Diplomarbeit

Comparison of the multiplex eSensor® Respiratory Viral Panel to duplex real-time PCR assays for detection of viral respiratory pathogens

eingereicht von

Veronika Sinner

zur Erlangung des akademischen Grades

Doktor(in) der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt am

Institut für Hygiene, Mikrobiologie und Umweltmedizin

unter der Anleitung von

Univ.-Prof. Dr.med.univ. Harald H. Kessler

Graz am 02.12.2014

Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlichgemacht habe.

Graz am 02.12.2014 Veronika Sinner eh

Table of Contents

ABSTRACT

Background: Rapid and accurate diagnosis of acute respiratory tract infections is of major importance. Recently, the eSensor® Respiratory Viral Panel (RVP), a qualitative assay for simultaneous detection of 7 respiratory viruses and several viral subtypes has been introduced.

Objectives: To evaluate the accuracy of the new eSensor® RVP assay. To evaluate the clinical and laboratory performances of the new assay and to compare results to those obtained with duplex assays routinely used.

Study design: The accuracy of the eSensor® RVP assay was evaluated with panels of secondary reference material. For comparison of the clinical performances, 94 residual respiratory specimens that remained following routine clinical testing were included. Additionally, times-to-result for both testing methods were compared.

Results: When eSensor® RVP accuracy was tested with proficiency panels, all results were found to be concordant with the expected results. When analyzing 94 routine samples with the eSensor® RVP, results obtained from 87 samples (92.6%) were found to be concordant with those obtained with the duplex standard assays. Seven samples (7.4%) gave discrepant results. When times-to-results were compared, the eSensor® RVP required 315 min compared to 160 min when using the routine duplex assays.

Conclusion: The eSensor® RVP showed excellent accuracy and concordance with proficiency panels. Comparison of the clinical performance revealed concordant results in the vast majority of samples. When employing this multiplex assay, more pathogens can be detected simultaneously and this may have an impact on patient management.

KURZFASSUNG

Hintergrund: Die schnelle und exakte Diagnose von Infektionen des Respirationstraktes ist äußerst wichtig. Kürzlich wurde der eSensor® RVP Test eingeführt, ein qualitiatives Testverfahren, das die gleichzeitige Detektion von 7 respiratorischen Viren und mehreren Subtypen ermöglicht.

Ziele der Studie : Die Evaluierung der Richtigkeit des neuen eSensor® RVP Tests. Die Leistungsbeurteilung in Labor und Klinik und der Vergleich der Ergebnisse des neuen Testverfahrens mit denen des in der Routinediagnostik verwendeten Tests.

Studiendesign: Die Genauigkeit des eSensor® RVP Verfahrens wurde durch die Testung von sekundärem Referenzmaterial ermittelt. Um die klinische Leistungsfähigkeit mit der des Standardverfahrens zu vergleichen, wurden 94 klinische Proben getestet. Zusätzlich wurde die Analysezeit der beiden Testmethoden verglichen.

Ergebnisse: Bei der Evaluierung der Richtigkeit des neuen Verfahrens, durch die Testung von sekundärem Referenzmaterial, entsprachen alle Ergebnisse den zu erwarteten Werten. Bei der Analyse der 94 klinischen Proben stimmten 87 Ergebnisse (92.6%) mit denen der Standard Duplex-Verfahren überein. 7 Proben (7.4%) ergaben diskrepante Ergebnisse. Der eSensor® RVP Test benötigte 315 Minuten Analysezeit, das in der klinischen Routine eingesetzte Duplex-Verfahren benötigte 160 Minuten.

Schlussfolgerung: Der eSensor® RVP Test zeigte eine ausgezeichnete Richtigkeit und Übereinstimmung bei der Testung des sekundären Referenzmaterials. Bei der klinischen Leistungsbeurteilung zeigte sich eine Übereinstimmung in der überwiegenden Anzahl der Ergebnisse. Durch den Einsatz dieses neuen Tests können mehrere Pathogene gleichzeitig detektiert werden und dadurch kann die Behandlung des Patienten beeinflusst werden.

1 Introduction

Upper and lower respiratory tract infections are very common and may proceed to severe illness. Furthermore, respiratory tract infections are the leading cause of medical visits and responsible for high expenditures on the healthcare system.

Acute respiratory tract infections may proceed lethally, especially in developing countries, but they are often associated with high morbidity and even mortality in developed countries, particularly in newborns, young children, elderly people, and immunosuppressed patients (Popowitch et al., 2013). Therefore, a rapid and accurate diagnosis is of major importance. It improves the patient's outcome, particularly if an antiviral therapy for this pathogen is available (Barenfanger et al 2000). Furthermore, through a rapid and sensitive detection, the necessity of additional testing and hospital care may be avoided. Besides, the cost for the healthcare system can be reduced. Additionally, the prescription of unnecessary antibiotics can be limited (Pierce et al., 2012; Templeton, 2007).

Diagnosis based on the clinical presentation alone is difficult because most of the respiratory viruses develop similar symptoms (Popowitch et al., 2013). In the last 20 years, nucleic acid amplification has become state of the art for rapid detection of viral infections (Kehl and Kumar, 2009; Mahony, 2008; Mahony et al., 2011). This technology has been shown to be superior to conventional diagnostic methods such as virus culture, direct antigen detection, and enzyme immunoassays. Molecular diagnostics provides a superior sensitivity and specificity, together with identification of a broader range of pathogens (Gharabaghi et al., 2011; Krause et al., 2014).

Pathogens relevant for respiratory tract infections include adenoviruses, coronaviruses, the human metapneumovirus, influenza viruses, parainfluenza viruses, the respiratory syncytial virus, and rhinoviruses (Fox 2007).

2 Viruses

2.1.1 Adenoviruses

Adenoviruses are members of the family *Adenoviridae*. They are medium-sized (90–100 nm), non-enveloped viruses with an icosahedral nucleocapsid containing a double stranded DNA genome (Fig. 1). Today, 51 subtypes are known. Adenoviruses show different sites of tropism including respiratory tract epithelial cells, the urothelium, and conjunctival cells.

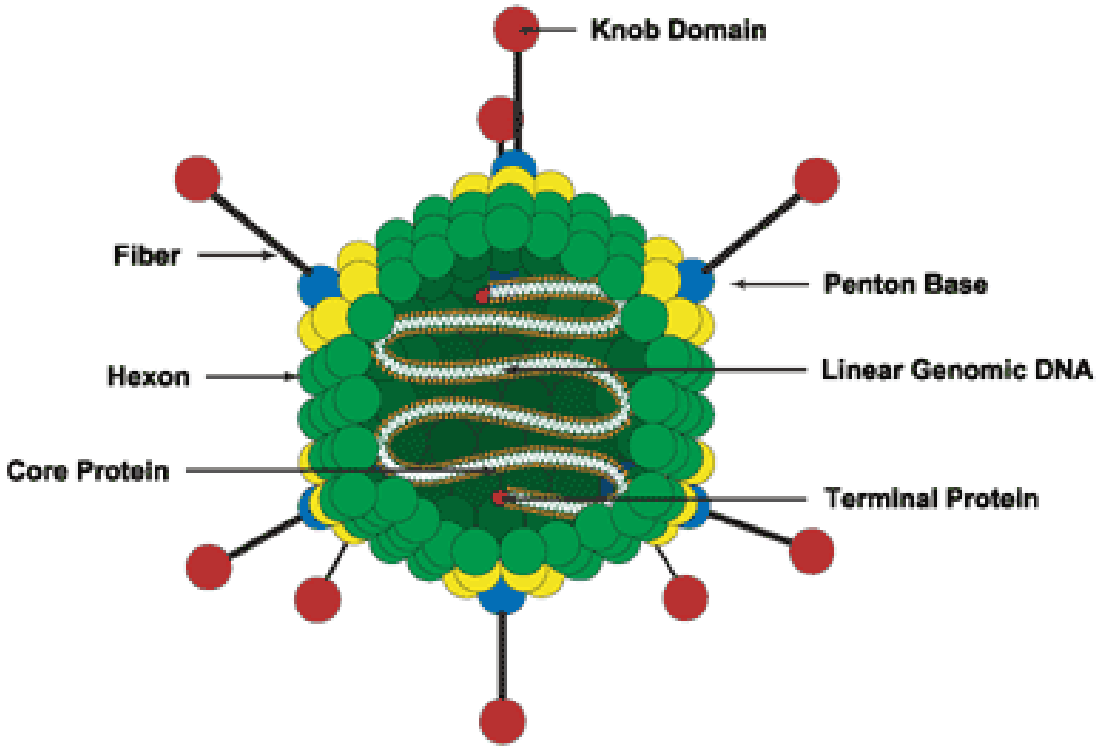

Fig.1. Structure of the adenovirus (from http://www.daviddarling.info/encyclopedia/A/adenovirus_infection.html).

Adenoviruses cause 5 percent of all respiratory tract infections in children younger than 5 years. Mild symptoms such as cough, fever, rhinitis, pharyngitis, and tonsillitis are common but the virus is also responsible for severe diseases such as croup, bronchitis, and pneumonia. At the age of 10, most of the children are infected with at least one adenovirus subtype, through droplet transmission or smear infection. Furthermore, adenoviruses can cause (hemorrhagic) cystitis and genital ulcera.

Finally, (hemorrhagic) conjunctivitis (often transmitted in swimming pools) may develop. Other manifestations of adenovirus infection include meningitis and gastroenteritis. Immunosuppressed persons are at high risk of adenovirus infection. Especially, in the early post-transplant phase, adenovirus infection is associated with a mortality of up to 60 percent.

Detection of adenoviruses through virus culture is complicated, adenoviruses grow very slow. Serological assays have been used for detection of antibodies. Recently, detection of adenovirus DNA has been introduced and has become the state of the art technique for detection of adenovirus infection in immunosuppressed patients. There is no antiviral therapy available but administration of antibiotics to prevent bacterial superinfection, especially in case of a conjunctival infection, may be indicated (Hof et al., 2009; Mahony et al., 2011).

2.1.2 Coronaviruses

Coronaviruses are members of the family *Coronaviridae*. They are medium-sized (80-120nm), enveloped viruses with a nucleocapsid of helical symmetry containing a positive-sense RNA genome (Fig. 2).

