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Evolution of direct diagnostic techniques in Virology; analytical performances and clinical input

Thesis submitted by Laurent BUSSON
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Résumé :

Le diagnostic virologique est un sujet d'actualité particulièrement du fait des récentes épidémies ou pandémies telles que la pandémie d'influenza A(H1N1) en 2009 ou la diffusion du virus Zika dans les Amériques et la région du Pacifique entre 2014 et 2017, associée à des cas de microcéphalie et des syndromes de Guillain Barré. Encore plus récemment, en août 2018, le ministre de la santé de la République Démocratique du Congo annonçait la 10^{e} épidémie de virus Ebola dans le pays et en décembre 2019, le coronavirus SARS-CoV-2 est à l'origine d'une pandémie au départ de la Chine. Avec le nombre croissant de migrants et de voyageurs favorisant la dissémination des maladies virales, les laboratoires diagnostiques doivent être parés à la fois pour l'identification des virus communs mais aussi de ceux importés.

Les techniques les plus anciennes de diagnostic virologique tendent à devenir obsolètes suite au développement rapide des techniques moléculaires depuis les années 90. Cependant, nous utilisons toujours un mélange de techniques moléculaires et non moléculaires au sein de notre laboratoire.

Les objectifs de ce travail sont de passer en revue les différentes techniques communément utilisées pour la détection directe des virus avec leurs avantages et leurs inconvénients et de fournir une réflexion sur la place de chaque technique, en 2020, dans un laboratoire diagnostique.

Nous aborderons tout d'abord les cultures cellulaires et nous insisterons sur leur polyvalence qui permet parfois de mettre en évidence des micro-organismes que l'on ne suspectait pas. Nous illustrerons ce point par un article relatant la mise en évidence de *Chlamydia trachomatis* du serovar L responsables de la lymphogranulomatose vénérienne dans des prélèvements envoyés pour suspicion d'infection herpétique.

Le travail se focalisera ensuite plus particulièrement sur le diagnostic des infections virales respiratoires. Nous verrons les principes des tests de détection antigéniques et discuterons de leurs limites en se basant sur un article qui traite du diagnostic des virus influenza A et B par 3 différents tests immunochromatographiques. Cet article montre que la sensibilité des tests varie en fonction de la charge virale dans le prélèvement ainsi que du sous-type de virus.

Nous poursuivrons avec les tests d'amplification d'acides nucléiques (tests moléculaires) en expliquant la technique de PCR (Polymerase Chain Reaction) et une technique d'amplification isothermique (Nicking Enzyme Amplification Reaction - NEAR). Nous illustrerons par un article portant sur l'évaluation du test Alere i influenza A&B (technique NEAR) en comparaison du test Sofia influenza A+B (immunochromatographie). Cet article montre un gain de sensibilité de l'Alere i par rapport au Sofia pour le diagnostic de l'influenza A mais pas pour l'influenza B. Il constitue également un travail préliminaire sur l'appréciation de l'utilité d'une technique PCR rapide dans la prise en charge des patients. La conclusion est qu'il pourrait y avoir un apport de ce type de technique pour la diminution des hospitalisations, de la prescription des examens complémentaires et des antibiotiques. Cela permettrait également une prescription plus adéquate de l'oseltamivir pour le traitement de la grippe. Le point important est que l'impact du résultat est d'autant plus grand qu'il est délivré précocement dans la prise en charge des patients, idéalement lorsqu'ils sont encore aux urgences.

Suite au travail sur l'Alere i, nous avons entrepris d'évaluer un test PCR multiplex (FilmArray Respiratory Panel) pour le diagnostic des virus afin de voir si la détection d'un plus grand nombre de pathogènes pourrait avoir un impact plus grand sur la prise en charge des patients. Cette évaluation a donné lieu à deux articles. Le premier détaille les avantages et inconvénients des différents outils de diagnostic pour la détection des virus respiratoires et sert d'état des lieux sur les tests utilisés actuellement dans les laboratoires de virologie. Le deuxième article porte plus particulièrement sur l'apport du FilmArray dans la prise en charge des patients. La conclusion est que ce n'est pas le résultat du test qui a un impact sur cette prise en charge mais plutôt d'autres facteurs notamment l'âge ou des marqueurs inflammatoires biologiques.

Nous terminerons ce travail par un aperçu des techniques de séquençage qui seront sans aucun doute de plus en plus utilisées pour le diagnostic en virologie.

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List of abbreviations

CMV: cytomegalovirus

CoV: coronavirus

CPE: cytopathic effect

DNA: deoxyribonucleic acid

EBV: Epstein-Barr virus

EEA: European Economic Area

EIA: enzyme immunoassay

ER: emergency room

FDA: Food and Drug Administration

HBV: hepatitis B virus

HCV: hepatitis C virus

HDA: Helicase-Dependent Amplification

HIV: human immunodeficiency virus

HSV: (human) herpes simplex virus

IFA: immunofluorescent assay

ILI: influenza-like illness

INAMI: Institut National d'Assurance Maladie-Invalidité

LAMP: Loop Mediated Isothermal Amplification

LOS: length of stay

LRTI: lower respiratory tract infection

NAAT: nucleic acid amplification test

NEAR: Nicking Enzyme Amplification Reaction

NGS: next-generation sequencing

NIH: National Institute of Health

PCR: polymerase chain reaction

RNA: ribonucleic acid

SD: standard deviation

SDA: Strand Displacement Amplification

URTI: upper respiratory tract infection

VZV: varicella zoster virus

WGS: whole genome sequencing

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1 Introduction

Due to recent epidemics or pandemics, namely, the 2009 influenza A(H1N1) pandemic [1] or the spread of zika virus, associated with microcephaly and Guillain-Barré syndrome, in the Americas and the Pacific region from 2014 to 2017 [2], viral diagnosis has become a very topical issue. More recently, in August 2018, the Ministry of Health of the Democratic Republic of the Congo declared the 10th outbreak of Ebola virus disease in the country [3] In December 2019, first cases of coronavirus SARS-CoV-2 in China led to a pandemic. [4] With increasing number of migrants and travellers facilitating the spread of viral diseases, diagnostic laboratories have been facing the prospect of devising methods of identification targeting known routine viruses as well as more "exotic" ones.

Viral detection can be performed in various sample types. In the blood, the presence of a virus (viremia) can be measured with the viral load and is useful to set a diagnosis and follow a treatment such as for viral hepatitis or HIV infection. It can also screen for viral recurrences in immunosuppressed patients namely for EBV, CMV or HHV6. The viremia can sometime be appreciated by the detection of the excretion of the virus in urines namely for congenital CMV infection. The analysis of cerebrospinal fluid is crucial for the diagnosis of viral meningitis and encephalitis. For gastro-enteritis, viruses can be detected directly in a faeces sample. Skin infections can be diagnosed by testing swab samples and for respiratory tract infections the analysis of various respiratory samples permit to detect one or more viruses. Viral detection can be performed on virtually any type of samples provided it is technically feasible and medically indicated.

The fast development of molecular techniques since the 90s' has pushed non-molecular techniques towards obsolescence. However, both molecular and non-molecular techniques are still performed in our laboratory.

The objectives of this work are to review the different techniques commonly available for direct detection of viruses with their pros and cons and to provide a reflection on the place of each technique, in 2019, in a diagnostic laboratory.

This work will chronologically present the discovery of the different diagnostic tools for the direct detection of viruses and explain their basic principles. Articles relating our experience with the different techniques will be integrated throughout the text. A state of art of the different available techniques will then be presented and their respective usefulness in patients' management will be discussed based on respiratory viruses revealed by the tests used. Afterward, an insight on the perspectives in direct viral diagnosis will be exposed. It was chosen to appreciate the impact of respiratory viruses' detection in the management of patients as this point is still controversial in the literature. Indeed, contrary to other sample types where viral detection is clearly associated with a disease or an abnormal finding requiring handling or follow-up (namely for blood or cerebrospinal fluid samples), the detection of viruses in respiratory samples, although abnormal, is not always associated with symptoms and won't automatically necessitate a treatment. This work will try to contribute in bringing some light on this issue.

2 Material and methods

2.1 Study site

Analyses were performed in Iris-Lab which is a multisite clinical laboratory gathering samples from 5 university hospitals. The following hospitals contributed to the studies reported in this work:

- Saint-Pierre University Hospital: a 582-bed teaching hospital located in Brussels (Belgium). [5]

- Jules Bordet Institute: a 160-bed teaching hospital located in Brussels and specialized in the management of oncological patients. [6]

Iris-Lab became the LHUB-ULB (Laboratoire Hospitalier Universitaire de Bruxelles – Universitair Laboratorium Brussel) in September 2016, a top 5 European laboratory regarding the number of analyses performed. [7]

2.2 Population

Included population is described in each article. Patients visited one of the abovementioned hospitals between April 2010 and Mars 2016.

2.3 Techniques

Laboratory techniques used are described in the "Material and Methods" section of each article.

2.4 Statistical analysis

When statistical analysis of data was required, tests used are described in the "Material and Methods" section of each article.

3 Evolution of diagnostic virology

3.1 A short history of Virology: discovery of viruses

The discovery of viruses is indissociable from that of bacteria. As early as 1546, Girolamo Fracastoro, an Italian physician, suggested that epidemics were caused by the dissemination of minutes particles. In 1676, Anton van Leeuwenhoek, a Dutch businessman and scientist, was the first to observe bacteria through his microscope and the first culture of bacteria was performed in 1775 by Lazarro Spallanzani, an Italian biologist. In 1892, Dimitri Ivanovsky, a Russian scientist, and then in 1898, Martinus Beijerinck, a Dutch soil microbiologist, observed that the agent causing mosaic disease in tobacco plants could go through the unglazed porcelain ultrafilter developed by Charles Chamberland; this ultrafilter could retain bacteria and was used to sterilize water and other fluids. Thus, the existence of infectious agents smaller or other than bacteria was suspected; they were named viruses meaning poison in Latin. Friedrich Loeffler and Paul Frosch correctly assumed that these ultrafilterable infectious agents were submicroscopic particles; they proved their hypothesis by studying the cause of foot-and-mouth disease of cattle. Several diseases were then demonstrated to be caused by ultrafilterable agents such as the first adenovirus discovered in 1930 or the first influenza virus discovered in 1931. In 1933, Ernst Ruska and Max Knoll invented the electron microscope and what could only be suspected through experimentations, was revealed with the first electron micrographs of viruses in 1938. Figure 1 gathers a selection of electron microscopy photographs of viruses. Although the electron microscope was a convenient diagnostic tool in virology at that time, it was only suitable for clinical samples with high concentration of viruses, that is at least 10^6 virions per millilitre or milligram, which is mainly solely the case for faeces or vesicle fluids. Therefore, new diagnostic tools had to be developed. [8] [9]

