were detected by the Flu A/B ASR kit (Cepheid, CA, USA) according to the manufacturer's instructions.

The second aliquot of these respiratory samples were retrospectively screened by real-time PCR with the Respiratory Multi Well System (MWS) r-gene™ assay (bioMerieux S.A., Marcy l'Etoile, France). At the time of the study, the commercial assay included five kits for the simultaneous detection by duplex real-time PCR of the following respiratory pathogens: Influenza A, Influenza B, Respiratory Syncytial Virus, Bocavirus, Adenovirus, Metapneumovirus, Rhinovirus/Enterovirus, *Chlamydomphila pneumoniae*, and *Mycoplasma pneumoniae*. A cellular control kit for the validation of the presence/absence of cells is included, and inhibition within each reaction tube is controlled by the inclusion of an internal control (RICO-gene). Amplification was carried out following the manufacturer's instructions.

DNA/RNA extraction was performed using the NucliSENS® easyMAG™ extraction kit on the automated Easy Mag extractor (bioMerieux S.A., Marcy l'Etoile, France) according to the manufacturer's instructions.

The distribution of the specific viral agents by month of diagnosis and age of the participant was evaluated. Odds ratios (OR) and their 95% confidence intervals (95% CI) were calculated to evaluate the association between each variable (i.e., age, month of diagnosis) and the most common viral infections at the univariate level. The statistical analysis was conducted using IBM SPSS 20.0.

Screening of the 178 respiratory samples by Flu A/B ASR kit (Cepheid, CA, USA) revealed the presence of the influenza B virus in three samples (1.7%) and the absence of influenza A virus. When the same samples were retrospectively re-evaluated by the MWS r-gene™ kit (bioMerieux S.A., Marcy l'Etoile, France) which targets a wider spectrum of respiratory pathogens, the detection rate for influenza virus types A and B rose to 6.7% (12/178), while the overall detection rate of respiratory pathogens was 54% (96/178). Adenovirus was the pathogen detected most frequently (17.4%, 31/178) followed by respiratory syncytial virus (16.8%, 30/178). Most of the patients infected by these two viruses were stem cell transplant patients or patients from the emergency medicine ward. Table 1 summarizes all respiratory pathogens detected.

Co-infections were detected in 4.5% (8/178) of the samples analyzed. Five samples were positive for bocavirus and adenovirus; one for adenovirus + bocavirus + *mycoplasma pneumoniae*; one for human metapneumovirus and influenza B virus; and one for respiratory syncytial virus + adenovirus.

Considering the samples by respiratory pathogen, it was found that the risk of being infected by respiratory syncytial virus is almost three-fold higher in patients older than 65 years compared to the younger age group (OR:2.7, 95% CI: 1.2-6.2).

TABLE 1 - Respiratory pathogens detected most frequently using the Respiratory Multi Well System (MWS) r-gene™ assay.

	Positive samples		Negative samples	
	No.	%	No.	%
Adenovirus	31	17.4	147	82.6
Respiratory syncytial virus	30	16.8	148	83.1
Influenza virus*	12	6.7	166	93.3
Bocavirus	12	6.7	166	93.3
<i>Mycoplasma pneumoniae</i>	6	3.4	172	96.6
Rhinovirus/enterovirus	3	1.7	175	98.3
Human Metapneumovirus	2	1.1	176	98.9

*Influenza A virus: one single infections; Influenza B virus: nine single infections and two co-infections.

Samples were collected mainly in December (24.7%), January (32%), February (23%) and March (11.2%). Of the viruses detected most frequently, adeno and influenza viruses were detected mainly in December, while respiratory syncytial virus was detected mainly in January (Table 2).

Real-time PCR has greatly improved the laboratory diagnosis of viral respiratory infections due to its high sensitivity, specificity and ability

TABLE 2 - Detection of respiratory pathogens according to month of diagnosis.

	N. Positive samples (%)									
	Aug n. 3	Oct n. 2	Nov n. 4	Dec n. 44	Jua n. 57	Feb n. 41	Mar n. 20	Apr n. 5	May n. 3	Tot.
Adenovirus	1 (33.3)	0	2 (50)	10 (22.7)	6 (10.5)	7 (17.1)	2 (10)	1 (20)	2 (66.7)	31
Respiratory syncytial virus	0	0	0	8 (18.2)	15 (26.3)	4 (9.8)	3 (15)	0	0	30
Influenza virus	0	0	0	7 (15.9)	4 (7)	0	1 (5)	0	0	12
Bocavirus	0	1 (50)	0	4 (9.1)	3 (5.3)	3 (7.3)	1 (5)	0	0	12
<i>Mycoplasma pneumoniae</i>	0	0	0	2 (4.5)	2 (3.5)	1 (2.4)	0	0	1 (33.3)	6
Metapneumovirus					1 (1.7)		1 (5)			2
Rhino/enterovirus				2 (4.5)	1 (1.7)					3