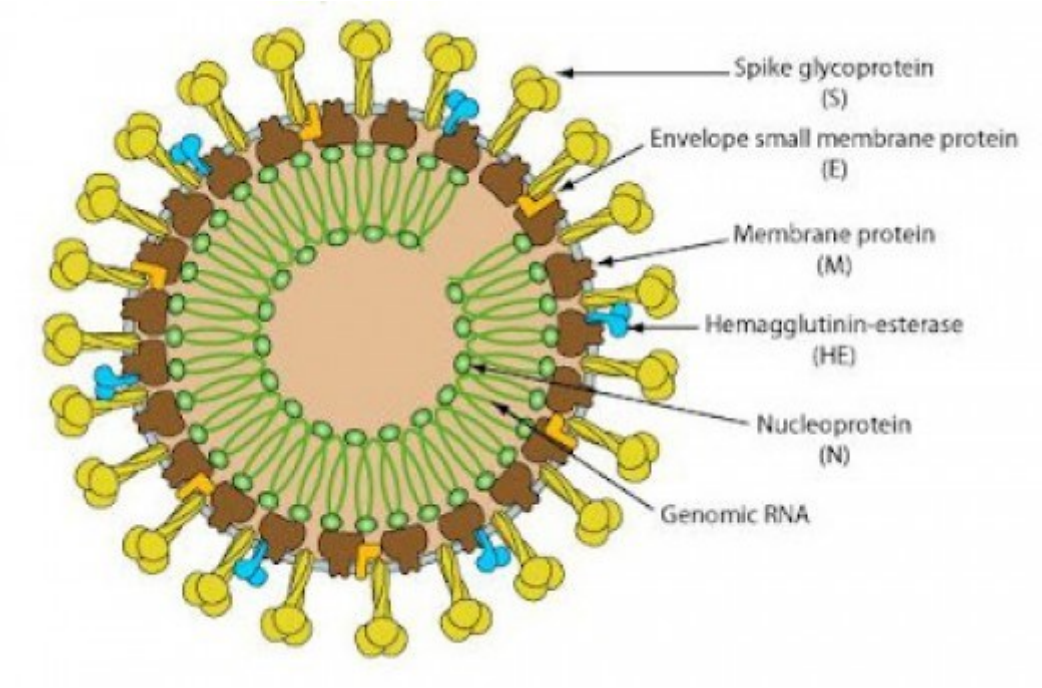

Fig.2. Structure of the coronavirus (from http://skperdon.hubpages.com/hub/Travel-Infections).

Today, five coronavirus strains infecting humans are known, the human coronaviruses OC43 and 229E produce upper and lower respiratory tract infections mainly in children, elderly people, newborns, and hospital employees. The human coronavirus NL63 causes mild upper respiratory tract infections, while HKU1 is associated with pneumonia, bronchiolitis, asthma exacerbation, fever, runny nose, and cough. The SARS coronavirus was identified in 2003 and causes a severe acute respiratory syndrome. It was responsible for the pandemic in 2003 with nearly 10000 infected people and a fatality rate of 9%.

Diagnosis of coronavirus infection has mainly been based on serology. Recently, detection of coronavirus RNA has been introduced in the routine diagnostic laboratory. Currently, there is no specific antiviral therapy existing (Hof et al., 2009; Mahony et al., 2011).

2.1.3 The human metapneumovirus

The metapneumovirus belongs to the family *Paramyxoviridae*. They are enveloped negative single-stranded RNA virus of approximately 150-300 nm in diameter, with a nucleocapsid of helical symmetry (Fig. 3).

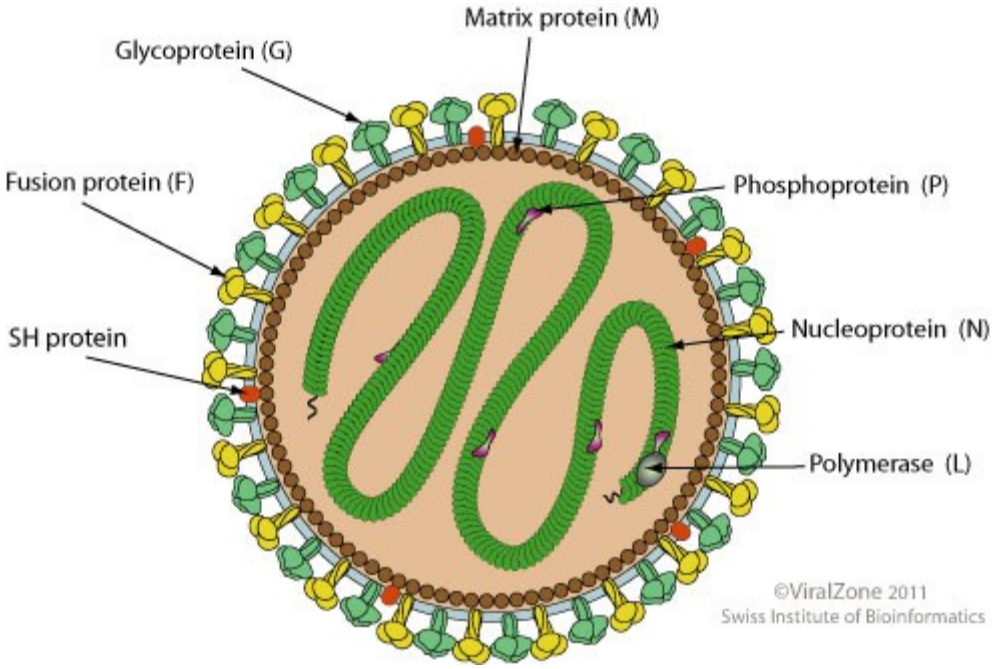

Fig.3. Structure of the human metapneumovirus (from http://viralzone.expasy.org/all_by_protein/89.html).

The human metapneumovirus causes upper and lower respiratory tract infections, especially in young children. Elderly people are at risk for a lethal course. Following respiratory syncytial virus, it is the second most prevalent cause of bronchiolitis (with or without pneumonia) in children. The symptoms are similar to those produced by respiratory syncytial virus infections such as high fever, cough, and wheezing. Transmission occurs through droplet infection.

Detection of metapneumovirus is possible through virus culture and antigen detection but the most sensitive and specific diagnosis is provided by PCR. There is no successful causal treatment known but ribavirin may be helpful (Hof et al., 2009; Mahony et al., 2011).

2.1.4 Influenza viruses

Influenza viruses are members of the family *Orthomyxoviridae*. They are medium-sized (80-120nm), enveloped viruses with a nucleocapsid of helical symmetry, containing a negative single-stranded and segmented RNA genome (Fig. 4).

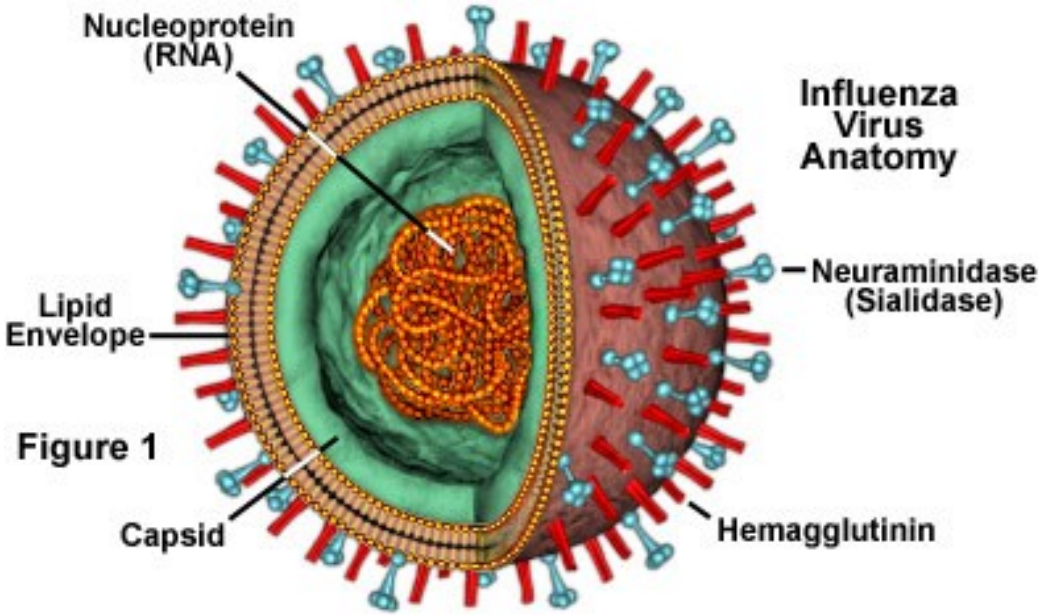

Fig.4. Structure of the influenza virus (from http://micro.magnet.fsu.edu/cells/viruses/influenzavirus.html).

Influenza viruses can be classified into the genera A, B, and C. All of them are able to infect humans. Additionally, influenza A virus may infect other mammals. Due to the high mutation rate subtypes can be distinguished according to the different surface antigens, neuraminidase and hemagglutinin. Thus, a considerable number of different subtypes (e.g. influenza A H1N1, influenza A H1N1, influenza A H2N3 etc.) exist. The influenza B virus shows a lower mutation frequency. Influenza C virus is able to infect humans but the progress of the disease is very mild, so that there is almost never a need of medical treatment. Influenza A and Influenza B can cause annual epidemics and antigen shifts (new combinations of the surface antigens) may lead to pandemics. Influenza, also commonly known as flu, is an infectious disease with major symptoms such as fever, cough, headache, weakness, and muscle aches.

It is especially dangerous for elderly and immunosuppressed people. Influenza is typically transmitted through droplet infection. Symptoms and clinical appearance are very similar among the different subtypes.

Molecular tests are the standard method for detection of viral RNA, especially important in elderly and immunosuppressed people. In the majority of cases a symptomatical therapy is sufficient but immunosuppressed patients may be treated with an antiviral drug. Vaccination against influenza viruses is recommended for elderly and immunosuppressed people and exposed personnel (Harder et al., 2013; Hof et al., 2009; Mahony et al., 2011).

2.1.5 Human parainfluenza viruses

Parainfluenza viruses are members of the family *Paramyxoviridae*. They are enveloped negative single-stranded RNA viruses, approximately 150-300 nm in diameter, with a nucleocapsid of helical symmetry (Fig. 5).