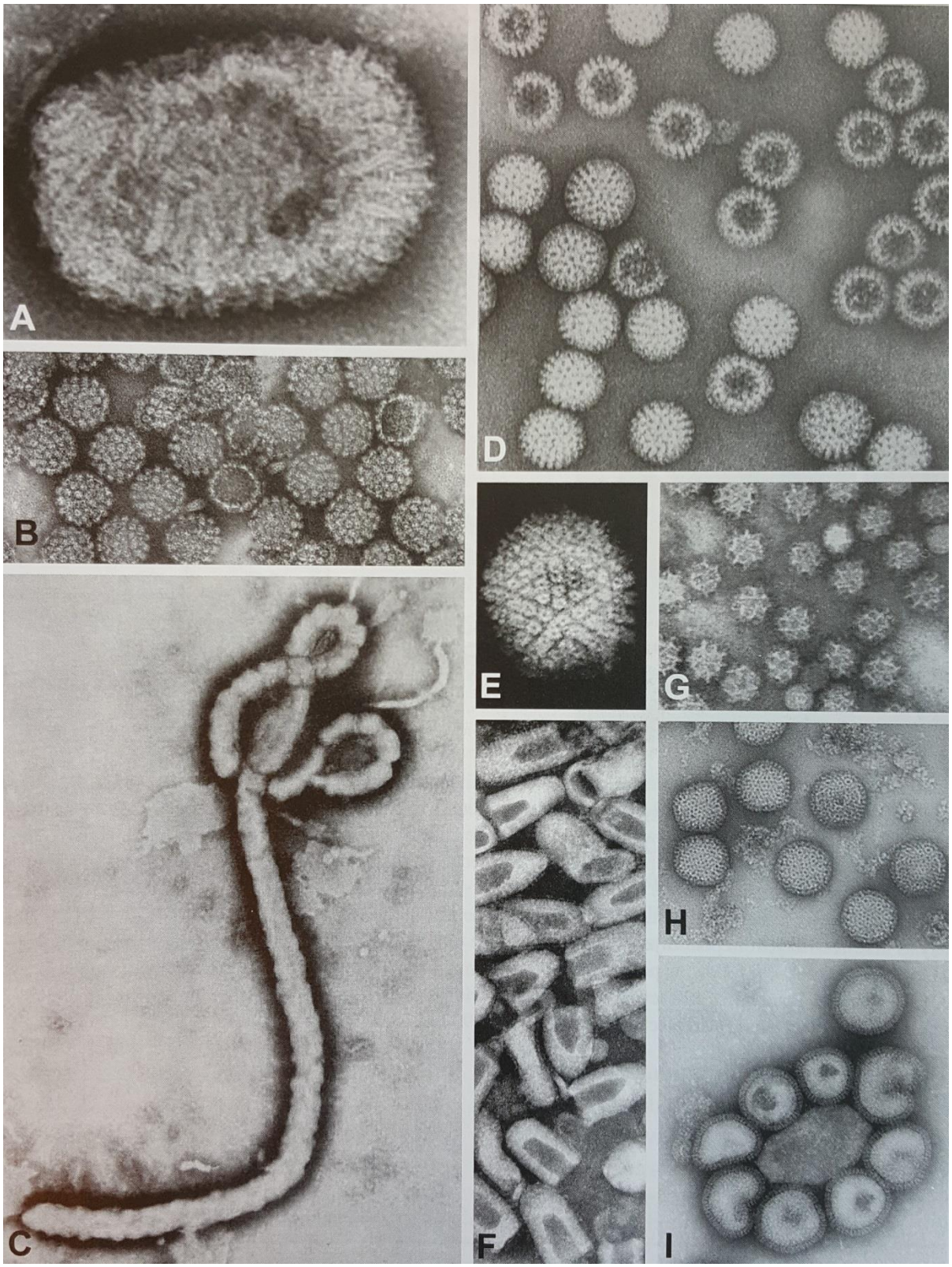

Figure 1: Negative contrast electron microscopy of selected viruses. (A) Family *Poxviridae,* genus *Orthopoxvirus,* vaccinia virus. (B) Family *Papillomaviridae,* genus *Papillomavirus,* human papillomavirus. (C) Family *Filoviridae,* Ebola virus. (D) Family *Reoviridae,* genus *Rotavirus,* human rotavirus. (E) Family *Herpesviridae,* genus *Simplexvirus,* human herpesvirus 1 (capsid only, envelope not shown). (F) Family *Rhabdoviridae,* genus *Lyssavirus,* rabies virus. (G) Family *Caliciviridae,* genus *Norovirus,* human norovirus. (H) Family *Bunyaviridae,* genus *Phlebovirus,* Rift Valley fever virus. (I) Family *Orthomyxoviridae,* genus *Influenzavirus A,* influenza virus A/Hong Kong/1/68 (H3N2). Adapted from *[8]*

3.2 Cell cultures: the first revolution for viral diagnostic

After the demonstration of transmissible viruses only capable of growing in plant cells or in bacteria (bacteriophages), many viruses able to replicate only in living animal tissues were discovered such as yellow fever virus (1900), rabies virus (1903), dengue virus (1907) or poliovirus (1909). Between 1948 and 1955, several major steps transformed animal virology into a laboratory science. At first, viruses had to be cultured in animals such as the mouse, on embryonated eggs or on tissue cultures, but Katherine Sanford and her associates from the National Institute of Health (NIH) overcame the difficulty of culturing single cells, thus simplifying the process of viral culture. Georges Gey and his colleagues at John Hopkins Medical School cultured and passaged human cells from a cervical carcinoma for the first time (HeLa cell line) while Harry Eagle at the NIH developed an optimal medium for the culture of single cells. Finally, as a demonstration of these findings, John Enders and his colleagues showed that poliovirus could replicate in non-neural human explant of embryonic tissues. Cell cultures, besides being the first broadly usable diagnostic tool, thus led to great scientific advances such as the development of vaccines (poliovirus, smallpox, influenza …) and antitumoral drugs. [9] [10]

The principle of tube cultures as a diagnostic tool in clinical virology laboratories is as follows; a monolayer of cells of human or animal origin is constituted on one side in the bottom third of round-bottomed tubes or in the flat area of tubes designed with a culture chamber. A monolayer of cells can also be constituted in multiwell plates. These cells are bathed in a suitable maintenance and growth medium. Subsequently, a portion of the prepared patient sample is inoculated on the monolayer. The culture are afterwards incubated in a humidified CO_2 (5 to 8%) atmosphere at 35 +/- 1°C. Culture should be assessed daily with a microscope for evidence of viral replication during at least 5 to 7 days. Viral replication will produce a cytopathic effect (CPE) resulting in morphologic changes of the monolayered cell cultures. All viruses do not grow or provoke CPE on every cell line; each laboratory will have to choose several cell lines in order to recover viruses of interest. Characterization of the virus present in the sample can be established depending on the type of CPE observed, on the cell line on which it is observed and on the speed of CPE appearance. [11] Table 1 describes the characteristic cytopathic effects of common human viruses. Table 2 presents a list of cell lines and virus sensitivity profiles. Figure 2 displays a selection of cell cultures photographs.

Virus	CPE	Development	Progression	Comment
Adenoviruses	Enlarged, rounded cells in tightly associated, grapelike clusters. Some isolates may produce a lattice-type arrangement of rounded cells	4-7 days	Moderate	CPE less characteristic in diploid fibroblasts
SARS-CoV-2	Refractile cells appearing above the monolayer	3-7 days	Moderate	
Cytomegalovirus	Plump, rounded cells in elongated foci parallel to the long axis of the cell	7-10 days	Slow	May take 2-3 weeks or longer; may develop rapidly if inoculum contained high viral concentration
Enteroviruses	Rounded, highly refractile cells in loose clusters or throughout monolayer	2-5 days	Moderate to rapid	
Herpes simplex	Clusters of rounded, ballooned cells with or without syncytia. Early CPE is focal, then progresses throughout monolayer	1-3 days	Moderate to rapid	May develop more slowly and be less characteristic in human fibrobalsts
Influenza	Variable, from no CPE to granular and vacuolated appearance or nonspecific degeneration	3-5 days	Moderate	
Measles	Syncytia develop by fusion of cells. Nuclei may encircle granular mass of giant cell. Extensive vacuolization may also be present	5-10 days	Slow to moderate	
Metapneumovirus	Variable CPE reported, including focal areas of rounded refractile cells and detachment and syncytia	10-12 days	Slow	
Mumps	Cell rounding and syncytia formation. May appear as non specific granularity with progressive degeneration	3-7 days	Moderate	
Parainfluenza viruses	Variable, increased rounding, granularity, progressive degeneration; syncytia formation associated with types 2 & 3	3-7 days	Moderate	
Respiratory Syncytial Virus	Syncytia develop in some cell lines. May also appear as granular progressive degeneration.	3-5 days	Moderate	
Rhinovirus	Enterovirus-like	5-7 days	Moderate	No or diminished CPE at 37°C as compared to CPE at 33°C suggests a rhinovirus rather than enterovirus isolate
Varicella-zoster virus	Foci of enlarged, rounded, refractile cells with or without syncytia. Cytoplasmic strands and granularity may be prominent as CPE progresses	4-7 days	Slow to moderate	

Table 1: Characteristic cytopathic effect (CPE) in tube cultures. Adapted from *[11]*

Cell line	Origin	Virus(es)
A-549	Human lung carcinoma	Adenovirus, HSV, influenza virus, measle virus, mumps virus, parainfluenza virus, poliovirus, RSV, rotavirus, VZV
AGMK	African green monkey kidney	Influenza virus, parainfluenza virus, enterovirus
AP61	Mosquito	Arboviruses
B95 or B95a	EBV-transformed lymphoblastoid	Measles virus, mumps virus
BGMK	Buffalo green monkey kidney	*Chlamydia spp.* , HSV, coxsackie B virus, poliovirus
C6/36	Mosquito	Arboviruses
Caco-2	Human epithelial colorectal adenocarcinoma	HCoV (NL63)
CV-1	African green monkey kidney	HSV, measles virus, mumps virus, rotavirus, SV40, VZV, some encephalitis viruses
Graham 293	Human embryonic kidney transformed with adenovirus type 5	Enteric adenoviruses
H292	Human mucoepidermoid pulmonary carcinoma	Adenovirus, coxsackie B virus, echovirus, HSV, mumps virus, parainfluenza virus, poliovirus, RSV, rubella virus
HEK	Human embryonic kidney	Adenovirus, BK polyomavirus, enterovirus, HSV, measles virus, mumps virus, rhinovirus
HEK 293	Human embryonic kidney transformed with adenovirus type 5	Enteric adenoviruses
HeLa	Human cervix adenocarcinoma	Adenovirus, coxsackie B virus, CMV, echovirus, HSV, poliovirus, rhinovirus, vesicular stomatitis virus (Indian strain), VZV
HeLa 229	Human cervix adenocarcinoma	Adenovirus, *Chlamydia spp.* , CMV, echovirus, HSV, poliovirus, rhinovirus, vesicular stomatitis virus (Indian strain), VZV
HEL	Human embryonic lung	Adenovirus, CMV, echovirus, HSV, poliovirus, rhinovirus, vesicular stomatitis virus (Indian strain), VZV
Hep-2	Human epidermoid carcinoma	Adenovirus, *Chlamydia spp* , coxsackie B virus, HSV, measles virus, parainfluenza virus, poliovirus, RSV
HNK	Human neonatal kidney	Adenovirus, HSV, VZV
Hs27 (HFF)	Human foreskin fibroblast	Adenovirus, CMV, echovirus, HSV, mumps virus, poliovirus, rhinovirus, VZV
HuH-7	Human hepatocyte	HCoVs (OC43, 229E), Ebolavirus
LLC-MK2	Original rhesus monkey kidney	Arboviruses (some), Chlamydia spp. (serovar L), enteroviruses (including coxsackie virus groups A and B, echovirus, poliovirus), HCoV (NL-63), HMPV, influenza virus, mumps virus, parainfluenza virus, poxvirus groups, rhinovirus
Mv1Lu	Mink lung	CMV, HSV, influenza virus
McCoy	Mouse fibroblast	*Chlamydia spp,* HSV
MDCK	Madin-Darby canine kidney	Adenovirus (some), coxsackie virus, influenza virus, reovirus
MNA	Mouse neuroblastoma	Rabies virus
MRC-5	Human fetal lung	Adenovirus, coxsackie A virus, CMV, echovirus, HSV, influenza virus, mumps virus, poliovirus, rhinovirus, RSV, VZV, cytotoxicity for *C. difficile*
NCI-H292	Human mucoepidermoid pulmonary carcinoma	Adenovirus, BK polyomavirus, enteroviruses (most), HSV, measles virus, reoviruses, rhinoviruses (most), RSV, vaccinia virus
RD	Human rhabdomyosarcoma	Adenovirus, coxsackie A virus, echovirus, HSV, poliovirus
RK	Rabbit kidney	HSV, paramyxoviruses
RhMK	Rhesus monkey kidney	Arbovirus, coxsackie A and B viruses, echovirus, influenza virus, parainfluenza virus, measles virus, mumps virus, polioviruses
SF	Human foreskin	Adenovirus, coxsackie A virus, CMV, echovirus, HSV, poliovirus, VZV
Vero	African green monkey kidney	Adenovirus (some), arboviruses (some), *Chlamydia spp.* , coxsackie B virus, HSV, HMPV, measles virus, mumps virus, poliovirus type 3, rotavirus, rubella virus
Vero E6	African green monkey kidney	Adenovirus, Ebolavirus, coxsackie B virus, HSV, measles virus, mumps virus, poliovirus type 3, rotavirus, rubella virus, SARS-CoVs
Vero 76	African green monkey kidney	Adenovirus, coxsackie B virus, HSV, measles virus, mumps virus, poliovirus type 3, rotavirus, rubella virus, West Nile virus
WI-38	Human lung	Adenovirus, coxsackie A virus, CMV, echovirus, HSV, influenza virus, mumps virus, poliovirus, rhinovirus, RSV, VZV

Table 2: List of cell lines and virus susceptibility profiles. CMV: cytomegalovirus; HCoV: human coronavirus; HMPV: human metapneumovirus; HSV: herpes simplex virus, RSV: respiratory syncytial virus; SARS-CoV: severe acute respiratory syndrome coronavirus; SV40: simian virus 40; VZV: varicella zoster virus. Adapted from [12]