to detect a wide spectrum of respiratory pathogens. Nowadays, these molecular methods are able to target up to 21 respiratory pathogens including viruses and bacteria (*Chlamydomphila pneumoniae*, *Bordetella pertussis*, and *Mycoplasma pneumoniae*) and this ability changed our view of the respiratory infections and their etiology. Co-infections are often detected suggesting that more than one pathogen can replicate at the same time in the respiratory tract and likely contribute to the appearance of the clinical symptoms and severity of disease. Although the clinical meaning of multiple infections is not clear yet, co-detection of viruses has been associated with a longer hospital stay, higher fever, and more frequent use of antibiotics with respect to a single viral infection (Calvo *et al.*, 2008).

In this study, we used a wide spectrum real-time PCR approach to demonstrate the usefulness and importance of this type of screening for detecting respiratory pathogens that otherwise could be missed by traditional screening (i.e., cell culture isolation or PCR targeting one single agent). For this purpose, respiratory samples of hospitalized adult patients already screened by real-time PCR for influenza viruses A and B were screened with the MWS r-gene™ kit which has been recently approved (CE marked) for in vitro diagnostic use in Europe. With this second screening, the detection rate for influenza viruses increased from 1.7% to 6.7% likely due to a higher sensitivity of the Biomerieux assay compared to the Cepheid assay. In addition, the wider screening disclosed other respiratory pathogens including rhino/enteroviruses, bocavirus, human metapneumovirus and *mycoplasma pneumoniae*. The low detection rate of these pathogens is likely related to the age of the selected patients (median age 61.5 years). Improved diagnosis may have major clinical implications in terms of implementation of control measures and treatment.

Co-detection of respiratory pathogens may also be relevant. Multiple viral infections have been detected in approximately 10% of respiratory specimens (Mahony 2008), especially in immunocompromised patients and young children. In this work, co-infections were detected in 4.5% of critically ill patients. The clinical meaning of these observations is still a matter of debate.

The concomitant presence of multiple replicating agents may represent an aggravating factor for the patient, resulting in more severe clinical symptoms (Paranhos-Baccala *et al.*, 2008; Babakir-Mina *et al.*, 2013). Measurement of the viral load of the single agents detected may be useful in the assessment of their contribution to the clinical symptoms (Babakir-Mina *et al.*, 2013; Utokaparch *et al.*, 2011; Campanini *et al.*, 2007; Allander *et al.*, 2007b).

Adenovirus was the virus detected most frequently followed by respiratory syncytial virus. The two viruses were detected mainly in HSCT patients and in those admitted to the emergency medicine ward.

Considering the age of the patients, respiratory syncytial virus was detected mainly in the elderly and this finding was statistically significant. Indeed, respiratory syncytial virus is recognized as a significant problem in older adults (Falsey *et al.*, 2005a). Attack rates in nursing homes are approximately 5-10% per year with significant rates of pneumonia (10-20%) and death (2-5%) (Falsey and Walsh, 2005b). In the United States, respiratory syncytial virus infection causes approximately 10,000 all-cause deaths annually among persons >64 years of age (Falsey and Walsh, 2005b). Older adults with underlying pulmonary and heart diseases and immunocompromised patients are at highest risk of developing respiratory syncytial virus-related pneumonia and death. An early diagnosis by sensitive real-time PCR methods and immediate supportive therapy may improve the survival rate in immunocompromised patients.

Finally, the two viruses were detected mainly in winter which was the period during which the majority of the samples were collected.

In conclusion, respiratory infections are an important cause of morbidity and mortality in patients with underlying chronic diseases. Wide spectrum screening carried out by sensitive and specific real-time PCR methods (Pillet *et al.*, 2013) represents a major advantage for a prompt diagnosis and for planning early therapeutic interventions. In addition, this approach can shorten the hospital stay and save money. MWS r-gene™ assay combining the sensitivity of a real-time PCR assay with the ability of screening for a wide range of respiratory viruses

and atypical bacteria fulfills these requirements and appears to be a useful tool in the routine diagnostic setting.

Considering the practical aspects of the workflow, a limitation of the MWS r-gene assay is the manual setup of the multiwell plate. The turnaround time from the extraction to result is 3h and 30 min. On the other hand, the use of the same thermal profile for all pathogens detected allows wide spectrum screening in one run on the same thermocycler, thereby reducing the response time and facilitating the staff's workload.

Taken together, the technical features of the assay and the broad respiratory panel make the MWS r-gene assay suitable for current clinical practice.

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