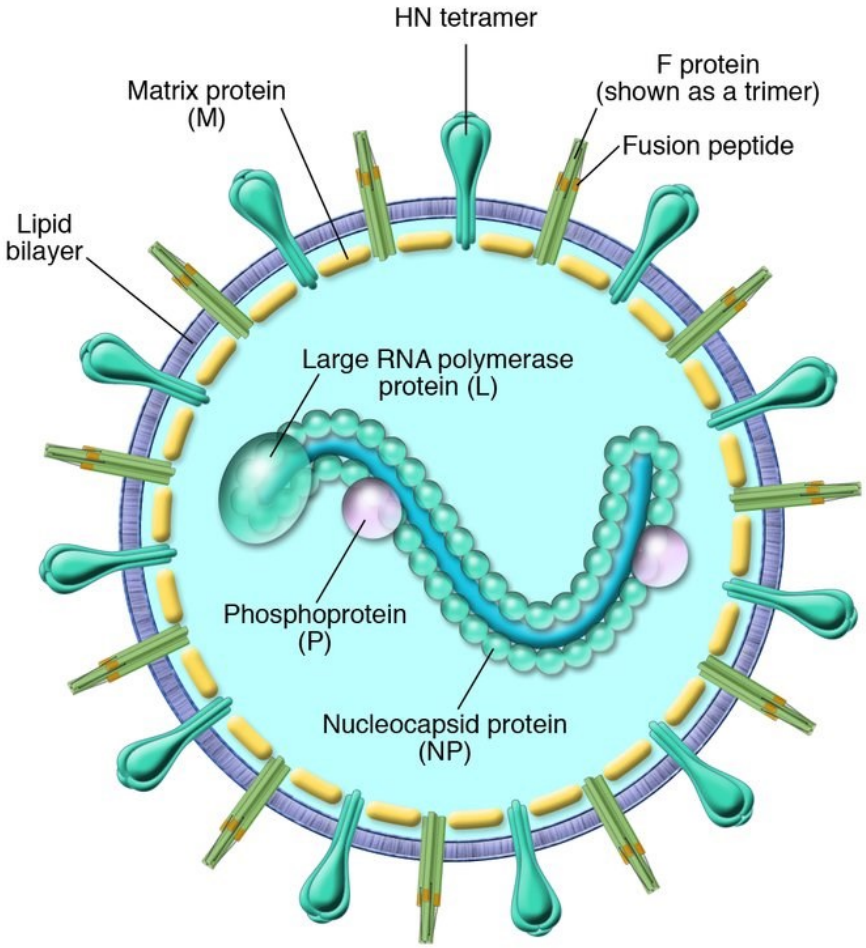

Fig.5. Structure of the parainfluenza virus (from http://blog.naver.com/PostView.nhn?blogId=flu1918h1n1&logNo=100093617602).

Parainfluenza viruses cause 15-30% of nonbacterial respiratory diseases in children requiring hospitalization. It is classified into four subtypes. Parainfluenza virus 1 is the most common cause of acute croup in children and it is responsible for upper respiratory tract infections. Parainfluenza virus 2 may also cause croup but in a milder and less infectious way compared to parainfluenza virus 1 and parainfluenza virus 3. Parainfluenza virus 3 is the second most common cause of croup in infants and children. It is associated with severe lower respiratory tract infections such as

pneumonia and bronchiolitis, especially in children younger than one year. Most infections occur in winter and spring. Parainfluenza virus 4 is not as important as previous mentioned subtypes. It causes only a few mild infections. Transmission occurs through droplet infection.

The respiratory symptoms are mostly nonspecific. Diagnostic approaches include antibody detection and detection of viral RNA. There is no efficient causal therapy known (Hof et al., 2009; Mahony et al., 2011).

2.1.6 The respiratory syncytial virus

The respiratory syncytial virus belongs to the genus *Pneumoviridae*, which are members of the family *Paramyxoviridae*. The respiratory syncytial virus is an enveloped negative single-stranded RNA virus of approximately 150-300 nm in diameter with a nucleocapsid of helical symmetry (Fig. 6).

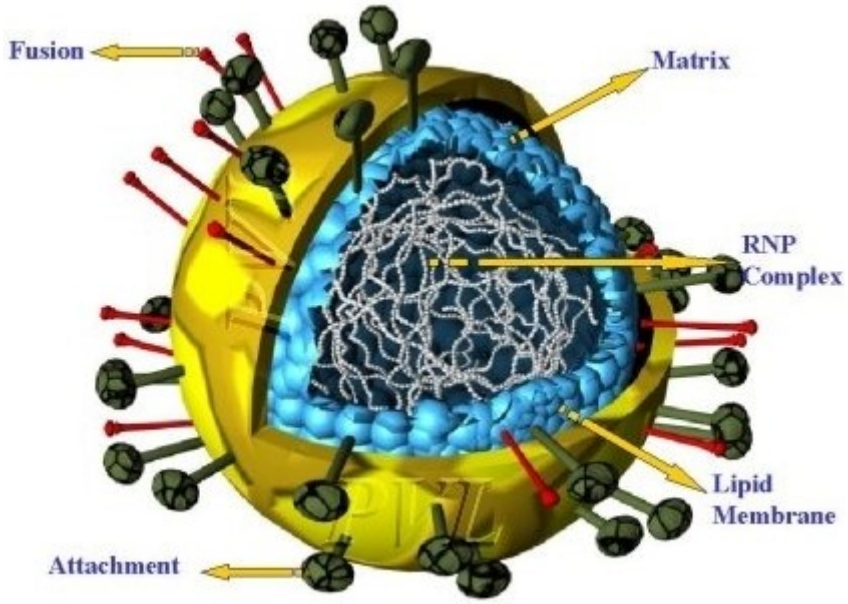

Fig.6. Structure of the respiratory syncytial virus (from http://bcrt.ca/2008/09/30/respiratory-syncytial-virus-rsv/).

Due to its extreme high infectivity, the respiratory syncytial virus is the leading cause of respiratory tract infections and a major cause of hospital visits during infancy. The infection is often mild but may cause severe complications such as pneumonia and bronchiolitis. Respiratory syncytial virus infections can be found worldwide and transmission occurs through droplet infection.

Over the last decades, a several approaches have been performed to diagnose respiratory syncytial virus infection including rapid antigen detection, antibody detection, and, most recently, nucleic acid detection. Molecular assays provide increased sensitivity and specificity. Mild infections are treated symptomatically, while severe infections may be treated with ribavirin. Premature newborns are treated prophylactically with palivizumab, a monoclonal antibody (Hof et al., 2009; Mahony et al., 2011).

2.1.7 Rhinoviruses

Rhinoviruses belong to the genus *Enterovirus* and are a member of the *Picornavirus* family. They are very small (medium size 20-30 nm), non-enveloped viruses with an icosahedral nucleocapsid containing a single stranded RNA genome.

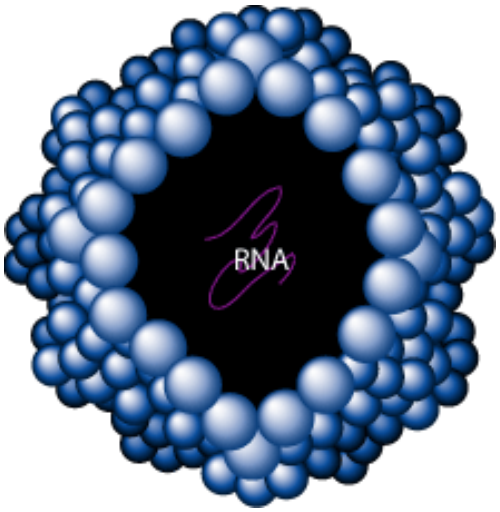

Fig.7. Structure of the human rhinovirus (from http://www.scq.ubc.ca/rhinovirus-an-unstoppable-cause-of-the-common-cold/).

The typical disease associated with rhinovirus infection is the common cold with its typical symptoms such as sneezing, cough, runny nose, headache, myalgia, and fatigue. However, in immunosuppressed patients and those with chronic obstructive pulmonary disease, rhinoviruses may cause more severe disease including bronchitis and even pneumonia. Transmission of this virus is possible through contaminated surfaces or droplets. Infections occur predominantly in winter and early spring. Rhinoviruses are responsible for two thirds of upper respiratory tract infections.

Antigen detection is difficult because there are more than 110 serotypes known, without any common and recognizable antigen. Nowadays, PCR is the most sensitive technique for rhinovirus detection. There is no efficient causal therapy known (Hof et al., 2009; Mahony et al., 2011).

2.2 Nucleic acid-based tests

Nucleic acid-based tests (NATs) have become state of the art for detection of pathogens in patients at-risk for acute respiratory disease. They offer a lot of advantages such as quicker results, increased sensitivity and specificity, specific differentiation of subtypes, and avoidance of inappropriate treatment. NATs encompass a broad spectrum of different molecular technologies. All of them are based on three basic steps: nucleic acid extraction, target amplification and detection (Kessler, 2014).

2.2.1 Nucleic acid extraction

The nucleic acid extraction is a separation and purification process using chemical and physical methods. It is an essential step prior to DNA amplification. Adequate and accurate extraction of nucleic acids is the premise for correct results. Manual nucleic acid extraction protocols are time consuming and associated with a high risk of contamination. Recently, automated platforms have been commercially available. They provide increased hands-off time and a lower contamination rate due to less manipulation. Most of the nucleic acid extraction techniques are based on the silica adsorption, also called magnetic glass particle technology. This technique involves four major steps (Fig. 8). At first, the lysis buffer breaks the cell open and releases the nucleic acid. Under defined conditions (high salt concentration and neutral pH value) the DNA molecules bind to silica surfaces. With repeat washing steps, the nucleic acids are purified from potential PCR inhibitors and are conserved by an elution buffer finally (Kessler, 2014).