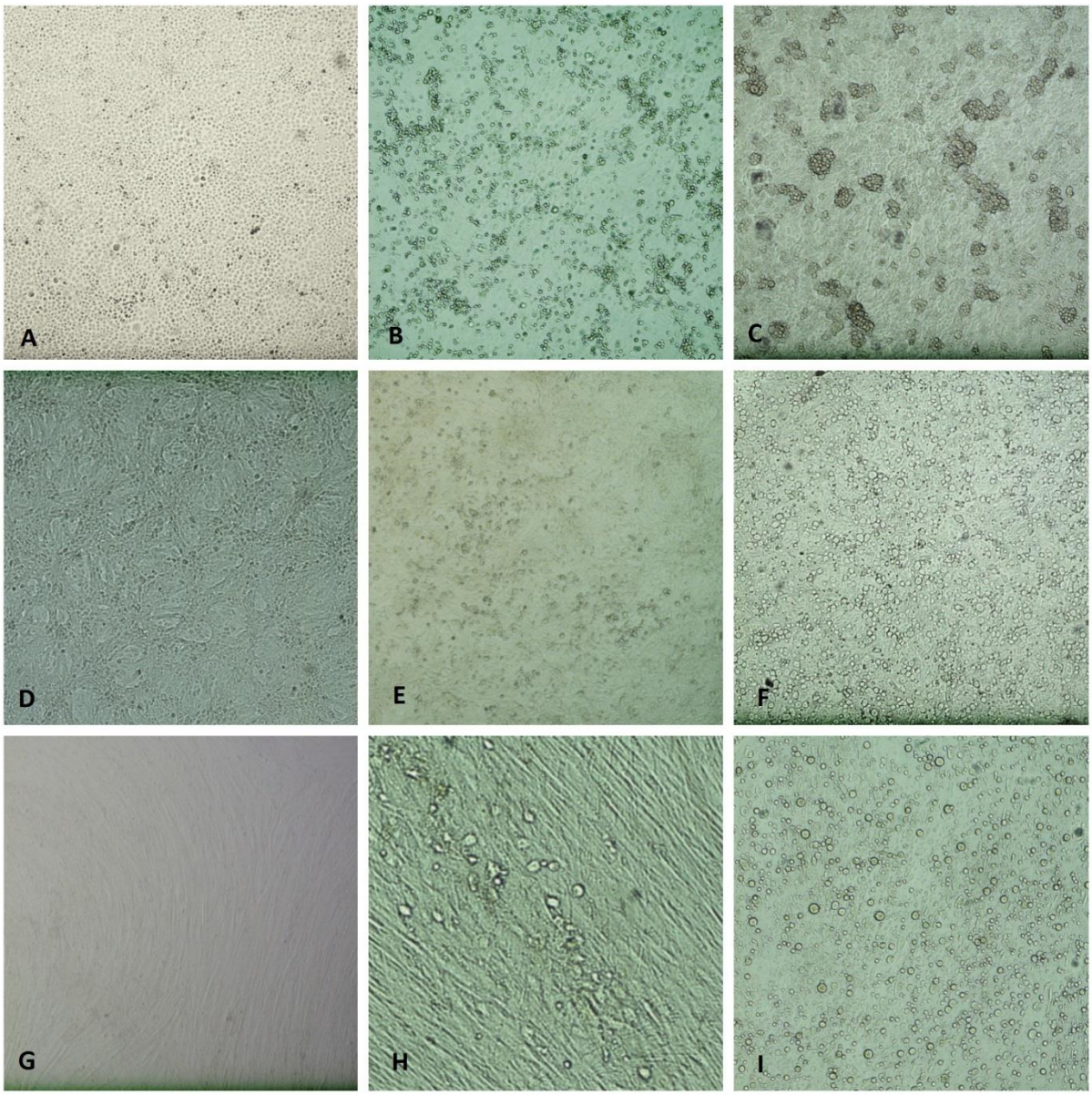

Figure 2: Cell culture photographs (various magnifications); (A) non-inoculated LLC-MK2 cell line, (B) influenza A CPE on LLC-MK2 , (C) Respiratory Syncytial Virus CPE on LLC-MK2, (D) non-inoculated Vero cell line, (E) adenovirus CPE on Vero cell line, (F) *Chlamydia trachomatis* serovar L CPE on Vero cell line (see article further), (G) non-inoculated MRC5 cell line, (H) varicella-zoster virus CPE on MRC5 cell line, (I) herpes simplex virus CPE on MRC5 cell line. Courtesy of Marc De Foor.

Nowadays, cell cultures for viral diagnostic tend to be replaced by newer techniques. Main disadvantages of cell cultures are that they are slow, time-consuming, labour-intensive and lack the sensitivity needed to have an appreciable impact on clinical decision making. [13]

The shell-vial technique, performed on cell cultures, improves turnaround time by detecting the presence of viruses in the cells with the use of monoclonal antibodies rather than waiting for the CPE to appear. Results are however not delivered before 1 to 4 days. [11] Moreover, some viruses are not culturable or grow poorly such as group C rhinoviruses, metapneumovirus, coronaviruses or some parainfluenza viruses.

Lastly, viral culture systems have not been standardized to the same extent as other techniques, notably molecular tests, and results could vary between laboratories depending upon the

selection of appropriate cell lines and media as well as the adequate collection, transport and handling of specimens to ensure virus viability. Nevertheless, in a point/counterpoint article, Kaiser advocates toward an interest to maintain viral cultures in diagnostic virology for the detection of new viruses or variants of well-recognized viruses that may be missed by molecular methods. [13] Leland and Ginocchio suggest their use to monitor the sensitivity and specificity of other laboratory techniques while encouraging their use to test samples with negative rapid antigen tests results during high-prevalence seasons for patients with clinical signs and symptoms of infection, and to confirm positive rapid antigen results obtained during periods of low viral prevalence. [14]

Cell cultures are still in use in our laboratory mainly for the diagnosis of respiratory viruses and for the recovery of some *Herpesviridae* (herpes simplex viruses [HSV], varicella-zoster virus [VZV] and cytomegalovirus [CMV]). Enteric viruses, mainly rotavirus, group F adenoviruses and norovirus, do not grow in cell cultures or require specific cell lines.

In our experience, reasons why cell cultures are still in use are beyond mere performance issues. First of all, the actual organization of our laboratory could not sustain the workload of analyses performed in cell cultures with molecular tests; in 2018, we performed more than 18,000 cell cultures for 5 university hospitals. Adapting our practice would require an important financial investment. Moreover, still about this financial aspect, reimbursement of molecular tests in Belgium depends on rules enacted by the social welfare system (INAMI: Institut National d'Assurance Maladie-Invalidité). Molecular tests for respiratory viruses are recently reimbursed by the social welfare system (since April 2019) but only in broncho-alveolar lavages for solid organ transplant patients. For *Herpesviridae*, molecular tests are reimbursed only for ophthalmic or neurologic affections (HSV and VZV), for gastro-intestinal or respiratory infections in immunosuppressed patients (HSV) and for neonatal HSV infections. Congenital CMV infection can be diagnosed with molecular techniques, free of charge, at the Belgium National Reference Centre for Congenital Infections. For the other conditions, molecular tests for *Herpesviridae* cannot be reimbursed and are charged to the patients. However, the cost of these tests cannot be supported by all patients.

Moreover, concerning respiratory viruses, the cost-effectiveness of molecular tests is not obvious and seems to depend on the population in which it is used. [15] Cell cultures are reimbursed by the social welfare system and are thus an interesting alternative to molecular tests, especially for mildly diseased or all-comers patients. It allows us to propose a diagnostic tool for these patients for whom nothing else would have been performed because their condition did not require expensive molecular testing. Indeed, results of cell cultures are available often too late in the course of patient management, especially for influenza viruses as, if a treatment is needed, it has to be administered in less than 48 hours of symptoms. [16] [17] [18] The characterization of infections is however an important task for a laboratory analysing samples from university hospitals, otherwise, most infectious episodes would be categorized as "possible viral disease" in the patients' files. In 2018, 18156 cell cultures for viral recovery were performed in our laboratory among which 22% yielded a positive result. In addition, for respiratory viruses, these results are sent anonymously to the Belgian Institute of Public Health (Sciensano) to participate in the national surveillance system.

Regarding *Herpesviridae*, having a positive cell culture is very useful when the determination of susceptibility toward antiviral drugs is required. Indeed, the positive culture allows for determination of actual sensitivity toward antiviral drugs in vitro (phenotyping). Without a positive cell culture, only genotyping of the virus can be performed in order to search for known genetic mutations conferring resistance toward antiviral drugs. In case an unknown mutation is displayed, its association with resistance cannot be established without the realization of a phenotyping.

Another reason to maintain cell cultures for viral diagnosis is that it occasionally allows the recovery of an unsuspected agent. Actual multiplex molecular techniques usually target a broad panel of micro-organisms causing diseases with similar symptoms; this approach is called the syndromic diagnosis. However, in some occasions, cell cultures recover a micro-organism which is not included in the panel of the molecular test. In our experience, it is not uncommon to recover CMV or HSV in a respiratory sample of a toddler admitted for fever. Less often, a measles virus is isolated in a respiratory sample of patients for whom the diagnostic was not suspected either because the disease has become less frequent thanks to vaccination or because the clinical symptoms were not specific. CMV, HSV and measles virus are generally not included in commercial molecular tests targeting respiratory viruses. To highlight this point, an article on the fortuitous recovery of *Chlamydia trachomatis* serovar L in patients suspected of having HSV infection is provided. [19]

SHORT REPORT

Observation of a cytopathogenic effect on cell lines used for routine viral cultures led to the diagnosis of lymphogranuloma venereum

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ABSTRACT

Objectives This article reports the fortuitous recovery of nine *Chlamydia trachomatis* serovar L strains in cell cultures (Vero and LLC-MK_2 cell line) designed for viral culture.

Methods Nine ano-genital swabs were inoculated on confluent Vero, MRC5 and LLC-MK_2 cell cultures. They were collected from HIV-positive patients who were primarily men who have sex with men (MSM) presenting ulcerations that mimicked herpes simplex infections.

Results A cytopathogenic effect was observed on Vero and LLC-MK_2 cells on day 14. The presence of *C trachomatis* serovar L in the cell lines was confirmed by Real Time–PCR.

Conclusions *C trachomatis* serovar L can grow on Vero and LLC-MK_2 cell lines designed for viral cultures. Lymphogranuloma venereum must be considered as a differential diagnosis for herpes-like lesions, particularly in MSM with high-risk behaviours.

INTRODUCTION

Chlamydia trachomatis was identified in the mid-sixties; it causes several sexually transmitted diseases and trachoma. The D to K serovars of *C trachomatis* primarily cause urethritis, cervicitis, salpingitis and mild proctitis. Lymphogranuloma venereum (LGV) is caused by the L serovar. LGV is an inguinal syndrome, but in Europe, it typically presents as an anorectal syndrome among men who have sex with men (MSM). Unlike the other urogenital *Chlamydia* serovars, the L serovar is more invasive and affects the submucosal connective tissue layers.[1]

This bacterium was formerly thought to be a virus due to its intracellular replication and ability to grow on cell lines.[2] Currently, the McCoy (human synovial fluid), HeLa 229 (human cervical epithelial adenocarcinoma) and BGMK (Buffalo Green Monkey Kidney) cell lines are used for its isolation and identification from clinical specimens.[1 3]

Herein, we report the observation of an unexpected cytopathogenic effect (CPE) caused by *C trachomatis* serovar L on cell lines used for routine viral culture, which led to the identification of nine cases of LGV.

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MATERIALS AND METHODS

From April 2010 through September 2011, eight anorectal swabs and one penile swab using FLOQSwabs (Copan, Brescia, Italy) led to an unexpected and atypical CPE on cell lines used for viral cultures. Those swabs were stored in virus transport medium consisting of veal infusion broth and bovine albumin, supplemented with antibiotics (vancomycin, gentamycin and amphotericin B). As a part of routine viral culture procedure, portions of the eluted specimens were inoculated on confluent Vero (African Green Monkey Kidney), MRC5 (Human lung cells) and LLC-MK_2 (Rhesus Monkey Kidney) cell cultures (Vircell, Santa-Fé, Spain) in 24-well or 6-well tissue culture plates (Greiner-Bio One, Frickenhausen, Germany) and incubated at 36°C in a 5% CO_2 atmosphere for 2 weeks for Vero and LLC-MK_2 cells and 3 weeks for MRC5 cells. The media were replaced every week. The cultures were examined every 2–3 days using an inverted microscope. CPE was observed on the Vero and LLC-MK_2 cell lines on day 14, which is the final day of observation before discarding negative cell cultures. The CPE consisted of cell swelling and syncytia formation. No CPE was observed on MRC5 cells during the 3 weeks of observation.

Aliquots of the infected cell lines were sent to the Institute of Tropical Medicine, Antwerp (Belgium), which confirmed the *C trachomatis* L serovar in all nine samples using Real Time–PCR based on the publication by Chen *et al*.[4]

CASE DESCRIPTIONS

The clinical data, symptoms and laboratory findings of the nine patients are summarised in table 1.

It is noteworthy that patient D presented a penile lesion of approximately 1 cm diameter, erythematosus, lightly ulcerated and with recurrences over at least 5 years. The lesion was diagnosed as a herpes simplex infection and treated without success with acyclovir. The swab of this ulceration was positive for *C trachomatis* in cell culture and using the molecular technique.