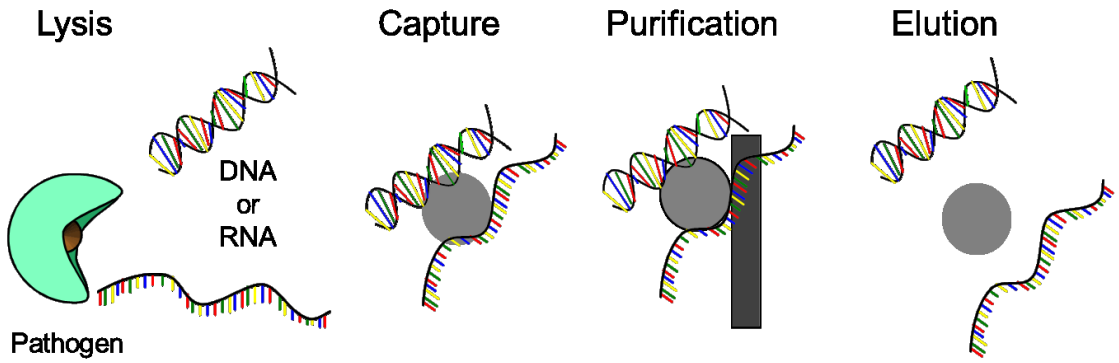

Fig. 8. The four major steps of the silica adsorption technology (from Kessler, molecular diagnostics of infectious diseases).

2.2.2 Amplification: polymerase chain reaction (PCR)

The PCR is an indispensable technique for molecular diagnostics. This technology uses specific primers to amplify particular nucleic acid sequences. It requires only a small amount of starting specimen to generate millions of copies of the desired nucleic acid segment. Because PCR is a DNA amplification technology, a reverse transcription step must be performed when RNA amplification is required. The enzyme *reverse transcriptase* generates a complementary DNA strand from the RNA to allow the PCR technology.

The PCR consists of 30-40 cycles of three consecutive steps (Fig. 9). The first step is heating of the extracted DNA (at 94-98°C) in order to break the hydrogen bonds and generate single-strand DNAs. This is followed by the annealing of the (forward and reverse) primers to the single-strand DNA at a temperature of 52-58°C. Finally, the polymerase synthesizes a complementary DNA strand at a temperature of 72°C using deoxynucleoside triphosphates (Lorenz et al., 2012).

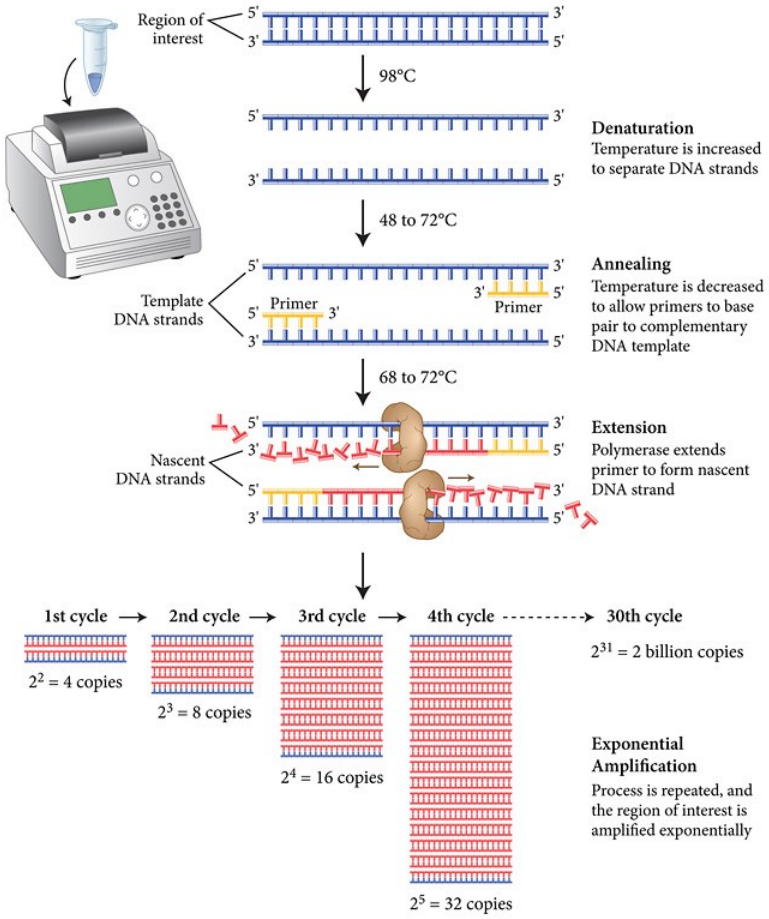

Fig. 9. PCR processing (from https://www.neb.com/applications/dna-amplification-and-pcr).

2.2.3 Detection of amplification products

For detection of amplification products, fluorescence dyes are used. Fluorescence dyes bind double-stranded DNA. After completion of endpoint PCR, the fluorescence generated from the amplified DNA is detected and reported. When real-time PCR (qPCR) is employed, the increase in fluorescence is reported throughout the whole amplification process online (Bustin and Kessler, 2014).

2.2.4 Duplex, multiplex PCR assays

If a PCR assay contains more than one primer system, it is called duplex, triplex, etc. assay. Multiplex PCR assays contain several primer systems to target different genome sequences and to detect different targets simultaneously. When compared to singleplex PCR assays, multiplex PCR assays may lack sensitivity (Gadsby et al., 2010; Pabbaraju et al., 2011).

3 Objectives

The aim of this study was to compare the new eSensor® RVP multiplex assay (GenMark Dx, Inc., Carlsbad, CA, USA) for detection of respiratory pathogens to the current duplex assays employed in the routine diagnostic laboratory (standard assays). The accuracy of the new assay was evaluated with panels of secondary reference material. The clinical performance of the new assay was compared to that of the standard assays. Additionally, the times-to-result including the automated and the manual times were compared.

4 Materials and Methods

4.1 Material

4.1.1 Secondary Reference Material

For evaluation of the accuracy of the new assay, Quality Control for Molecular Diagnostics (QCMD) proficiency panels were used (www.qcmd.org). Panels included programs for detection of adenovirus, coronavirus, human metapneumovirus, influenza virus, human parainfluenza virus, respiratory syncytial virus, and rhinovirus. Each panel consisted between 8 and 12 members. Characteristics of panels are shown in Tables 1 through 7.

Table 1
Members of the QCMD 2013 Adenovirus DNA External Assurance Programme.

Sample	Matrix	Sample content	Copies/ml[1]
ADV13-03	VTM	Adenovirus Serotype 1	11,776
ADV13-05	VTM	Adenovirus Serotype 1	3,334
ADV13-07	VTM	Adenovirus Serotype 1	3,221
ADV13-10	Plasma[2]	Adenovirus Serotype 1	3,192
ADV13-08	VTM	Adenovirus Serotype 5	1,660
ADV13-02	VTM	Adenovirus Serotype 5	206
ADV13-09	VTM	Adenovirus Serotype 4	22,182
ADV13-06	VTM	Adenovirus Serotype 4	2,339
ADV13-01	VTM	Adenovirus Serotype 14	392,645
ADV13-04	VTM	Adenovirus Negative	0

VTM, viral transport medium.
[1]Consensus concentration values calculated from all quantitative datasets returned by participants.
[2]Plasma, human plasma previously screened negative for ADV DNA.

Table 2
Members of the QCMD 2013 Coronavirus RNA External Assurance Programme.

Sample	Matrix	Sample content	Ct value[1]
CV13-09	VTM	Coronavirus-OC43	25.5
CV13-03	VTM	Coronavirus-OC43	28.5
CV13-01	VTM	Coronavirus-OC43	33.4
CV13-10	VTM	Coronavirus-229E	28.3
CV13-08	VTM	Coronavirus-229E	31.5
CV13-06	VTM	Coronavirus-229E	34.5
CV13-02	VTM	Coronavirus-NL63	25.4
CV13-07	VTM	Coronavirus-NL63	28.7
CV13-05	VTM	Coronavirus-NL63	31.7
CV13-04	VTM	Coronavirus Negative	0

VTM, viral transport medium.
[1]Provided by an external testing laboratory.

Table 3
Members of the QCMD 2013 Human Metapneumovirus RNA External Assurance Programme.

Sample	Matrix	Sample content	Ct value[1]
MPV13-03	VTM	Metapneumovirus (Type A2)	23.1
MPV13-08	VTM	Metapneumovirus (Type A2)	24.6
MPV13-07	VTM	Metapneumovirus (Type A2)	24.6
MPV13-02	VTM	Metapneumovirus (Type A2)	28.3
MPV13-04	VTM	Metapneumovirus (Type B2)	22.5
MPV13-01	VTM	Metapneumovirus (Type B2)	25.7
MPV13-06	VTM	Metapneumovirus (Type B2)	29.2
MPV13-05	VTM	Metapneumovirus Negative	0

VTM, viral transport medium.
[1]Provided by an external testing laboratory.

Table 4
Members of the QCMD 2013 Influenza virus A and Influenza virus (INF) B RNA External Assurance Programme.

Sample	Matrix	Sample content	Ct value[1]
INF-RNA13-09	VTM	Influenza virus H1N1 pdm09	33
INF-RNA13-06	VTM	Influenza virus H1N1 pdm09	36
INF-RNA13-03	VTM	Influenza virus H3N2	30
INF-RNA13-12	VTM	Influenza virus H3N2	33
INF-RNA13-07	VTM	Influenza virus H3N2	36
INF-RNA13-01	VTM	Influenza virus H5N1	30
INF-RNA13-04	VTM	Influenza virus B (Victoria)	30
INF-RNA13-10	VTM	Influenza virus B (Victoria)	33
INF-RNA13-02	VTM	Influenza virus (Yamagata)	33
INF-RNA13-11	VTM	Influenza virus (Yamagata)	36
INF-RNA13-08	VTM	Influenza virus B (Yamagata)	39
INF-RNA13-05	VTM	Influenza virus A & B negative	0

VTM, viral transport medium.
[1]Provided by an external testing laboratory.

Table 5
Members of the QCMD 2012 Parainfluenzavirus RNA External Assurance Programme.

Sample	Matrix	Sample content	Ct value[1]
PINF12-10	VTM	Parainfluenzavirus Type 1	30.1
PINF12-01	VTM	Parainfluenzavirus Type 1	34.5
PINF12-05	VTM	Parainfluenzavirus Type 1	35.1
PINF12-07	VTM	Parainfluenzavirus Type 1	36.1
PINF12-03	VTM	Parainfluenzavirus Type 2	25.7
PINF12-09	VTM	Parainfluenzavirus Type 2	28.6
PINF12-02	VTM	Parainfluenzavirus Type 2	32.6
PINF12-04	VTM	Parainfluenzavirus Type 3	25.6
PINF12-08	VTM	Parainfluenzavirus Type 4	35.4
PINF12-06	VTM	Parainfluenzavirus Negative	0

VTM, viral transport medium.
[1]Provided by the production testing laboratory: real-time in-house PCR on the ABI7500.

Table 6
Members of the QCMD 2013 Respiratory syncytial virus RNA External Assurance Programme.

Sample	Matrix	Sample content	CT value[1]
RSV13-05	VTM	Respiratory syncytial virus Type A	31.4
RSV13-07	VTM	Respiratory syncytial virus Type A	34.6
RSV13-01	VTM	Respiratory syncytial virus Type A	39.6
RSV13-03	VTM	Respiratory syncytial virus Type B	30.3
RSV13-02	VTM	Respiratory syncytial virus Type B	33.8
RSV13-08	VTM	Respiratory syncytial virus Type B	34.0
RSV13-04	VTM	Respiratory syncytial virus Type B	37.7
RSV13-06	VTM	Respiratory syncytial virus negative	0

VTM, viral transport medium.
[1]Provided by an external testing laboratory.

Table 7
Members of the QCMD 2013 Rhinovirus RNA External Assurance Programme.

Sample	Matrix	Sample Content	Ct value[1]
RV13-05	VTM	Rhinovirus (A) 16	35.3
RV13-08	VTM	Rhinovirus (A) 16	41.7
RV13-03	VTM	Rhinovirus (A) 16	39.1
RV13-01	VTM	Rhinovirus (A) 16	44.3
RV13-09	VTM	Rhinovirus (A) 16	42.9
RV13-07	VTM	Rhinovirus (A) 16	27.8
RV13-02	VTM	Rhinovirus (A) 16	34.5
RV13-11	VTM	Rhinovirus (A) 16	30.8
RV13-04	VTM	Rhinovirus (A) 16	35.6
RV13-06	VTM	Enterovirus 68	0
RV13-10	VTM	Rhinovirus negative	0

VTM, viral transport medium.
[1]Provided by an external testing laboratory.

4.1.2 Specimens

For comparison of the clinical performance, 94 respiratory specimens that remained following routine clinical testing were tested. Specimens had been derived from patients (40 females, 54 males; mean age, 13.8 years; age range, 0 to 84 years) with a clinical presentation compatible to respiratory infection. Specimens had been collected from 94 patients presenting at different departments of the University Hospital Graz. Specimens included 69 (73.4%) nasopharyngeal aspirates, 14 (14.9%) bronchoalveolar lavages, 7 (7.4%) throat swabs, and 4 (4.3%) induced sputa. Nasopharyngeal aspirates, bronchoalveolar lavages, and induced sputa were collected in screw-cap sterile vessels, throat swabs in swab containers containing viral transport medium (Fig. 10). Immediately after collection, specimens had been transferred to the molecular diagnostics laboratory and stored at ms70° C until comparison studies were done.

A B

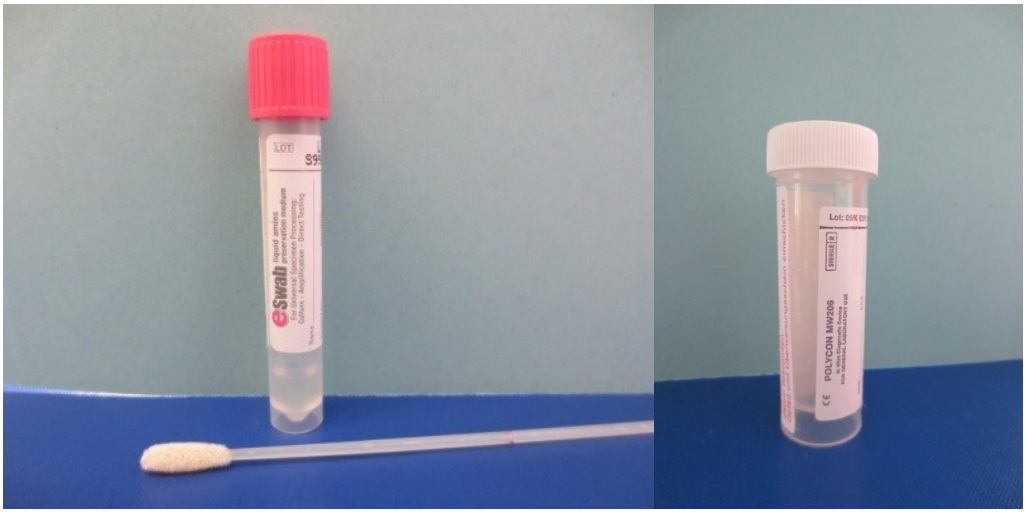

Fig. 10: Specimen containers used in this study. (A) Swab container for viral and bacterial pathogens containing viral transport medium, (B) Sterile container with screw-cap.

4.2 Molecular Methods

All experiments were performed in an International Standard Organization (ISO 9001:2008)-certified laboratory, the Molecular Diagnostics Laboratory, IHME, Medical University of Graz.

4.2.1 The eSensor® RVP multiplex assay

The multiplex eSensor® RVP includes detection of seven viruses and several viral types:

- Adenovirus type B/E
- Adenovirus type C
- Coronavirus type NL63
- Coronavirus type 229E
- Coronavirus type OC43
- Coronavirus type HKU1
- Human metapneumovirus
- Influenza virus type A
- Influenza virus type A H1
- Influenza virus type A H3
- Influenza virus type A 2009 H1N1
- Influenza virus type B
- Parainfluenza virus type 1
- Parainfluenza virus type 2
- Parainfluenza virus type 3
- Parainfluenza virus type 4
- Respiratory syncytial virus type A
- Respiratory syncytial virus type B
- Rhinovirus

In this study, the GenMark eSensor RVP multiplex assay was performed according to the manufacturer's instructions. The assay consists of nucleic acid extraction followed by reverse transcription and PCR amplification and detection by the eSensor® technology.

Nucleic acid extraction was performed on the NucliSens® easyMAG™ (bioMerieux, Marcy l'Etoile, France) platform using the generic protocol. This instrument uses the magnetic particle technology to capture nucleic acids. The input volume was 200 µl and the extracted DNA was automatically eluted with 60 µl of elution buffer. Additionally, 10 µl of internal control (Bacteriophage MS2), included in the kit, was added prior to extraction.

Reverse transcription and amplification was performed on an Applied Biosystems 2720 thermal cycler (Life Technologies, Carlsbad, CA, USA). After amplification, the double stranded DNA was converted into single stranded DNA by addition of an exonuclease followed by another cycling procedure on the thermal cycler. After this, detection reagents were added and the mixture was loaded onto a specially designed cartridge. The cartridge contains a serpentine disposal of gold electrodes with single stranded oligonucleotide capture probes, where the targets hybridize specifically (Fig. 11, 12). The voltage and therefore intensity of the signal is detected at the surface of the gold electrode and transformed into an electrical signal. If this signal is above a certain threshold, the specific viral type is reported as positive. This detection step was performed on the eSensor® XT-8 (GenMark) instrument (Fig. 13).

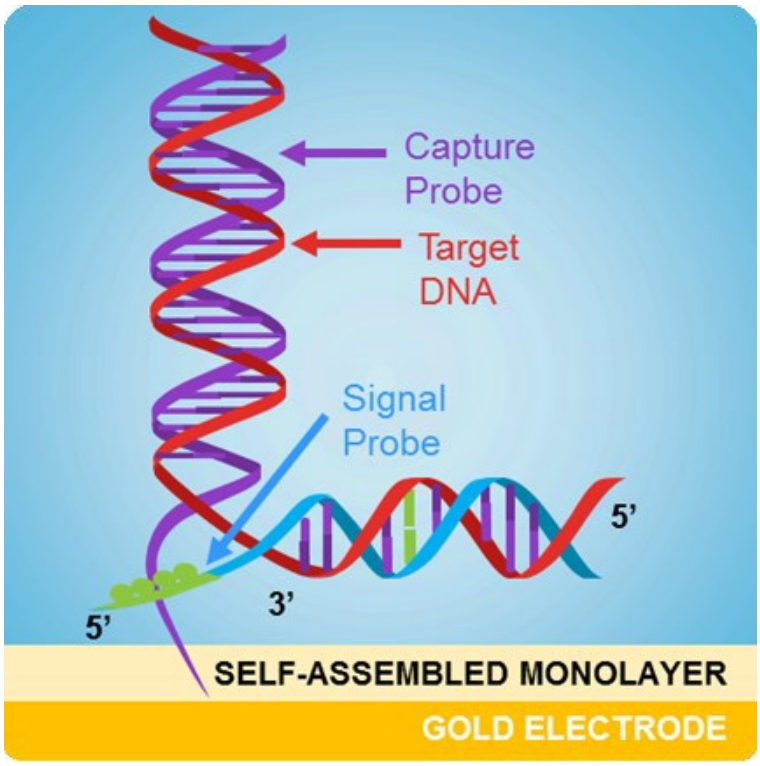

Fig. 11. Hybridization schedule (from GenMark brochure).

Fig. 12. Cartridge (from GenMark brochure).

Fig. 13. The eSensor® XT-8 instrument.

4.2.2 The duplex (standard) assays

In this study, duplex assays of the respiratory multi well system (MWS) r-gene (bioMerieux) were performed according to the manufacturer's instructions. Assays included the AdV/hBoV R-gene® kit, the hCoV/PIV R-gene® kit, the Influenza A/B R-gene® kit, the RSV/hMPV R-gene® kit, and the Rhino&EV/Cc R-gene® kit.

Nucleic acid extraction was performed on the NucliSens® easyMAG™ (bioMerieux) instrument using the specific B protocol (Fig.14). An input volume of 200 µl and an elution volume of 50 µl were used. Real-time PCR amplification and detection were performed on the LightCycler 480 II (Roche Diagnostics, Rotkreuz, Switzerland) instrument (Fig.15). The kits contain several controls. The negative amplification and extraction control verifies the absence of contamination during the amplification and extraction process. The positive control validates a proper outcome

of the amplification step. To verify the presence of cells in the sample, a specially designed cell control is co-amplified and detected.