DISCUSSION

LGV used to occur primarily in Asia, Africa, South America and the Middle East. Prior to 2003, it was unusual in Western Europe, with most cases being imported. According to the Belgian Institute of Public Health, a total of 43 cases of LGV were reported in Belgium between 2004 and 2008 and were exclusively due to *C trachomatis* serovar L2. Of those 43 cases, 99.7% were MSM, including 1 who was bisexual, 95.3% were HIV positive, 14% had a co-infection with syphilis and 11.6% were

Table 1 The clinical data and laboratory findings for the nine patients

Patient	Age	Gender	Sexual orientation	Localisation of the lesion	Symptoms	HIV serology	HIV viral load (copies/ml)	Chlamydia serology	Co-infection
A	21	M	MSM	Anorectal	Haematochezia Proctitis	Positive	77 300	Strongly positive for IgA and IgG	*Neisseria gonorrhoea*
B	48	M	Unknown	Anorectal	Haematochezia Proctitis Diarrhoea	Positive	Unknown	Not performed	CMV colitis
C	38	M	Unknown	Anorectal	Proctitis	Positive	Unknown	Not performed	
D	36	M	MSM	Penis	Recurrent penile ulceration	Positive	<20	Not performed	Syphilis
E	50	M	MSM	Anorectal	Haematochezia Proctitis	Positive	<20	Not performed	Past syphilis
F	52	M	MSM	Anorectal	Haematochezia Proctitis	Positive	<20	Strongly positive for IgA and IgG	HPV (rectum) Cured/healed hepatitis C
G	35	M	MSM	Anorectal	Haematochezia Proctitis	Positive	14 200	Strongly positive for IgA and IgG	Past syphilis
H	50	M	MSM	Anorectal	Proctitis	Positive	<20	Strongly positive for IgA and IgG	Past syphilis Cured/healed hepatitis C Anal HSV2
I	49	M	MSM	Anorectal	Proctitis Anal stricture Anal discharge	Positive	<20	Strongly positive for IgA and IgG	

CMV, cytomegalovirus; HPV, human papilloma virus; HSV2, herpes simplex virus 2; M, male; MSM, men who have sex with men.

co-infected with gonorrhoea. The mean age was 38 years (range 20–58 years).[5]

The emergence of LGV in Europe appears to be restricted to some networks of MSM with high-risk behaviours. The 'new' L2b serovar was found in a cluster of MSM in Rotterdam, The Netherlands, in 2003 and led to the awareness of the problem in Europe. It was retrospectively retrieved in samples collected between 1979 and 1985 from patients living in San Francisco who had been diagnosed with LGV.[6] The hypothesis of a latent and endemic disease or a slowly evolving epidemic rather than a new outbreak was evoked, particularly because no systematic surveillance for LGV was available in Europe before 2003.[6–8]

Consistent with those data, seven of the nine patients diagnosed with LGV in our hospital were MSM; the sexual orientation of the other two was not documented. All patients had a positive HIV serology and some were co-infected with syphilis (1/9), cured or healed hepatitis C (positive serology with undetectable viral load) (2/9), gonococcal urethritis (1/9), anal human papillomavirus (1/9) and anal herpes simplex virus 2 (1/9).

The fortuitous detection of *C trachomatis* serovar L in cases in which the diagnosis of LGV was not suspected highlights the lack of awareness of this disease. Moreover, some presentations may mimic other pathologies, particularly in penile ulcerations or inguinal nodes. A previous case report mentions the detection of *C trachomatis* serovar L in the cell culture of an inguinal lymph node from a patient with a suspicion of cat scratch disease.[9]

CONCLUSION

LGV has been an emerging disease in Europe since 2003, particularly in some networks of MSM with high-risk behaviours. The methods of diagnosing and declaring LGV could be improved, as highlighted in the 2010 surveillance report of the European Centre for Disease Prevention and Control.[10] Virology laboratories using cell cultures should be aware of the possible growth of *C trachomatis* serovar L in cell cultures used for viral detection and should therefore be more vigilant when confronted with an unusual CPE for an ano-genital sample. This observation also demonstrates that cell cultures are still useful in university hospitals in addition to molecular techniques. Indeed, with molecular techniques, you only find what you seek, whereas with cell cultures you can sometimes find what you did not expect.

Key messages

- *Chlamydia trachomatis* serovar L can be grown in the Vero and LLC-MK_2 cell lines, which are typically used for viral cultures.
- Lymphogranuloma venereum can have atypical presentations (recurrent penile ulceration).
- The diagnosis and declaration systems for lymphogranuloma venereum could be improved.

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Contributors LB wrote the draft of the article and coordinated subsequent revisions. TC confirmed the *Chlamydia trachomatis* serovar L in her laboratory, critically reviewed the manuscript and corrected some parts of the text. MDF was involved as well as his team in the technical aspects of this work and in the data collection. He also critically reviewed the article and wrote the material and methods section. SVDW is a participating investigator of this study. OV critically reviewed this work and helped for the layout of the article.

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3.3 Antigen detection tests: a compromise between speed and sensitivity

Antigen detection tests are based on antibody-antigen reactions. They are available to detect viruses having a limited number of serotypes. The first techniques to be used for viral diagnosis were immunofluorescence and immunoperoxydase staining. Immunofluorescence was first applied to the diagnosis of influenza by C. Liu in the mid-fifties and was then extensively developed and applied to most respiratory viruses by P.S. Gardner and Joyce Mc-Quillin in the late sixties. It can also be used to detect and distinguish HSV from VZV in vesicle fluids as well as to spot CMV in various types of samples. The sensitivity of this technique is usually lower than that of cell cultures and depends on the quality of the specimen, on the skill of the operator for the realization and the reading of the slides on the microscope as well as on the specificity of the antibodies used. [9]

Immunofluorescence technique in virology implies the direct detection of viruses, using specific antibodies, in patients' cells harvested during the sampling. These antibodies can be labelled with a fluorochrome (direct immunofluorescence) or, if they are unlabelled, they must in a second step be detected by secondary labelled antibodies (indirect fluorescence). The reading of the test will be made on a light microscope using a powerful ultraviolet/blue light source. (Figure 3)

For immunoperoxydase technique, the principle is the same except that antibodies are labelled with horseradish peroxidase; the addition of hydrogen peroxide and benzidine derivative will form a coloured insoluble precipitate which can be seen with the use of an ordinary light microscope. [8] [20]

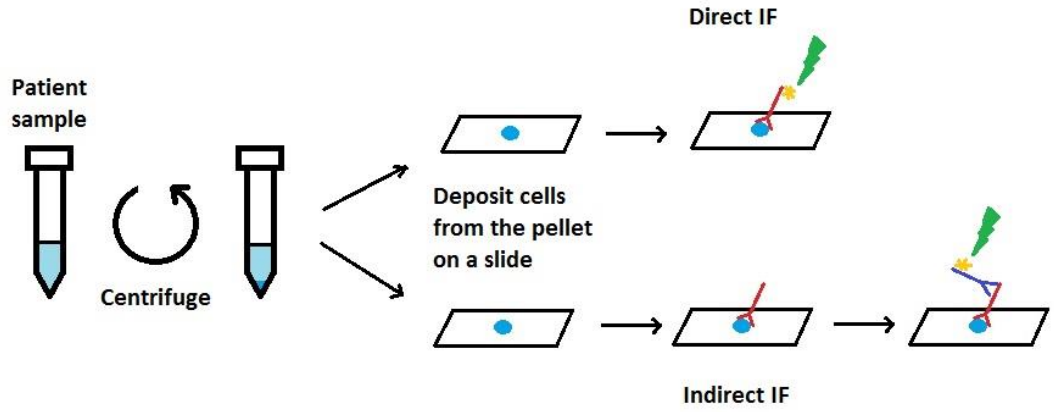

Figure 3: Principle of direct and indirect immunofluorescence (IF) for diagnostic in virology

Immunofluorescence can also be used to detect the growth of viruses in cell cultures before the apparition of the CPE (shell vial technique). [11] [20] Finally, both immunofluorescence and immunoperoxydase can be used for the examination of tissue specimens in order to detect viral inclusions. [9]

Membrane immunoassay is another broadly used antigen detection technique. Its principle is a variant of the enzyme immunoassay (EIA) which is more commonly used for serologic diagnostic tests. One type of membrane immunoassay is the lateral flow immunochromatographic assay. In these tests, a sample is applied, directly or after dilution in a buffer solution, to a membrane and is drawn across the membrane by capillary action. The reagents of the test contain antibodies specific from an epitope of the virus to be detected. These antibodies are either conjugated to a detector label such as

gold particles or fluorescein. On the membrane, two reactive zones will be crossed by the flowing sample. The first one is labelled with antibodies specific of another epitope of the virus to be detected. In case the virus is present in the sample, it will be trapped between antibodies conjugated with the detector label and antibodies on the membrane. The second zone is labelled with antibodies specific from the antibodies conjugated with the label. The unbound conjugated antibodies will be captured on this zone. The detection of a coloration or a fluorescence in the first zone indicates the presence of the expected virus in the sample whereas the coloration or fluorescence in the second zone attests that the sample has migrated through the whole length of the membrane. In case no coloration or fluorescence is detected in the second zone, the test in not valid. (Figure 4)

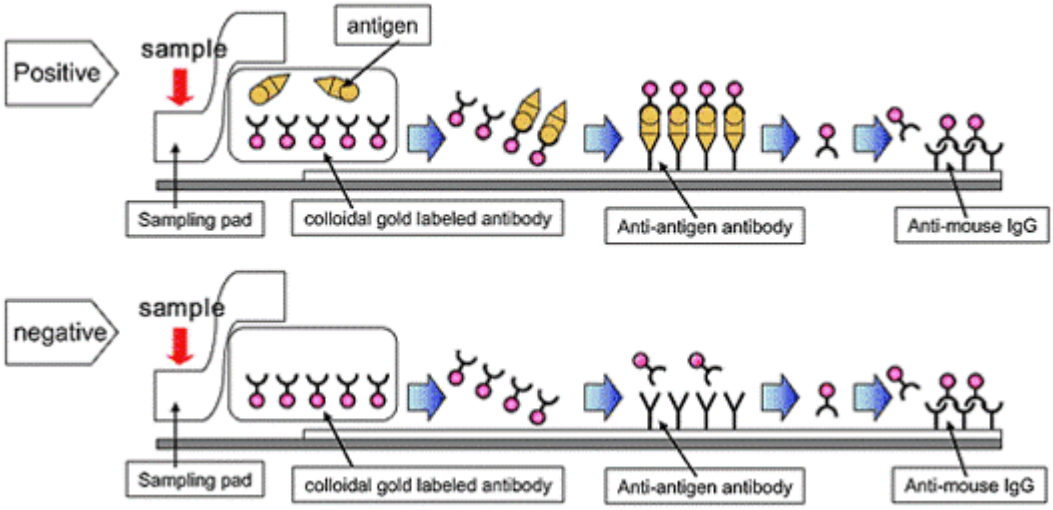

Figure 4: Principle of immunochromatographic tests. Adapted from *[21]*

The advantages of lateral flow immunochromatographic tests are that they give rapid results in 5 to 20 minutes and they do not require a lot of expertise to perform and to interpret. Their sensitivities are however lower than cell cultures or molecular techniques. [8] [9] In our laboratory, immunofluorescence is used for the diagnosis of human metapneumovirus and parainfluenza viruses. Lateral flow chromatography tests are employed for the diagnosis of influenza viruses, RSV, respiratory and enteric adenoviruses, rotavirus and norovirus.

The following article compares the performances of 3 rapid lateral flow immunochromatographic influenza diagnostic tests in comparison to cell cultures and molecular tests. It highlights that the sensitivity of the tests is influenced by the viral load in the samples and by the virus type. [22]

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Evaluation of 3 rapid influenza diagnostic tests during the 2012–2013 epidemic: influences of subtype and viral load

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ABSTRACT

This article evaluates the performance of 3 rapid influenza diagnostic tests (RIDTs), in correlation with the influenza subtypes and the viral load. A total of 236 samples were prospectively analyzed with BinaxNOW Influenza A/B, Directigen EZ Flu A and B, and bioNexia Influenza A+B. The results were compared to cell cultures and real-time polymerase chain reaction. Positive samples were further subtyped. Thirty-seven samples were positive for influenza A, and 57, for influenza B. For A(H1N1), the sensitivities were 71.42% for BinaxNOW, 78.57% for Directigen, and 67.85% for bioNexia. Eight samples were positive for A(H3N2), and only the bioNexia test had 1 false-negative result. Lowest sensitivities were observed for influenza B/Yamagata, (56.86% for BinaxNOW and Directigen and 39.21% for bioNexia). The 3 evaluated RIDTs were more efficient at detecting influenza A (H3N2) than for A(H1N1) and B/Yamagata. Highest viral loads in the samples were associated with better rate of detection.

1. Introduction

Rapid diagnosis of influenza infections is critical during epidemic season to allow treatment initiation and patient isolation. The diagnosis of influenza can be made using several methods. Cell culture is relatively sensitive for recovering influenza viruses, but this method needs viable viruses in the sample and is rather slow; the delay in obtaining results often varies between 3 and 14 days. Molecular methods are the most sensitive techniques available and can detect non-viable viruses by targeting a conservative area of the matrix gene (WHO Global Influenza Network, 2011); however, these techniques have high financial costs, are not available in all facilities, and require a well-optimized workflow during epidemics to be able to obtain results in a clinically relevant timeframe. Rapid influenza diagnostic tests (RIDTs) offer a fair alternative; these tests based on the principle of immunochromatography are easy and rapid to perform, as they generally yield a result in 10–15 minutes. They can detect non-viable viruses as they are targeted at their nucleoprotein. Additionally, these tests are very specific. However, they lack the sensitivity of the cell culture and molecular methods (Kumar and Henrickson, 2012). Moreover, an evaluation of some Food and Drug Administration–approved RIDTs published in November 2012 stated that some subtypes of influenza viruses could be less detectable than others depending on the RIDT used (Beck et al., 2012). In the present study, the performances of 3 RIDTs were evaluated in comparison to the cell culture method and influenza A/B real-time polymerase chain reaction (PCR) analysis during the 2012–2013 influenza epidemic. All influenza A– and influenza B–positive samples were retrospectively subtyped to determine if some subtypes were less detectable than others.