Fig. 14. The NucliSens® easyMAG™ instrument.

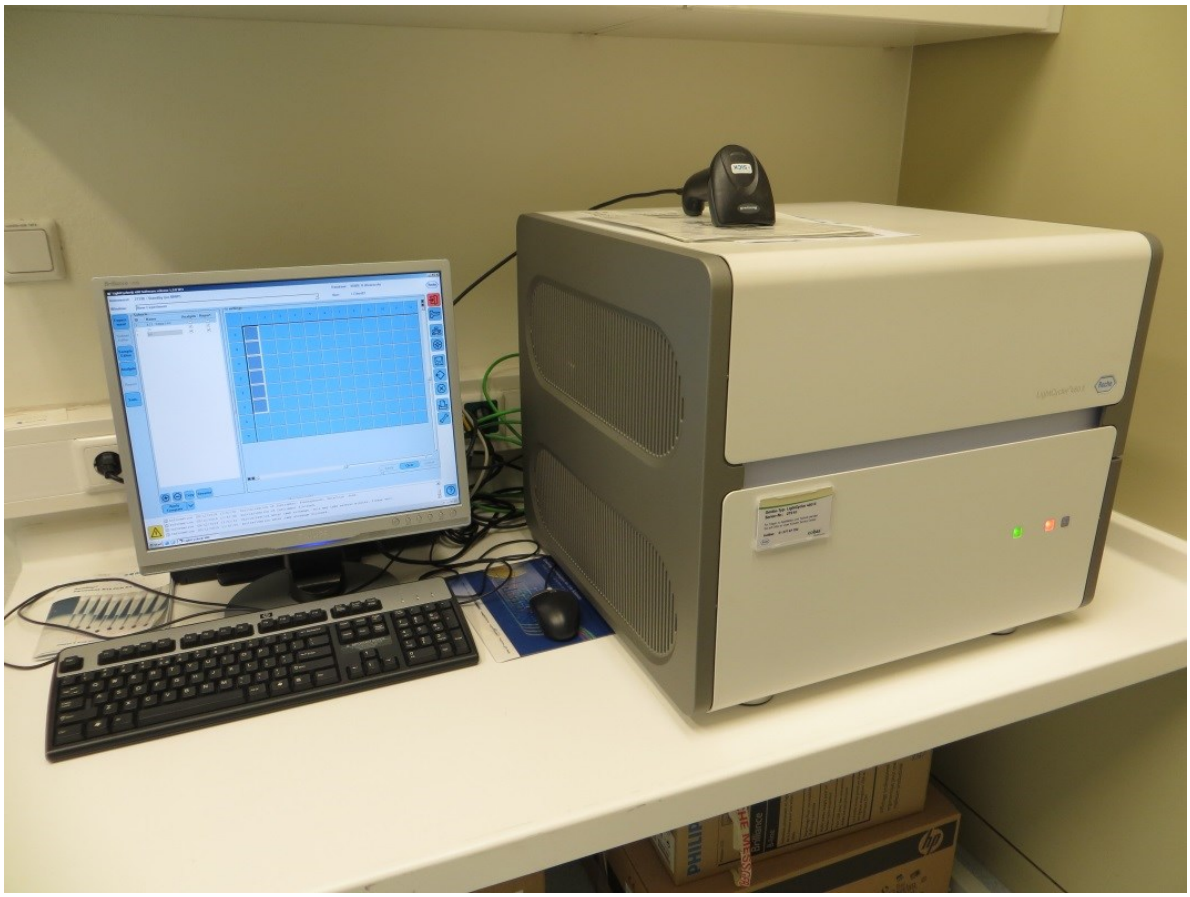

Fig. 15: LightCycler 480 II instrument.

The AdV/hBoV R-gene® kit

The AdV/hBoV R-gene® kit enables the detection of the Adenovirus and the Bocavirus genomes. After DNA extraction, carried out by the NucliSens® easyMAG™ platform, a real time duplex PCR is performed. It provides the detection of 52 different Adenovirus types and 4 Bocavirus types due to the 5' nuclease technique.

The hCoV/HPIV R-gene® kit

The hCoV/HPIV R-gene® kit enables the detection of the Coronavirus and the Parainfluenzavirus genomes. After RNA extraction, carried out by the NucliSens® easyMAG™ platform, a real time duplex PCR is performed. It provides the detection of the 4 Coronavirus types and the 4 Parainfluenza types due to the 5' nuclease technique

The Influenza A/B R-gene® kit

The Influenza A/B R-gene® kit enables the detection of the Influenza virus A and Influenza virus B genomes After RNA extraction, carried out by the NucliSens® easyMAG™ platform, a real time duplex PCR is performed. It provides the detection due to the 5' nuclease technique

The RSV/hMPV R-gene® kit

The RSV/hMPV R-gene® kit enables the detection of the respiratory syncytial and the human metapneumovirus genomes. After RNA extraction, carried out by the NucliSens® easyMAG™ platform, a real time duplex PCR is performed. It provides the detection due to the 5' nuclease technique

The Rhino&EV/Cc R-gene® kit

The Rhino&EV/Cc R-gene® kit enables the detection of the Rhinovirus and the Enterovirus genomes After RNA extraction, carried out by the NucliSens® easyMAG™ platform, a real time duplex PCR is performed. It provides the detection due to the 5' nuclease technique

5 Results

5.1 Results obtained from testing QCMD proficiency panels

When accuracy was tested with 7 QCMD proficiency panels, all results were found according to the expected results.

When 10 members of the QCMD 2013 Adenovirus DNA External Assurance Programme were analyzed, positive samples tested positive and the negative sample tested negative (Table 8). Six members were found to contain adenovirus type C and 3 members were found to contain adenovirus type B/E.

Table 8
Results of the QCMD 2013 Adenovirus DNA External Assurance Programme.

Sample	Matrix	Sample content	Copies/ml[1]	Result eSensor® RVP
ADV13-03	VTM	Adenovirus serotype 1	11,776	Positive
ADV13-05	VTM	Adenovirus serotype 1	3,334	Positive
ADV13-07	VTM	Adenovirus serotype 1	3,221	Positive
ADV13-10	Plasma[2]	Adenovirus serotype 1	3,192	Positive
ADV13-08	VTM	Adenovirus serotype 5	1,660	Positive
ADV13-02	VTM	Adenovirus serotype 5	206	Positive
ADV13-09	VTM	Adenovirus serotype 4	22,182	Positive
ADV13-06	VTM	Adenovirus serotype 4	2,339	Positive
ADV13-01	VTM	Adenovirus serotype 14	392,645	Positive
ADV13-04	VTM	Adenovirus negative	0	Target not detected

VTM, viral transport medium.
[1]Consensus concentration values calculated from all quantitative datasets returned by participants.
[2]human plasma previously screened negative for ADV DNA.

When 10 members of the QCMD 2013 Coronavirus RNA External Assurance Programme were analyzed, positive samples tested positive and the negative sample tested negative (Table 9). Three members were found to contain coronavirus type OC43, three members were found to be coronavirus type 229E, and three more were found to contain type NL63.

Table 9
Results of the QCMD 2013 Coronavirus RNA External Assurance Programme.

Sample	Matrix	Sample content	Ct value[1]	Result eSensor® RVP
CV13-09	VTM	Coronavirus-OC43	25.5	Positive
CV13-03	VTM	Coronavirus-OC43	28.5	Positive
vCV13-01	VTM	Coronavirus-OC43	33.4	Positive
CV13-10	VTM	Coronavirus-229E	28.3	Positive
CV13-08	VTM	Coronavirus-229E	31.5	Positive
CV13-06	VTM	Coronavirus-229E	34.5	Positive
CV13-02	VTM	Coronavirus-NL63	25.4	Positive
CV13-07	VTM	Coronavirus-NL63	28.7	Positive
CV13-05	VTM	Coronavirus-NL63	31.7	Positive
CV13-04	VTM	Coronavirus negative	0	Target not detected

VTM, viral transport medium.
[1]Provided by an external testing laboratory.

When 8 members of the QCMD 2013 human Metapneumovirus RNA External Assurance Programme were analyzed, positive samples tested positive and the negative sample tested negative (Table 10).

Table 10
Results of the QCMD 2013 Human Metapneumovirus RNA External Assurance Programme.

Sample	Matrix	Sample content	Ct value[1]	Result eSensor® RVP
MPV13-03	VTM	Metapneumovirus (Type A2)	23.1	Positive
MPV13-08	VTM	Metapneumovirus (Type A2)	24.6	Positive
MPV13-07	VTM	Metapneumovirus (Type A2)	24.6	Positive
MPV13-02	VTM	Metapneumovirus (Type A2)	28.3	Positive
MPV13-04	VTM	Metapneumovirus (Type B2)	22.5	Positive
MPV13-01	VTM	Metapneumovirus (Type B2)	25.7	Positive
MPV13-06	VTM	Metapneumovirus (Type B2)	29.2	Positive
MPV13-05	VTM	Metapneumovirus negative	0	Target not detected

VTM, viral transport medium.
[1]Provided by an external testing laboratory.

When 12 members of the QCMD 2013 Influenza virus A and Influenza virus B RNA External Assurance Programme were analyzed, positive samples tested positive and the negative sample tested negative. Additionally, the eSensor® RVP could differentiate between influenza A and B in all members and detected also samples containing types A H3 and A H1N1.

Table 11
Results of the QCMD 2013 Influenza virus A and Influenza virus (INF) B RNA External Assurance Programme.

Sample	Matrix	Sample content	Ct value[1]	Result eSensor® RVP
INF-RNA13-09	VTM	Influenza virus H1N1 pdm09	33	Positive
INF-RNA13-06	VTM	Influenza virus H1N1 pdm09	36	Positive
INF-RNA13-03	VTM	Influenza virus H3N2	30	Positive
INF-RNA13-12	VTM	Influenza virus H3N2	33	Positive
INF-RNA13-07	VTM	Influenza virus H3N2	36	Positive
INF-RNA13-01	VTM	Influenza virus H5N1	30	Positive
INF-RNA13-04	VTM	Influenza virus B (Victoria)	30	Positive
INF-RNA13-10	VTM	Influenza virus B (Victoria)	33	Positive
INF-RNA13-02	VTM	Influenza virus (Yamagata)	33	Positive
INF-RNA13-11	VTM	Influenza virus (Yamagata)	36	Positive
INF-RNA13-08	VTM	Influenza virus B (Yamagata)	39	Positive
INF-RNA13-05	VTM	Influenza virus A & B negative	0	Target not detected

VTM, viral transport medium.
[1]Provided by an external testing laboratory.

When 10 members of the QCMD 2012 Parainfluenza Virus RNA External Assurance Programme were analyzed, positive samples tested positive and the negative sample tested negative (Table 12). Additionally the eSensor® RVP could differentiate between parainfluenza virus types 1, 2, 3, and 4.

Table 12
Results of the QCMD 2012 Parainfluenzavirus RNA External Assurance Programme.

Sample	Matrix	Sample content	Ct value[1]	Result eSensor® RVP
PINF12-10	VTM	Parainfluenzavirus type 1	30.1	Positive
PINF12-01	VTM	Parainfluenzavirus type 1	34.5	Positive
PINF12-05	VTM	Parainfluenzavirus type 1	35.1	Positive
PINF12-07	VTM	Parainfluenzavirus type 1	36.1	Positive
PINF12-03	VTM	Parainfluenzavirus type 2	25.7	Positive
PINF12-09	VTM	Parainfluenzavirus type 2	28.6	Positive
PINF12-02	VTM	Parainfluenzavirus type 2	32.6	Positive
PINF12-04	VTM	Parainfluenzavirus type 3	25.6	Positive
PINF12-08	VTM	Parainfluenzavirus type 4	35.4	Positive
PINF12-06	VTM	Parainfluenzavirus negative	0	Target not detected

VTM, viral transport medium.
[1]Provided by the production testing laboratory: real-time in-house PCR on the ABI7500.

When 8 members of the QCMD 2013 Respiratory Syncytial Virus RNA External Assurance Programme were analyzed, positive samples tested positive and the negative sample tested negative (Table 13). The eSensor® RVP could differentiate between respiratory syncytial virus type A and type B correctly.

Table 13
Results of the QCMD 2013 Respiratory syncytial virus RNA External Assurance Programme.

Sample	Matrix	Sample content	Ct value[1]	Result eSensor® RVP
RSV13-05	VTM	Respiratory syncytial virus type A	31.4	Positive
RSV13-07	VTM	Respiratory syncytial virus type A	34.6	Positive
RSV13-01	VTM	Respiratory syncytial virus type A	39.6	Positive
RSV13-03	VTM	Respiratory syncytial virus type B	30.3	Positive
RSV13-02	VTM	Respiratory syncytial virus type B	33.8	Positive
RSV13-08	VTM	Respiratory syncytial virus type B	34.0	Positive
RSV13-04	VTM	Respiratory syncytial virus type B	37.7	Positive
RSV13-06	VTM	Respiratory syncytial virus negative	0	Target not detected

VTM, viral transport medium.
[1]Provided by an external testing laboratory.

When 11 members of the QCMD 2013 Rhinovirus RNA External Assurance Programme were analyzed, 11 members tested positive and two, the rhinovirus-negative member and that containing enterovirus 68, negative (Table 14). In one of the rhinovirus-positive members of this panel, adenovirus type B/E was detected additionally.

Table 14
Results of the QCMD 2013 Rhinovirus RNA External Assurance Programme.

Sample	Matrix	Sample Content	Ct value[1]	Result eSensor® RVP
RV13-05	VTM	Rhinovirus (A) 16	35.3	Positive
RV13-08	VTM	Rhinovirus (A) 16	41.7	Positive
RV13-03	VTM	Rhinovirus (A) 16	39.1	Positive
RV13-01	VTM	Rhinovirus (A) 16	44.3	Positive
RV13-09	VTM	Rhinovirus (A) 16	42.9	Positive
RV13-07	VTM	Rhinovirus (A) 16	27.8	Positive
RV13-02	VTM	Rhinovirus (A) 16	34.5	Positive
RV13-11	VTM	Rhinovirus (A) 16	30.8	Positive
RV13-04	VTM	Rhinovirus (A) 16	35.6	Positive
RV13-06	VTM	Enterovirus 68	0	Positive
RV13-10	VTM	Rhinovirus negative	0	Target not detected

VTM, viral transport medium;
[1]Provided by an external testing laboratory.

5.2 Results obtained from clinical samples

Analysis of 94 routine clinical samples with the eSensor® RVP confirmed results obtained from routine analyses with the duplex standard assays in 87 samples (92.6%). Viruses detected by both methods included respiratory syncytial virus (n=57) followed by rhinovirus (n=19), human metapneumovirusPV (n=13), influenza (n=9), adenovirus (n=4), coronavirus (n=3), and parainfluenza virus (n=1; Table 15). These results include double and triple infections (Table 16). When these results were compared to those obtained according to the original clinicians' orders, lower numbers of positive results were observed: respiratory syncytial virus 53 positives vs 57 positives, rhinovirus 6 vs 19, 13 vs 13, influenza 6 vs 9, adenovirus 3 vs 4, coronavirus 0 vs 3, and parainfluenza virus 0 vs 1.

Table 15
Concordant results obtained from clinical samples (n=87).

	Concordant positive results detected in this study[1]	Positive results that had been obtained with the routine assay according to the original order by the clinician
RSV	57	53
Rhinovirus	19	6
HMPV	13	13
Influenzavirus	9	6
Adenovirus	4	3
Coronavirus	3	0
Parainfluenzavirus	1	0

HMPV, human metapneumovirus; RSV, respiratory syncytial virus.
[1]Including multiple infections.

Table 16
Double (n=23) and triple (n=1) infections (concordant results) found in this study.

No. of samples (n)	Viruses detected
12	Rhinovirus and RSV
3	Influenza A virus and RSV
2	Coronavirus and RSV
1	Adenovirus and influenza A virus
1	Adenovirus and human metapneumovirus
1	Adenovirus and rhinovirus
1	Adenovirus and RSV
1	Human metapneumovirus and RSV
1	Parainfluenzavirus and RSV
1	Coronavirus and rhinovirus and RSV

RSV, respiratory syncytial virus.

Seven samples (7.4%) gave discrepant results (Table 17). Six of these samples gave positive results for two viruses when tested with the eSensor® RVP, whereas only one of these viruses was detected with the duplex standard assays. Additional positive results with the eSensor® RVP included human metapneumovirus (n=3), rhinovirus (n=2) and adenovirus (n=1). One sample tested positive for influenza A virus with the routine duplex standard assay but was not detected with the eSensor® RVP.

Table 17
Discrepant results (n=7) obtained in this study.

Sample ID	**Result obtained by**	
	eSensor® RVP	**duplex standard assay**
10	RSV pos, Adenovirus pos	RSV pos, Adenovirus TND
16	Influenza A pos, HMPV pos	Influenza A pos, HMPV TND
18	RSV pos, HMPV pos	RSV pos, HMPV TND
45	HMPV pos, Rhinovirus pos	HMPV pos, Rhinovirus TND
61	RSV pos, HMPV pos	RSV pos, HMPV TND
68	RSV pos, Rhinovirus pos	RSV pos, Rhinovirus TND
72	Influenza A TND	Influenza A pos

ID, identification number; RSV, respiratory syncytial virus; pos = positive, TND = target not detected; HMPV, human metapneumovirus.

5.3 Comparison of workflows and time required for assays used in this study

When times-to-result were compared, the eSensor® RVP required 315 minutes compared to 160 minutes when using the routine duplex assays (Table 18). Time required for manual steps was similar for both assays. However, after amplification, two additional visits for manual steps including exonuclease digestion and preparation reagents for detection were required for the eSensor® RVP.

Table 18
Comparison of workflows and time required for assays used in this study.

Steps	eSensor RVP	Time/sample (min)	Duplex standard assay	Time/sample (min)
Sample preparation	NucliSens easyMAG, generic protocol	60	NucliSens easyMAG, specific B protocol	70
Amplification	Endpoint PCR	180	Real-time PCR on the LightCycler 480 II instrument	90
Single-stranded DNA generation	Exonuclease digestion	30	-	-
Detection	eSensor XT-8 instrument	45	- [1]	-
Total		**315**		**160**

[1]Detection is integral part of the real-time procedure.

6 Discussion

The eSensor® RVP, a qualitative assay for simultaneous detection of 7 respiratory viruses and several viral types has recently been brought on the market. In this study, this multiplex assay was evaluated in a routine clinical setting.

When accuracy of the new assay was evaluated with panels of secondary reference material, all results were found to be concordant with the results expected. Proficiency panels of the QCMD program included members with low viral loads as well as negative members. In recent studies, reduced sensitivity was reported as disadvantage of PCR assays based on multiplex testing (Gadsby et al., 2010; Pabbaraju et al., 2011). In the present study, results showed an excellent accuracy of the eSensor® RVP multiplex assay despite the broad menu of the panel. In addition, the negative result of the Enterovirus 68-positive sample included in the QCMD 2013 Rhinovirus RNA EQA Programme is based on the fact that the eSensor® RVP multiplex assay discriminates the rhinovirus species from the enterovirus species which may have an impact on the management of patients. In contrast, the majority of assays on the market using the conserved 5´-NCR region of the enterovirus genome as target for the amplification cannot distinguish between enteroviruses and rhinoviruses (Mahony 2008).

When the clinical performance of the new assay was evaluated, concordant results (92.6%) were found in the majority of samples. In a recent study, a similar overall agreement (89.5%) was observed when comparing the eSensor® RVP with the FilmArray Respiratory Panel (BioFire Diagnostics) (Ruggiero et al., 2014). In the present study, respiratory syncytial virus was the virus detected most frequently followed by rhinovirus and human metapneumovirus. For 57 samples, one pathogen was detected and for 23 samples, two pathogens. The main combination was respiratory syncytial virus and rhinovirus. In one sample, a combination of coronavirus, respiratory syncytial virus, and rhinovirus was found. For 6 samples, no target was detected with both assays. These data correspond to those observed recently (Popowitch et al., 2014). When the results obtained from the present study were compared to those according to the original clinicians' orders, 25 positives would have been missed. The lack of more adequate orders may be caused by the

very similar symptomatology shared by several respiratory pathogens followed by insufficient orders, which may have an impact to the management of patients (Hammond et al., 2012).