2. Materials and method

2.1. Samples and population

The threshold of flu epidemic 2012–2013 in Belgium was crossed in week 52 of 2012, and the epidemic was declared in week 1 of 2013 and lasted 12 weeks (Thomas et al., 2013). From January 18th to February 18th 2013 (week 3 to week 8 of 2013), a total of 236 clinical samples were prospectively collected. The samples were taken from 118 female and 111 male patients between the ages of 8 days and 86 years old (mean age: 13.25 years; median: 1.35 years). All samples for which the practitioners prescribed a test for influenza were included whether the patients' symptoms met the case definition of influenza like illness or not (ECDC, 2005–2014). The interval between the time of collection of the sample and the onset of the first symptoms was not standardized, and patients coming later to the hospital could still have a sample taken if the practitioner estimated it could be useful. The samples were taken during 2 separate periods, and the gap between the 2 periods corresponded to a shortage of RIDTs due to insufficient production by the suppliers. The samples included 154 nasopharyngeal aspirates, 71 nasopharyngeal swabs, 5 throat swabs, 3 bronchoalveolar washes, 1 tracheal aspirate, 1 sputum, and 1 nasal swab. Swabs were collected with

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FLOQSwab (Copan, Brescia, Italy) and transported in 1.5 mL of veal infusion broth (Difco, Becton Dickinson, Sparks, MD, USA) supplemented with bovine albumin (Sigma Aldrich, St. Louis, MO, USA).

2.2. RIDTs

The 3 RIDTs evaluated were BinaxNOW Influenza A/B (Alere Inc., Waltham, MA, USA), Directigen EZ Flu A and B (Becton Dickinson, Franklin Lakes, NJ, USA), and bioNexia Influenza A+B (bioMérieux, Marcy l'Etoile, France). These tests are only validated for nasopharyngeal swabs and aspirates, which constitute 95.7% of the samples in this study, but are routinely performed on other types of samples with a warning concerning the reliability of the result. The samples were analyzed only once with each test according to the recommendations of the manufacturers. Viral transport medium with the nasopharyngeal swabs as well as the nasopharyngeal aspirates were diluted up to 3 mL with fresh viral transport medium to allow sufficient sample volume to perform all of the routine analyses.

2.3. Cell cultures

Portions of the diluted specimens were used to inoculate confluent Vero (African green monkey kidney), MRC5 (human lung), and LLC-MK_2 (rhesus monkey kidney) cell cultures (Vircell, Santa-Fé, Spain) in 24-well or 6-well tissue culture plates (Greiner-Bio One, Frickenhausen, Germany); these cultures were incubated at 36 °C in a 5% CO_2 atmosphere for 2 weeks for the Vero and LLC-MK_2 cells and 3 weeks for the MRC5 cells. The media was replaced weekly. The cultures were examined every 2–3 days using an inverted microscope. The combination of these 3 cell lines allows the recovery of most of the significant human respiratory viruses (Ginocchio and Harris, 2011). LLC-MK_2 cell line is utilized in our laboratory mainly for the recovery of influenza virus, whereas the use of MDCK cell line (canine kidney) is more common. In our experience, both cell lines perform as well for the recovery of influenza virus but LLC-MK_2 is better for parainfluenza virus, which motivated our choice (Frank et al., 1979). Hemadsorption was performed on the LLC-MK_2 cells at the end of the second week of incubation.

2.4. Molecular testing

An Influenza A/B real-time PCR was performed on the frozen aliquot of every culture-negative sample and on the samples that tested positive for a non-influenza virus (Fig. 1). First, 400 μL of the frozen aliquot was purified according to the QIAsymphony automated extraction protocol using the QIAsymphony DSP Virus/Pathogen Midi extraction kit (Qiagen, Germantown, MD, USA). Then, analyses was performed on a Lightcycler 480 using the Taqman EZ RT-PCR kit (Applied Biosystems, Paisley, United Kingdom); the primer and probe sequences were designed by Ward et al. (2004).

Finally, for all positive samples (culture-positive and culture-negative/PCR-positive samples), the frozen extracts were sent to the National Reference Centre for Influenza to confirm the typing and for subtyping analysis. The typing of influenza A/B was performed with an in-house duplex quantitative real-time PCR (qRT-PCR) using an Mx3005p qPCR System (Agilent Technologies, Stockport, United Kingdom) and the SuperScript III Platinum One-Step qRT-PCR kit (Invitrogen, Carlsbad, NM, USA). The primers and probes were these used for universal detection of influenza A in the CDC protocol for influenza A(H1N1)pdm09 (CDC, 2009), and the primers and probes used to detect influenza B were based on the works of van Elden et al. (2001). The subtyping of influenza A was performed using qRT-PCR and the SuperScript III RT/Platinum Taq Mix, with primers and probes for H1 (CDC, 2009) and H3 (Overduin et al., 2012). To determine the lineage of the

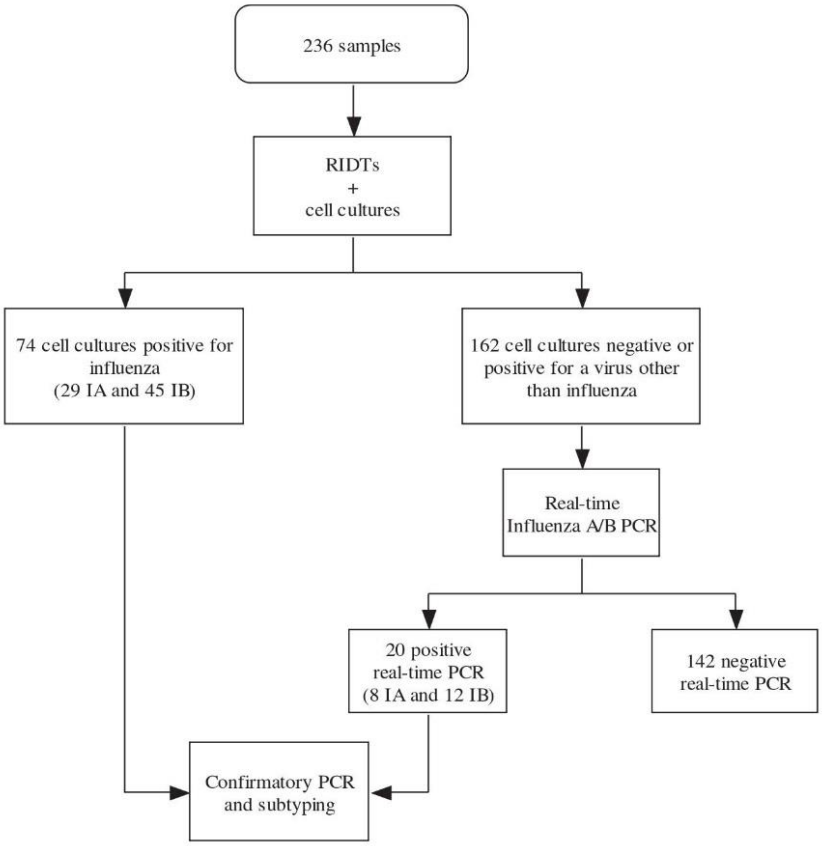

Fig. 1. Study work scheme. IA = influenza A; IB = influenza B.

influenza B viruses, a qRT-PCR protocol was adapted from the works of Hungnes (2011).

2.5. Work scheme

Upon receipt of the samples, the 3 RIDTs were simultaneously performed according to the manufacturers' instructions. A portion of each sample was used to inoculate the cell cultures. The rest of the sample was then stored in a freezer at −80 °C (−112 °F). Influenza A/B RT-PCR was performed using the frozen samples whenever the cell cultures yielded a negative result or were positive for a non-influenza virus (Fig. 1).

2.6. Statistical methods

In case of discrete variables, comparisons between proportions were performed with exact chi-square tests. In case of continuous variables, comparisons of more than 2 means were performed with analysis of variance, followed in case of statistical significance by Sidak tests for multiple comparisons. In case of too small sample sizes, non-parametric tests (Mann–Whitney tests) were used. The statistical tests were performed using the IBM-SPSS V20.0 software. The calculation of the 95% confidence intervals for sensitivities and specificities was performed with MedCalc Statistical Software version 13.3 (MedCalc Software bvba, Ostend, Belgium).

3. Results

Of the 236 samples, 29 were positive for influenza A, and 45 were positive for influenza B according to the cell culture method. A total of 162 Influenza A/B RT-PCRs were performed, and 8 samples were positive for influenza A, while 12 were positive for influenza B. The total number of positive samples was 37 for influenza A and 57 for influenza B. These results were confirmed by the National Reference Centre for Influenza. No influenza A and B co-infections were diagnosed. All positive samples were on sample types recommended by the manufacturers (nasopharyngeal swabs or aspirates) at the exception of 1 throat swab positive for influenza B in cell culture and PCR but negative with all RIDTs. One hundred forty-two samples were negative by both the cell culture and PCR methods.

3.1. Overall sensitivity and specificity of the RIDTs

The sensitivities of the RIDTs for influenza A were 75.68% (28/37), 81.08% (30/37), and 70.27% (26/37) for the BinaxNOW Influenza A/B, Directigen EZ Flu A, and B and bioNexia Influenza A+B kits, respectively. The specificities were 100%, 100%, and 96.48% (192/199), respectively (Table 1).

Concerning influenza B, the sensitivities were 52.63% (30/57), 52.63% (30/57), and 36.84% (21/57) for the BinaxNOW Influenza A/B, Directigen EZ Flu A and B, and bioNexia Influenza A+B kits, respectively. The specificities were 100%, 100%, and 100% (Table 1).

Of the 236 samples, 34 were positive for viruses other than influenza using the cell culture method, including 16 adenoviruses (2 co-infections with influenza A), 9 rhinoviruses, 4 parainfluenza viruses, 3 enteroviruses, and 3 cytomegaloviruses (1 co-infection with influenza A). No false positives were observed with RIDTs in these samples, indicating no obvious cross reaction with those viruses.

3.2. Influence of subtype and viral load

Table 2 summarizes the number of positive tests and cell cultures, the patients' mean ages, and the repartition of the sample types for each subtype. Five influenza B strains and 1 influenza A strain could not be subtyped due to low viral load. Because there was only 1 sample that was positive for B/Victoria lineage, it was not included in the statistical analysis.

There was no significant difference in the repartition of the sample types between the A(H1N1)pdm09, A(H3N2), and B/Yamagata lineage groups ($P = 0.890$; exact chi-square test). There was no age difference between the group with A(H1N1)pdm09 and the group with B/Yamagata lineage ($P = 0.683$; Sidak test for multiple comparisons). By contrast, the patients from the group with A(H3N2) were statistically older than those in the groups with A(H1N1)pdm09 ($P = 0.004$) or B/Yamagata lineage ($P = 0.012$).

The cycle thresholds (Cts) of the confirmatory PCRs for the positive samples were analyzed, as the Ct indirectly and semi-quantitatively reflects a sample's viral load. As expected, the Cts obtained for the culture-positive samples were significantly lower than those observed for the culture-negative PCR-positive samples ($P < 0.001$). Similarly, the Cts obtained for the RIDT-positive (at least 1 out of 3) samples were also lower than those obtained for the RIDT-negative samples, for both the A(H1N1)pdm09 (Fig. 2) and the B/Yamagata lineage (Fig. 3) subtypes ($P < 0.001$).

4. Discussion

The sensitivities of the RIDTs have been reported to range from 20% to 90%. (Chartrand et al., 2012; Kumar and Henrickson, 2012). They can vary from one epidemic season to another, depending on the circulating influenza strains (Weinberg et al., 2005), on the studied population (children or adults) (Aoki and Boivin, 2009; Welch and Ginnochio, 2010) and on the reference method to which they are compared (cell culture or molecular methods) (Hurt et al., 2007). In our hospital, an algorithm for the management of patients with influenza-like illness includes the realization of an RIDT then a molecular technique for hospitalized patients. If the RIDT is positive, the molecular method is not performed resulting in a saving of money for the hospital. A good sensitivity of the RIDT used is thus important.

In this work, the sensitivities for influenza A were quite high when compared to the real-time PCR results; the sensitivities ranged from 70.27% for bioNexia Influenza A+B to 81.08% for Directigen EZ Flu A and B. In contrast, for influenza B, the sensitivities were rather low and ranged from 36.84% for bioNexia Influenza A+B to 52.63% for BinaxNOW Influenza A/B and Directigen EZ Flu A and B. The RIDTs have already been shown to be more sensitive in the detection of influenza A compared to influenza B (Chartrand et al., 2012; Hurt et al., 2007; Welch and Ginnochio, 2010).

For A(H1N1)pdm09, the sensitivities of the RIDTs were slightly lower in comparison to real-time PCR (71.42% for BinaxNOW Influenza A/B and 67.85% for bioNexia Influenza A+B) than that of the cell culture method (75% sensitivity), except for Directigen EZ Flu A and B (78.57% sensitivity), which detected 1 more sample. This sample, which was positive by all RIDTs and negative in cell culture, was taken on a Sunday and processed on the next day. The Ct for this sample was 26.47, which is quite low in comparison to the mean Ct for the negative cell cultures (33.14). In this sample, the questionable viability of the virus could explain the negative cell culture.

Eight samples were positive for the A(H3N2) subtype in this study, and only the bioNexia Influenza A+B test missed 1 positive sample. The 2 other RIDTs and the cell culture method were positive for all of the samples, including the samples with low viral loads ("high" Cts of confirmatory PCR). The mean age of the population with A(H3N2) infection was statistically higher than the one with A(H1N1)pdm09 and B/Yamagata lineage, which is usually associated with a weaker viral excretion and a lower detection rate. However, paradoxically, a higher rate of detection in the A(H3N2) group was observed. RIDTs and the cell culture method seem to be particularly efficient in diagnosing A(H3N2) infections. Could those good results be explained by the choice of antibodies used in the RIDTs, which could bind A(H3N2) more easily than other subtypes?

Table 1
Overall sensitivities and specificities of the evaluated tests in percentage with 95% confidence interval.

	Influenza A		Influenza B	
	Se	Sp	Se	Sp
BinaxNOW	75.68 (55.80–88.23)	100 (98.16–100)	52.63 (38.97–66.02)	100 (97.96–100)
Directigen	81.08 (64.84–92.04)	100 (98.16–100)	52.63 (38.97–66.02)	100 (97.96–100)
bioNexia	70.27 (53.02–84.13)	96.48 (92.89–98.57)	36.84 (24.45–50.66)	100 (97.96–100)

Se = sensitivity; Sp = specificity.

Table 2
Sensitivities depending on subtypes.

Subtype	n	Positives Binax	Positives Directigen	Positives bioNexia	Positives viral cultures	Mean Ct for negative cultures	Mean Ct for positive cultures	Mean Ct for false negatives with all RIDTs	Mean Ct for true positives with at least one RIDT	Age (mean ± SEM)	Number of NPA/ NPS/TS
A/H1N1	28	20 (71.42%)	22 (78.57%)	19 (67.85%)	21 (75%)	32.43 (n = 7)	21.74 (n = 21)	33.43 (n = 6)	21.95 (n = 22)	4.78 ± 1.77	21/7/0
A/H3N2	8	8 (100%)	8 (100%)	7 (87.50%)	8 (100%)	NA	23.53 (n = 8)	NA	23.53 (n = 8)	29.47 ± 11.96	5/3/0
B/Yamagata	51	29 (56.86%)	29 (56.86%)	20 (39.21%)	44 (86.27%)	33.25 (n = 7)	27.03 (n = 44)	31.59 (n = 20)	25.50 (n = 31)	28.48 ± 2.79	34/16/1
B/Victoria	1	1 (100%)	1 (100%)	1 (100%)	1 (100%)	NA	25.59	NA	25.59	71.07	1/0/0

NA = not applicable; NPA = nasopharyngeal aspirate; NPS = nasopharyngeal swab; SEM = standard error of the mean; TS = throat swab.

For influenza B strains of the Yamagata lineage, the sensitivity of the RIDTs was the lowest (56.86% for BinaxNOW Influenza A/B and Directigen EZ Flu A and B and 39.21% for bioNexia Influenza A+B). Here, the mean Ct in the samples that were negative by all 3 RIDTs was statistically higher than that of a sample in which at least 1 RIDT was positive, demonstrating the influence of viral load on the sensitivity of the test. Fig. 3 shows an overlap of Cts between the group with at least 1 positive RIDT and the group with all RIDTs being negative. This could be explained by the fact that the viral load in the sample is not the only parameter influencing the detection of the virus and the affinity of the chosen antibodies in the test could also have an impact. It might also mean that the correlation of Cts with viral load is not very good for influenza B. By contrast, this virus appeared to grow well in LLC-MK_2 cells, as the cell culture method was 86.27% sensitive compared to real-time PCR analysis.

Because only 1 influenza B in this study was of the Victoria lineage and was positive with the 3 RIDTs and the cell culture method, no conclusions could be drawn from this observation.

Altogether, the viral loads (estimated by the Cts of the confirmatory PCRs) of the culture-positive samples were significantly higher than those observed for the culture-negative/PCR-positive samples, and the same phenomenon was observed for the RIDT-positive samples (at least 1 out of 3) compared to the RIDT-negative samples, confirming that viral load influences the sensitivity of both RIDTs and cell culture.

Strains from this study were not further subtyped than A(H1N1) pdm09, A(H3N2), B/Yamagata lineage, and B/Victoria lineage, but some collected strains from 2012–2013 epidemic were sequenced by Belgian National Reference Center for Influenza within the scope of its virological surveillance function and in order to know which ones were circulating (Thomas et al., 2013). It is very probable that the strains included in the study were reflecting the same epidemic.

The circulating influenza A(H1N1)pdm09 strains in Belgium during the 2012–2013 epidemic season were A/St-Petersburg/27/2011-like and A/St-Petersburg/100/2011-like, which are antigenically related and similar to vaccine strain A/California/7/2009. Thus, the imperfect sensitivities of the RIDTs could not be explained by the circulation of several antigenically unrelated strains, with some strains being more easily detected than others. For influenza A(H3N2), the sensitivities were excellent, and most of the circulating A(H3N2) strains were related to the cell-cultured reference strain A/Victoria/361/2011. Concerning influenza B/Yamagata lineage, the majority of the circulating strains were related to B/Estonia/55669/2011 and belonged to clade 2. A few viruses belonged to clade 3 and were related to the strain B/Stockholm/12/2011 and to the vaccine virus B/Wisconsin/1/2010. Due to its design, this study could not determine if one of these clades was less detectable than the other, which could explain the low sensitivity observed for influenza B/Yamagata lineage. The circulation of 2 different clades may explain the overlap of the Cts observed on the Fig. 3. Maybe one of the clades even with low Cts could be less detected than the other. Just to be mentioned for epidemiological information, the B/Victoria lineage circulating strains during this season were related to the reference strain B/Brisbane/60/2008.

5. Conclusions

In conclusion, the 3 evaluated RIDTs seemed more efficient at detecting influenza A(H3N2) than for the 2 other circulating viruses A(H1N1) and B/Yamagata lineage. This has to be balanced as there were only 8 positive samples for A(H3N2). These results are paradoxical, as the group of patients with A(H3N2) infection was statistically older than the 2 other groups, and older age is usually associated with lower viral excretion and a lower detection rate. These lower sensitivities for the detection of influenza A(H1N1)pdm09 and influenza B/Yamagata lineage can be confidently attributed to the subtype of the virus, as the sample types and ages of the 2 groups of patients were not different. The

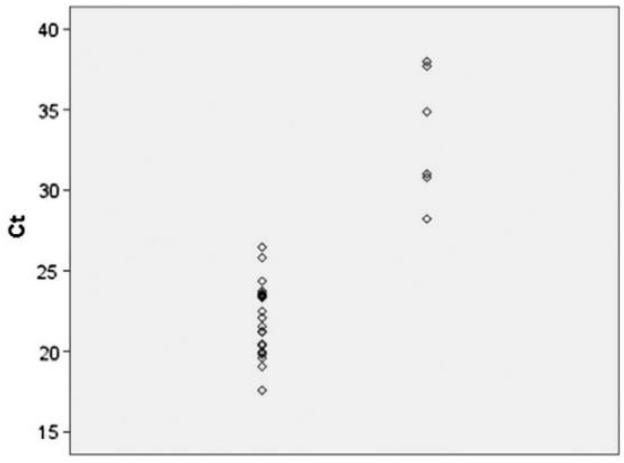

Fig. 2. Repartition of Cts of the positive samples for which at least 1 RIDT was positive (on the left) and for which all RIDTs were negative (on the right) for A(H1N1)pdm09.

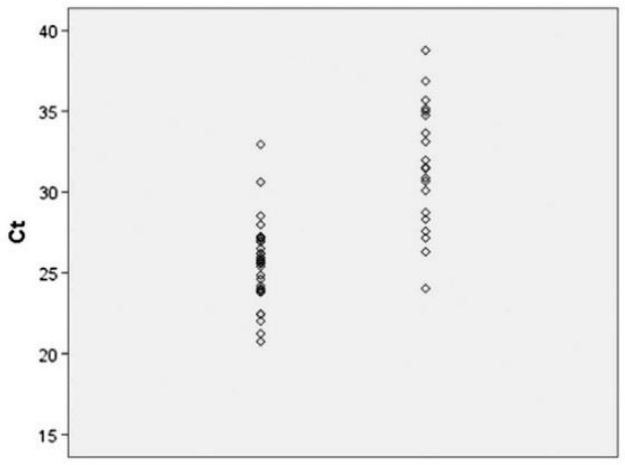

Fig. 3. Repartition of Cts of the positive samples for which at least 1 RIDT was positive (on the left) and for which all RIDTs were negative (on the right) for influenza B/Yamagata lineage.

viral load in the samples, indirectly reflected by the Cts of the PCR, also had an impact on the detection of these 2 viruses when using RIDTs.

In the past few years, the proportion of the viral strain subtypes circulating during influenza epidemics was changed. During the 2012–2013 epidemic in Europe, type A and type B viruses were detected in similar proportions, and A(H1N1)pdm09 viruses were detected at approximately twice the level of A(H3N2) viruses (Daniels et al., 2013). If the seemingly better results for A(H3N2) can be explained by the choice of the antibodies in the assays, the manufacturers of the RIDTs should adapt these tests to make them more suitable for the detection of virus subtypes that were previously less prevalent.

Conflict of interest

None.

Funding

None.

Competing interest

None declared.

Ethical approval

Not required.

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3.4 Nucleic acid amplification tests (NAATs): an ongoing revolution

Diagnostic virology has been revolutionized by the application of nucleic acid amplification techniques, which can detect specific DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) sequences of virtually any virus. [9]

Most of the molecular tests are based on the PCR (polymerase chain reaction) technique developed in 1985. Its principle relies in the enzymatic amplification of a gene sequence. The enzyme used, *Taq* polymerase, is thermostable and originates from the bacteria *Thermus aquaticus*. It can replicate DNA and thus for the detection of RNA viruses, a reverse transcription step is required to convert RNA into DNA before the PCR. However, some recombinant *Taq* polymerase able to carry out reverse transcription as well as DNA polymerase reactions are now available. The amplification requires two primers, sometimes known as “forward” and “reverse” primers, in order to provide initiation points to which additional nucleotides can be attached by the *Taq* polymerase. These primers are specific of the gene sequence to be amplified.

The PCR comprises of three main steps which are repeated for at least 30 cycles: (1st) melting the target DNA at 95°C thus causing its denaturation, (2nd) cooling to around 50-60 °C to allow binding of the two primers (annealing), and (3rd) synthesis of the DNA strands by extension of the primers thanks to the polymerase.

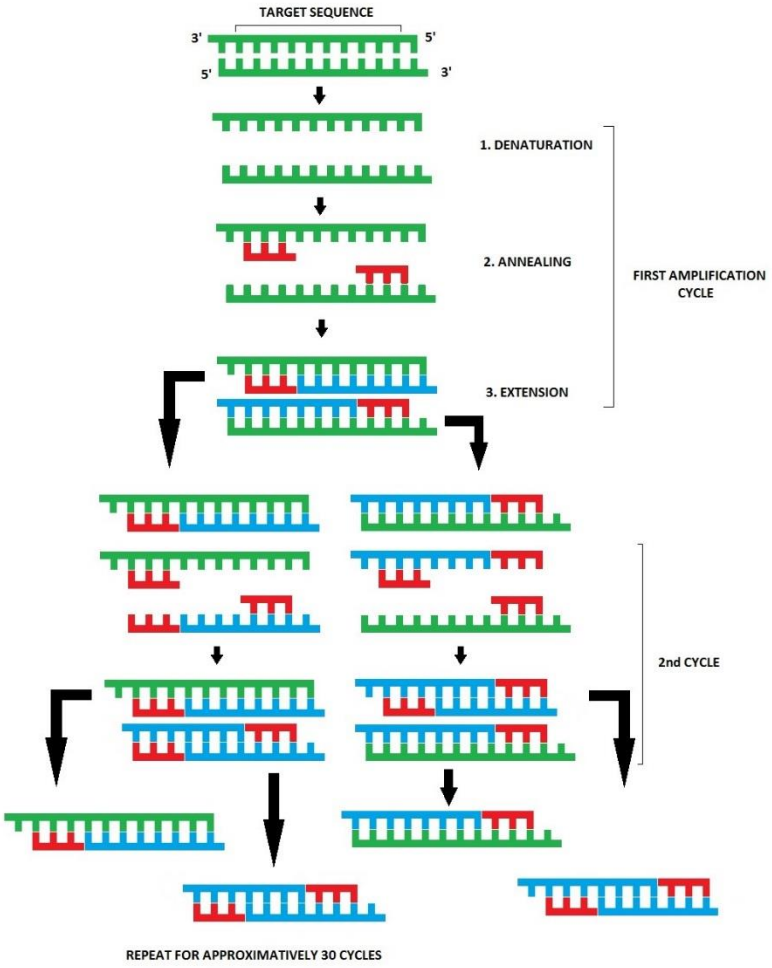

Figure 5: Principle of PCR technique describing the 3 main steps, denaturation, annealing and extension.

After 30 cycles, a single copy of the target sequence theoretically becomes 2^{30} copies. (Figure 5) The product of the amplification then must be detected. This can be achieved at the end of the PCR process where amplified DNA can be detected either as a stained band of correct molecular weight on agarose gel electrophoresis or by hybridization with labelled DNA or RNA probes. [8] However, nowadays, most techniques perform a real-time detection by adding labelled probes emitting fluorescence throughout the PCR (real-time PCR).