The discordant samples (n=7) showed differences which may be the result of low viral load of the pathogens missed. All additional positive results obtained by the eSensor® RVP showed nanoamper (nA) values below 100. The threshold for positive samples is 3 nA; however, the vast majority of positive results show values higher than 100 nAs. The threshold cycle value calculated for the additional positive result obtained by the duplex assay routinely used was 36.9.

The total time required for the eSensor® RVP per sample was found to be almost two times longer in comparison to that for the routinely used duplex assays (315 min vs. 160 min). Time required for manual steps of the eSensor® RVP multiplex assay is similar with the routinely used duplex assays. However, more visits are needed during analysis. This drawback will be improved with the next generation ePlex® system which combines nucleic acid extraction, amplification, and detection in a single cartridge with a turnaround time of 60-90 minutes.

In conclusion, the eSensor® Respiratory Viral Panel (RVP) showed excellent accuracy. Comparison of the clinical performance revealed concordant results in the vast majority of samples. When employing this multiplex assay, more pathogens can be detected simultaneously and this may have an impact on the patients' management. Further automation and shorter times-to-results are necessary for future use in the routine diagnostic laboratory.

7 References

Barenfanger J, Drake C, Leon N, Mueller T, Troutt T. Clinical and financial benefits of rapid detection of respiratory viruses: an outcomes study. J Clin Microbiol **2000**;38:2824-8.

Bustin AS and Kessler HH. Amplification and detection methods. In: Kessler HH (Ed.). Molecular diagnostics of infectious diseases, 3rd edition. Walter de Gruyter (Berlin/Boston) 2014: 63-83.

Fox JD. Respiratory virus surveillance and outbreak investigation. J Clin Virol **2007**;40 Suppl 1:S24-30.

Gadsby NJ, Hardie A, Claas EC, Templeton KE. Comparison of the Luminex Respiratory Virus Panel fast assay with in-house real-time PCR for respiratory viral infection diagnosis. J Clin Microbiol **2010**;48:2213-6.

Gharabaghi F, Hawan A, Drews SJ, Richardson SE. Evaluation of multiple commercial molecular and conventional diagnostic assays for the detection of respiratory viruses in children. Clin Microbiol Infect **2011**;17:1900-6.

Hammond SP, Gagne LS, Stock SR, et al. Respiratory virus detection in immunocompromised patients with FilmArray respiratory panel compared to conventional methods. J Clin Microbiol **2012**;50:3216-21.

Herbert Hof, Rüdiger Dörries. Medizinische Mikrobiologie. 4rd edition. Georg Thieme Verlag KG. **2009**. P.36-39.

Kehl SC, Kumar S. Utilization of nucleic acid amplification assays for the detection of respiratory viruses. Clin Lab Med **2009**;29:661-71.

Kessler HH. Extraction of nucleic acids. In: Kessler HH (Ed.). Molecular diagnostics of infectious diseases, 3rd edition. Walter de Gruyter (Berlin/Boston) 2014: 57-62.

Krause JC, Panning M, Hengel H, Henneke P. The role of multiplex PCR in respiratory tract infections in children. Dtsch Arztebl Int **2014**;111:639-45.

Lorenz TC. J Vis Exp. Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies.**2012** May 22;(63):e3998. doi: 10.3791/3998.

Mahony JB. Detection of respiratory viruses by molecular methods. Clin Microbiol Rev **2008**;21:716-47.

Mahony JB, Petrich A, Smieja M. Molecular diagnosis of respiratory virus infections. Crit Rev Clin Lab Sci **2011**;48:217-49.

Pabbaraju K, Wong S, Tokaryk KL, Fonseca K, Drews SJ. Comparison of the Luminex xTAG respiratory viral panel with xTAG respiratory viral panel fast for diagnosis of respiratory virus infections. J Clin Microbiol **2011**;49:1738-44.

Popowitch EB, O'Neill SS, Miller MB. Comparison of the Biofire FilmArray RP, Genmark eSensor RVP, Luminex xTAG RVPv1, and Luminex xTAG RVP fast multiplex assays for detection of respiratory viruses. J Clin Microbiol **2013**;51:1528-33.

Ruggiero P, McMillen T, Tang YW, Babady NE. Evaluation of the BioFire FilmArray respiratory panel and the GenMark eSensor respiratory viral panel on lower respiratory tract specimens. J Clin Microbiol **2014**;52:288-90.

Templeton KE. Why diagnose respiratory viral infection?. J Clin Virol **2007**;40 Suppl 1:S2-4.

Virginia M. Pierce, Richard L. Hodinka. Comparison of the GenMark Diagnostics eSensor respiratory viral panel to real-time PCR for detection of respiratory viruses in children. J Clin Microbiol. **2012** Nov;50(11):3458-65. doi: 10.1128/JCM.01384-12.

8 Table of figures

9 Adnex

Medical University of Graz

Comparison of the multiplex eSensor® Respiratory Viral Panel to duplex real-time PCR assays for detection of viral respiratory pathogens

Evelyn Stelzl, Michael Bozic, Veronika Sinner, Harald H. Kessler
Research Unit Molecular Diagnostics & Molecular Diagnostics Laboratory, IHMEM, Medical University of Graz, Austria

Background

Diagnosis of respiratory tract infections based on the clinical presentation alone is difficult because the majority of respiratory viruses develop similar symptoms. Rapid and accurate diagnosis is of major importance. It improves the patient's outcome, particularly if antiviral therapy is available. In recent years, nucleic acid amplification has become state of the art for rapid detection of viral infections. This technology has been shown to be superior to conventional diagnostic methods such as virus culture, direct antigen detection, and enzyme immunoassays.

Objectives

In this study, the new eSensor® RVP multiplex assay (GenMark) for detection of respiratory pathogens was compared to duplex assays used routinely in the diagnostic laboratory. The accuracy of the new assay was evaluated with panels of secondary reference material. The clinical performance of the new assay was compared to that of assays used routinely. In addition, times-to-result were compared.

Study design

The new eSensor® Respiratory Viral Panel (RVP) is a qualitative assay for parallel detection of 7 viruses including 20 types based on multiplex PCR followed by detection of amplification products on the eSensor® XT-8 instrument (GenMark Diagnostics, Carlsbad, CA; **Fig.1**). The duplex assays used routinely included several respiratory multi well system (MWS) r-gene (bioMerieux, Marcy-l´Etoile, France) kits: Influenza A/B R-gene®, RSV/hMPV R-gene®, Rhino&EV/Cc R-gene®, AdV/hBoV R-gene®, and HCoV/HPIV R-gene®.
For accuracy testing, 7 proficiency panels provided by the Quality Control for Molecular Diagnostics (QCMD) organization were analyzed with the new assay. For comparison of the clinical performance, 94 respiratory specimens that remained following routine clinical testing were analyzed. In addition, times-to-result including the automated and the manual times were compared.

Results

When the accuracy of the new assay was evaluated with 7 QCMD proficiency panels, all results were found in agreement with the expected results. Results obtained with the QCMD 2013 Respiratory Syncytial Virus RNA EQA Programme are shown in **Table 1**.
Comparison of clinical performance with 94 routine clinical samples revealed concordant results for 87 samples (92.6%). Viruses detected with both assays included respiratory syncytial virus (n=57), rhinovirus (n=19), human metapneumovius (n=13), influenza virus (n=9), adenovirus (n=4), coronavirus (n=3), and parainfluenza virus (n=1). Seven samples (7.4%) gave discrepant results (**Table 2**).
When comparing times-to-result, the eSensor® RVP required 160 minutes compared to 315 minutes for the routine duplex assays (**Table 3**). Time required for manual steps was similar for both assays. However, after amplification, two additional visits for manual steps (exonuclease digestion and preparation of hybridizations buffers for detection) were required for the eSensor® RVP.

Fig. 1: The eSensor® XT-8 instrument.

Table 1: Results of the QCMD 2013 Respiratory Syncytial Virus RNA External Assurance Program. RSV, respiratory syncytial virus; VTM, viral transport medium.

Sample	Matrix	Sample content	CT value[1]	Result eSensor® RVP
RSV13-05	VTM	RSV Type A	31.4	RSV A positive
RSV13-07	VTM	RSV Type A	34.6	RSV A positive
RSV13-01	VTM	RSV Type A	39.6	RSV A positive
RSV13-03	VTM	RSV Type B	30.3	RSV B positive
RSV13-02	VTM	RSV Type B	33.8	RSV B positive
RSV13-08	VTM	RSV Type B	34.0	RSV B positive
RSV13-04	VTM	RSV Type B	37.7	RSV B positive
RSV13-06	VTM	RSV negative	0	Target not detected

Table 2: Results of discrepant samples. ID, identification number; RSV, respiratory syncytial virus; pos, positive; TND, target not detected; HMPV, human metapneumovirus.

Sample ID	Result obtained by	
	eSensor® RVP	Duplex standard assay
10	RSV pos, adenovirus pos	RSV pos, adenovirus TND
16	Influenza A pos, HMPV pos	Influenza A pos, HMPV TND
18	RSV pos, HMPV pos	RSV pos, HMPV TND
45	HMPV pos, rhinovirus pos	HMPV pos, rhinovirus TND
61	RSV pos, HMPV pos	RSV pos, HMPV TND
68	RSV pos, rhinovirus pos	RSV pos, rhinovirus TND
72	Influenza A TND	Influenza A pos

Table 3: Comparison of times-to-result for assays used in this study.

Steps	eSensor® RVP	Time/sample (min)	Duplex standard assay	Time/sample (min)
Sample preparation	NucliSens easyMAG, generic protocol	60	NucliSens easyMAG, specific B protocol	70
Amplification	Endpoint PCR	180	Real-time PCR on the LightCycler 480II instrument	90
Single-stranded DNA generation	Exonuclease digestion	30	-	-
Detection	eSensor XT-8 instrument	45	- [a]	-
Total		**315**		**160**

[a]Detection is integral part of the real-time procedure.

Conclusions

The eSensor® Respiratory Viral Panel (RVP) showed excellent accuracy. Comparison of the clinical performance revealed concordant results in the vast majority of samples. Further automation and shorter times-to-result are necessary for future use of the new assay in the routine diagnostic laboratory.

Corresponding author: evelyn.stelzl@medunigraz.at