The whole process can be completed in less than an hour. However, an extraction of the DNA and RNA from the sample is usually necessary before performing the PCR. Moreover, a positive control, a negative control and an internal control for each sample must be executed in every run of analysis in order to attest of the proper execution of the PCR and to rule out any contamination. This explains why PCR are usually performed in batch processes, delaying the report of the results. The different steps of PCR techniques must be performed in separate rooms in order to prevent contamination of the reagents or of the samples. More modern, fully automated techniques performing all the steps of the PCR are available in single tests (integrated platforms). They can detect a single target (monoplex) or multiple targets at the same time (multiplex). With such techniques, the analysis can be run without delay, speeding up the report of results.

Latest nucleic acid amplification methods based on enzymatic isothermal amplification do not require high temperature cycling. Various techniques are available among which Loop Mediated Isothermal Amplification (LAMP), Strand Displacement Amplification (SDA), Helicase-Dependent Amplification (HDA) and Nicking Enzyme Amplification Reaction (NEAR). These methods are extremely fast, and their sensitivity is close to the one of PCR. [8] The NEAR mechanism is described in the figure 6. A rapid influenza diagnostic test using NEAR technique was evaluated in comparison to antigen detection test and conventional PCR in the following article. [23] This article also constituted a preliminary work in order to appreciate the contribution of rapid molecular diagnostic tests in patients' management.

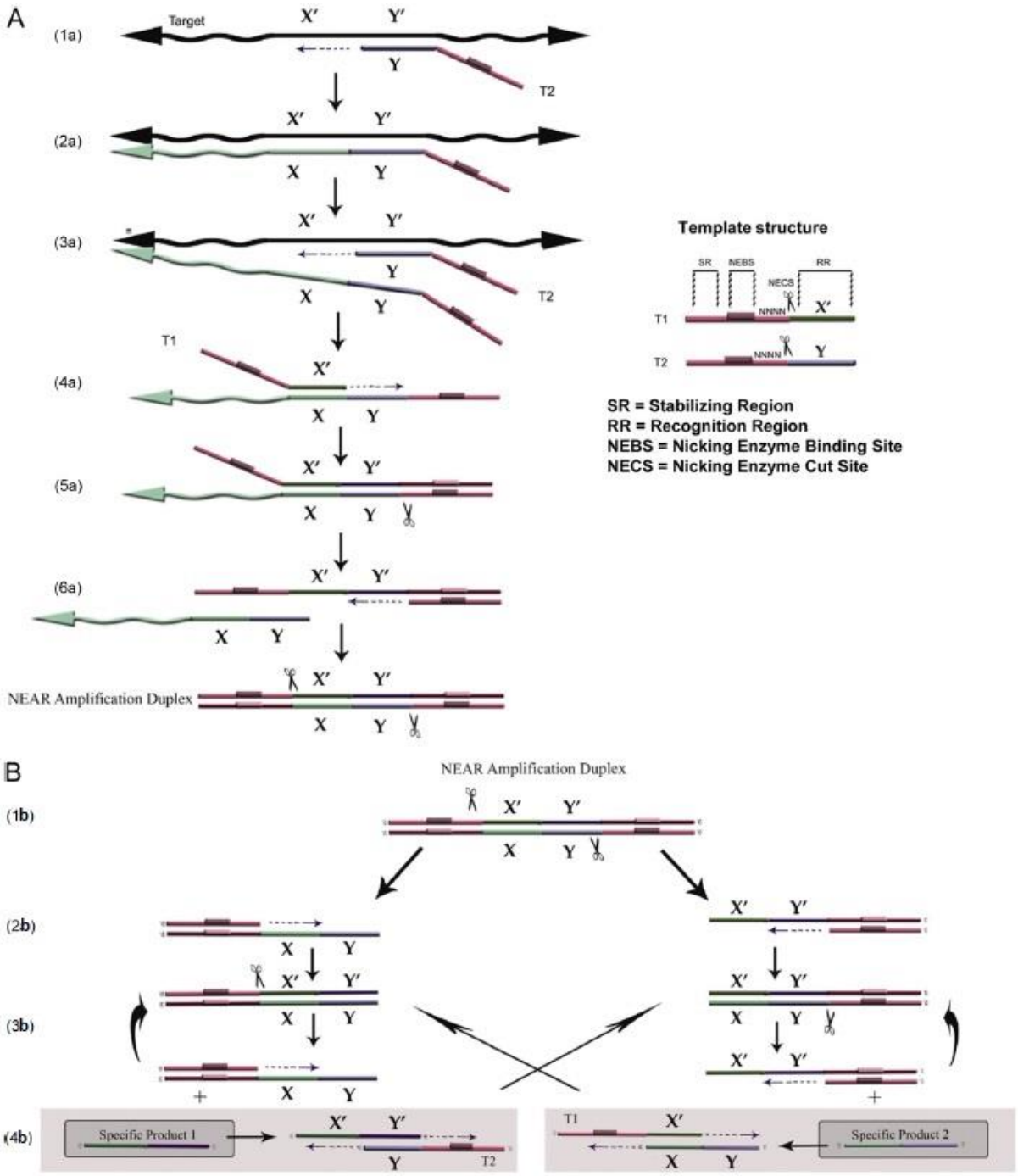

Figure 6: NEAR mechanism. (A) Mechanism of NEAR amplification duplex formation. (1a and 2a) The recognition region of T2 binds to the complementary target region and is extended by polymerase along the target. (3a) A second T2 binds to the same target and is extended, displacing the first T2. (4a) The recognition region of T1 binds to its complement in the released strand and is extended to the 5′ end, creating a double-stranded nicking enzyme recognition site. (5a) Nicking enzyme binds and nicks (indicated by scissors). (6a) polymerase synthesizes off the cleaved 3′ OH along T1, displacing the remaining target complement, and the final extended double-stranded complex is termed the NEAR amplification duplex. (B) Mechanism of product formation. (1b and 2b) Nicking enzymes bind to both nicking enzyme recognition sites on the NEAR duplex; cleavage and strand displacement amplification at both sites creates two complexes, each consisting of a duplex stability region, a nicking enzyme recognition region, and a single-stranded target. (3b and 4b) Repeated nicking, polymerization, and strand displacement result in the amplification of products 1 and 2. Cleaved complexes are regenerated (3b), while products 1 and 2 can anneal to T1 and T2, respectively (4b), resulting in bidirectional extension and creating duplexes that generate the opposite product upon cleavage. The products continue to recycle until the templates, deoxynucleoside triphosphates (dNTPs), or enzymes are depleted. Adapted from *[24]*

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Contribution of a rapid influenza diagnostic test to manage hospitalized patients with suspected influenza

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ABSTRACT

Aim: To evaluate the performances of the Alere i influenza A&B test and to appraise its contribution to patient management.
Methods: In total, 267 samples were tested. Influenza A and B PCR was performed as the reference. For each positive result, the supervising physician was contacted to collect data regarding patient management.
Findings: The overall sensitivity and specificity of the Alere i were 91.4% and 97.6% for influenza A and 54.5% and 98.8% for influenza B, respectively. More specifically, when used in the emergency room (ER), the test helped avoid 10.7% of hospitalizations, 46.4% of antibiotic prescriptions and 42.9% of additional investigations for positive patients. The test was also helpful in instituting the prescription of oseltamivir and patient isolation.
Conclusion: Alere i influenza A&B is a rapid, sensitive and specific diagnostic test for influenza A. Sensitivity for influenza B was poor. Its usefulness was more important when patients were still in the ER.

1. Introduction

The rapid management of influenza is critical during epidemics because although a treatment (oseltamivir) is available, it preferably must be administered within the first 48 hours following symptom onset; however it may retain some benefit in hospitalized patients beyond 48 h (Dutkowski, 2010; Peters et al., 2008). Furthermore, because the isolation of hospitalized infected patients, regardless of the pathogen causing the infection, is of utmost importance in preventing nosocomial spread among other patients and caregivers, the handling of such patients should be as efficient as possible. Although patient management should rely not only on the laboratory test results but also on the clinical symptoms exhibited by the patients, rapid diagnostic tests for influenza are nevertheless useful tools, despite often lacking sensitivity (Busson et al., 2014; Hurt et al., 2007; Peci et al., 2014). Notwithstanding, their clinical contribution is poorly documented (Moore, 2013). New techniques based on the principles of lateral flow chromatography with detection by fluorescence (Sofia Influenza A + B; Quidel, San Diego, CA, USA) (Lee et al., 2012; Leonardi et al., 2013; Rath et al., 2012) or on isothermal amplification (Alere i Influenza A&B, Alere, Waltham, MA, USA) (Bell and Selvarangan, 2014; Bell et al., 2014; Nie et al., 2014) have become available lately. These techniques offer superior sensitivity in comparison with conventional techniques based on lateral flow chromatography with visual interpretation and may still be performed in less than 20 minutes. The objectives of this study were (1) to compare the test performances of Alere i Influenza A&B to those of Sofia Influenza A + B routinely used in our setting and (2) to appraise the contribution of the use of Alere i Influenza A&B on the management of adult and pediatric patients in two university hospitals.

2. Patients and methods

2.1. Population and inclusion criteria

The study was conducted from January 2, 2015 (week 52 of 2014) to March 30, 2015 (week 13 of 2015). The epidemic threshold of ILI (influenza-like illness) in Belgium for the 2014–2015 season was set at 140 ILI/100,000 population. This threshold was crossed at week 3–2015, with a peak of 979 ILI/100,000 population reached at week 6–2015. The rate of ILI fell below the threshold during week 12–2015 according to the Belgian National Influenza Centre. During the period ranging from week 40–2014 to week 20–2015, 932 samples from patients with ILI were sent to the Influenza Centre by sentinel general practitioners participating in influenza surveillance. Among these samples, 485 (52%) tested positive for influenza A (16% A H1 and 84% A H3) and 8% for influenza B (92% Yamagata and 8% Victoria).

Patients were included from two different hospitals: the Jules Bordet Institute, which is a 160-bed hospital specializing in oncology

Abbreviations: ER, emergency room; ILI, influenza-like illness.

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management for adults and Saint Pierre University Hospital, which is a general 626-bed hospital.

The target patient eligibility criteria were determined by clinicians, with a focus on the most vulnerable populations.

Adult patients with suspected influenza were included if they were being treated with an immunosuppressive drug or chemotherapy or were exhibiting febrile neutropenia at the Bordet Institute. At Saint Pierre Hospital, any adult patient with suspected influenza and at risk of complications or requiring hospitalization was included. Suspected influenza was based on the definition of ILI by the World Health Organization.

Pediatric patients (<15 years old) were included upon admission in the emergency room (ER) of Saint Pierre University Hospital if (1) they were aged less than 3 months and presented with fever of undetermined origin or if (2) influenza was suspected and they would require potential hospitalization, irrespective of age or (3) had a pathology exposing them to complications (chronic respiratory diseases such as cystic fibrosis or asthma, sickle-cell disease, asplenia, neuromuscular diseases, severe neurological conditions, hereditary metabolic disorders including diabetes, congenital or acquired immunosuppressions, heart defects, chronic nephropathies and chronic liver diseases). In children, influenza presentation may be nonspecific, with symptoms including digestive disorders or febrile seizures, and thus the inclusion of these patients was left to the consideration of pediatricians, who were more experienced in detecting potential cases.

Four hospitalized patients who had been in close contact with confirmed influenza cases (a visitor or another hospitalized patient) were also included at the request of physicians to determine whether they had been infected. It was decided to include them even if they did not fulfill inclusion criteria as a point of interest to determine if Alere i could confirm suspected nosocomial influenza.

For the study design, each physician in charge was asked to determine the impact on patient management decisions based on the rapid turnaround time for a test result (<1 hour) versus no available test result. The study was approved by the ethics committee of Saint Pierre Hospital.

2.2. Processing of samples

Samples considered suitable for the study were nasopharyngeal swabs (NPS) (collected using FLOQSwab, Copan, Brescia, Italy), nasopharyngeal aspirates (NPA) and broncho-alveolar lavages. Samples arriving during working hours (Monday to Friday, from 8:00 am to 8:00 pm) were processed immediately upon receipt. Samples arriving outside working hours were stored between 2°C and 8°C and processed on the next working day.

Samples were diluted with viral transport medium composed of veal infusion broth (Difco, Becton Dickinson, Sparks, MD, USA) supplemented with bovine albumin (Sigma Aldrich, St. Louis, MO, USA) to a volume of 3 mL. Rapid influenza diagnostic tests (Sofia Influenza A + B and Alere i Influenza A&B) were then performed simultaneously on an aliquot of the diluted samples. In cases of invalid Alere i results, another aliquot was further diluted with an equal volume of viral transport medium and retested to mitigate the effect of a hypothetical inhibitory substance in the sample.

Another aliquot was sent to the molecular biology department to perform real-time qualitative PCR for the detection of influenza A and B as well as the betaglobin gene as a cellularity control (Argene, Biomérieux, Marcy L'Etoile, France). These PCRs, conducted twice a week, were used as the gold standards for evaluating the Sofia and Alere i performances. Extraction of nucleic acids was carried out with the QIAsymphony automated extraction protocol using the QIAsymphony DSP Virus/Pathogen Midi extraction kit (Qiagen, Germantown, MD, USA), amplification and detection were performed on an ABI 7500 automate (Applied Biosystem, Paisley, United Kingdom) as per manufacturer's instructions.

The last aliquot was frozen at −80°C and thawed to be retested by Alere i and gold- standard PCR in cases of discrepant results.

Table 1
Enrolled patients.

Populations (median age)	n patients (F/M)	n samples (NPA/NPS/BAL)
Children - Saint Pierre Hospital (1):	169 (84 / 85)	178 (102/76/0)
<3 months with FUO	51 (28 / 23)	58
Suspected influenza and comorbidity	34 (15 / 19)	35
Suspected influenza and hospitalization	83 (41 / 42)	83
Suspected nosocomial cases	2 (1 / 1)	2
Adults - Saint Pierre Hospital (63.5):	48 (26/ 22)	51 (0/49/2)
Intensive care units	34 (14 / 20)	35
Other units	12 (10 / 2)	14
Suspected nosocomial cases	2 (2 / 0)	2
Adults - Jules Bordet Institute (58.8):	36 (25 / 11)	38 (0/38/0)
Febrile neutropenia	5 (5 / 0)	6
Patients treated with immunosuppressive drug	7 (6 / 1)	7
Patients treated with chemotherapy	24 (14 / 10)	25

BAL = bronchoalveaolar lavage; FUO = fever of undetermined origin; NPA = nasopharyngeal aspirates; NPS = nasopharyngeal swabs.

The Alere i and gold-standard PCR testing were free of charge for the patients. The other tests used for routine practice were billed as usual.

2.3. Management of positive results

Each time a sample was positive by Alere i, the microbiologist immediately informed the physician in charge of the patient and collected information regarding the contribution of the positive result on the short-term management of the patient. The parameters recorded were the contribution of the result on (1) the decision to hospitalize the patient, (2) the fulfillment of additional investigations, (3) the decision to administer or withhold oseltamivir, (4) the decision to administer or withhold antibiotics, and (5) patient isolation. For samples arriving outside working hours, because the results were delivered on the next working day when the management of patients with respect to additional tests, hospitalization and isolation had already been decided, data on the theoretical impact of the results, had they been available earlier, were collected.

Positive Argene PCR results were also communicated when both Sofia and Alere i were negative. The same potential parameters were retrospectively evaluated.

3. Results

A total of 267 samples from 253 patients were analyzed: 163 were NPS, 102 were NPA and 2 were broncho-alveolar lavages. The population comprised 118 male and 135 female patients. The median age

Table 2
Sensitivity (Se) and specificity (Sp) of the tests (%).

	Overall		Children		Adults	
Influenza A						
n positives	58		35		23	
Se/Sp	Se	Sp	Se	Sp	Se	Sp
Sofia	70.7	99.5	82.9	100	56.5	98.5
Alere i	91.4	97.6	91.4	100	91.3	92.4
Alere i retest	94.8	99.5	97.1	100	91.3	98.5
Influenza B						
n positives	11		6		5	
Se/Sp	Se	Sp	Se	Sp	Se	Sp
Sofia	54.5	98.8	66.7	98.8	40	97.6
Alere i	54.5	98.8	66.7	99.4	40	100
Alere i retest	54.5	100	66.7	100	40	100

Table 3
Impact of results on the management of patients depending on the time of day and the hospitalization status.

	Number of cases	Hospitalization				Additional tests				
	(Children/Adults)	Prescribed anyway	Trigerred	Avoided	Not indicated	Avoided (sometimes several tests for the same patient)				No change
						LP	Blood test	X-ray	Urinalysis	
Working hours (still in ER)	28 (22/6)	15 (14/1)	1 (1/0)	3 (1/2)	9 (6/3)	4 (4/0)	4 (3/1)	7 (6/1)	2 (2/0)	16 (11/5)
Working hours (hospitalized)	18 (6/12)	18 (6/12)	0	0	0	0	0	0	0	18 (6/12)
Working hours (discharged home)	4 (3/1)	0	0	0	4 (3/1)	0	2 (2/0)	0	0	2 (1/1)
Outside working hours (hospitalized)	12 (6/6)	11 (6/5)	0	1 (0/1)*	0	1 (0/1)*	1 (0/1) * 1 (1/0)**	1 (1/0)	1 (0/1)* 1 (1/0)**	8 (4/4)
Outside working hours (discharged home)	7 (4/3)	0	0	0	7 (4/3)	0	2 (1/1)	0	0	5 (3/2)
Overall impact	69 (41/28)	44 (26/18) 63.8%	1 (1/0) 1.4%	4 (1/3) 5.8%	20 (13/7) 29%	5 (4/1) 29%	10 (7/3)	8 (7/1)	4 (3/1)	49 (25/24) 71%

ER= emergency room; LP lumbar puncture.
* Would have been avoided provided the result had arrived earlier.
** Avoided.
*** Stopped.
**** Would have been prescribed provided the result had arrived earlier.

was 3.5 years (range 1 week–91 years) for an enrolled population of 169 children and 84 adults (Table 1).

3.1. Analytical performances of Sofia and Alere i

Over the 267 tested samples, 69 were positive by PCR, that is, 58 for influenza A (23 adults, 35 children) and 11 for influenza B (5 adults, 6 children). The sensitivity and specificity of the other tests were calculated based on the assumption that PCR was the gold standard. The influenza viruses were not subtyped; however, according to the surveillance of ILI in Belgium during the 2014–2015 epidemic, influenza A(H3N2) was the dominant subtype with Yamagata the dominant influenza B lineage.

For influenza A, Sofia's sensitivity and specificity were 70.7% and 99.5%, respectively. Alere i showed 91.4% sensitivity and 97.6% specificity (Table 2).

For influenza B, Sofia's sensitivity and specificity were 54.5% and 98.8%, whereas Alere i showed sensitivities and specificities of 54.5% and 98.8%, respectively. Both tests showed greater sensitivity and specificity in the pediatric cohort (Table 2).

Nine invalid results (3.4%) were obtained with Alere i (7 NPA and 2 NPS), signifying the analysis could not give a positive or negative result due to an undetermined technical issue; samples with such results were retested after dilution. No invalid result was obtained for these nine retested samples.

Frozen aliquots of samples showing discrepant results between Alere i and PCR were also retested using these two techniques. For these samples, the PCR results were unchanged. For Alere i, a gain of sensitivity was achieved for influenza A (from 91.4 to 94.8%) because 2 of the 5 false negative samples retested as positive. We further analyzed the reference PCR Ct (cycle threshold), which is an approximation for viral load (the lower the Ct, the higher the viral load), of all samples. The mean PCR Ct of these 5 false negative samples was 34.62, whereas the mean Ct of the true-positive samples with Alere i was 25.05 (Wilcoxon–Mann–Whitney test; $P < 0.0001$).

Three of the 4 false-positive samples for influenza A and 3 of 3 for influenza B retested as negative.

Two samples gave positive results for both influenza A and B, which, according to manufacturer's instructions, indicates that either both viruses are concomitantly present in the sample or a "non-typeable" influenza virus is present. In one case, the sample was positive for influenza B by PCR and retested positive for influenza B with the Alere i. The other sample was negative for influenza A and B by PCR and retesting indicated a positive result for influenza A and an invalid result for influenza B.

3.2. Contribution to patient management

In all, 207 samples were received during working hours (77.5%), of which 50 were positive for influenza (44 influenza A and 6 influenza B). We received 60 samples outside working hours (22.5%), among which 14 were positive for influenza A and 5 for influenza B.

Of positive results obtained and communicated during working hours, 28 patients were still in the ER, 18 were hospitalized and 4 had left the hospital. For cases with samples arriving outside working hours, 12 patients were hospitalized and 7 had already left the hospital (Table 3). Excluding patients for whom hospitalization was not deemed necessary irrespective of the influenza test result ($n = 20$), 6.1% of hospitalizations were avoided and another 2% could have been avoided had the results been obtained earlier, whereas 2% of hospitalizations (1 patient) were triggered by the test results. The last case involved a child of 20 months with gastroenteritis and mild weight loss who could have been discharged home; however, the positive influenza test resulted in the pediatrician preferring to hospitalize the child for monitoring. Concerning additional investigations, the influenza tests helped avoid their realization for 29% of patients (or 42.9% of patients if we focus on those still in the ER when the results were delivered). Oseltamivir therapy was given to all patients fulfilling administration criteria who were still in the ER when the result was delivered. For hospitalized patients, a positive test result triggered oseltamivir administration in 50% of cases for which it was indicated; therapy was already prescribed for the other 50%. Antibiotics were avoided or stopped for 30.4% of patients and could have been avoided for an additional 5.8% had results been available earlier. More specifically, for hospitalized patients under medical observation, the influenza results prevented the administration of antibiotics in 16.7% of positive cases and allowed them to be discontinued in 3.3%. Concerning patient isolation, of the 28 patients who remained in the ER, hospitalization had been planned for 16 and for 10 of them (62.5%), the result was delivered before the decision to isolate had been made, thereby confidently aiding its establishment. Isolation was already planned for the 6 remaining patients. Of the 30 already-hospitalized patients, isolation was established in light of the positive result for 11 cases (36.7%) when it already should have been done based on clinical signs.

Oseltamivir				Antibiotics			Isolation		
Instituted after result	No indication	Result outside time limit	Already prescribed	Prescribed anyway	Avoided	Not deemed necessary	Instituted after result	Already instituted	Discharged home
10 (4/6)	18 (18/0)	0	0	4 (3/1)	13 (12/1)	11 (7/4)	10 (9/1)	6 (6/0)	12 (7/5)
6 (2/4)	1 (1/0)	4 (3/1)	7 (0/7)	8 (2/6)	4 (2/2)	6 (2/4)	6 (3/3)	12 (3/9)	0
3 (2/1)	1 (1/0)	0	0	1 (0/1)	2 (2/0)*	1 (1/0)	0	0	4 (3/1)
3 (1/2)	5 (5/0)	2 (0/2)	2 (0/2)	5 (1/4)	2 (2/0)* 1 (1/0)** 1 (0/1)***	3 (2/1)	5 (4/1)	7 (2/5)	0
3 (0/3)****	3 (3/0)	1 (1/0)	0	1 (0/1)	2 (1/1)*	4 (3/1)	0	0	7 (4/3)
25 (9/16)	28 (28/0)	7 (4/3)	9 (0/9)	19 (6/13)	25 (20/5)	25 (15/10)	21 (16/5)	25 (11/14)	23 (14/9)
36.2%	40.6%	10.1%	13%	27.5%	36.2%	36.2%	30.4%	36.2%	33.3%

3.3. Contribution to the management of nosocomial epidemics

During the evaluation period, two nosocomial influenza outbreaks were suspected: one in a pediatric unit and the other in a functional rehabilitation unit. A total of 4 contacts of the index cases were tested. Three were positive for influenza A by PCR (the fourth was positive for metapneumovirus). All 3 cases were diagnosed using Alere i, whereas none were diagnosed using the Sofia. All patients were already isolated because they were contacts of cases and were suspected of having influenza.

3.4. Impact of the higher sensitivity of Alere i

The Alere i had better sensitivity than the Sofia test considering that it detected 14 additional positive samples (5 children, 5 adults from the general hospital and 4 adults from the oncological hospital). Among these 14 cases, 3 were patients included as having had close contact with confirmed influenza cases. Treatment was modified by the addition of oseltamivir in one of the 3 cases and antibiotics were avoided in one patient. For the 11 remaining patients, additional investigations were avoided in 4 patients (including 2 lumbar punctures), oseltamivir was added to the treatment regimen of 6 patients, antibiotics were avoided in 4 patients and isolation, which had been overlooked, was implemented for 1 patient.

4. Discussion

In the present evaluation, although the Alere i showed better sensitivity for the diagnosis of influenza A than the Sofia test, the former was less sensitive than the PCR method used as the gold standard. Low viral load in the samples missed by the Alere i might explain these results as suggested by the late Cts observed by real-time PCR in the Alere i false-negative samples. The gain in sensitivity was particularly obvious for adult patients (+34.8%). For children, the gain in sensitivity was subtle but still noteworthy (+8.6%). Regarding influenza B, Sofia and Alere i showed the same suboptimal overall sensitivity (54.5%), which could also be attributed to the low viral loads observed in false-negative samples with Alere i. However, a reliable conclusion could not be drawn for influenza B because only 11 samples were positive. Specificity was greater than 97% overall for both techniques.

False-positive results occurred in 7 cases (2.6% of all cases, 11% of Alere i-positive cases). All but one were "resolved" by retesting, for unclear reasons. The device does not permit the visualization of raw results data to determine whether such false-positive results could have been due to nonspecific signals. In the present evaluation, the communication of these false-positive results had no impact on the hospitalization decision or the prescription of antibiotics. However, treatment with oseltamivir was begun in 3 patients.

The result of the rapid test alone did not solely motivate the modification in patient management; instead, such action resulted from the sum of the clinical evaluation together with the results of imaging and laboratory tests such as CRP (C-reactive protein) and urinary sediment. Hence, the rapid communication of an influenza-positive result contributed to the decision to hospitalize in a non-negligible proportion of patients, particularly when they remained in the ER because hospitalizations were avoided for more than 10% of positive patients, whereas additional investigations were avoided in more than 40% of the positive patients, mainly children. Notably, some of these investigations were lumbar punctures, which are invasive procedures that may engender complications. It has already been shown in other publications that the administration of oseltamivir has a positive impact on patient outcome (Dutkowski, 2010; Peters et al., 2008; Rodríguez et al., 2011). In this study, rapid diagnostic tests aided in treatment modification decisions by the addition of oseltamivir for all the ER cases for which it was indicated and for hospitalized patients for whom it was not initially prescribed. A rapid positive influenza result also helped avoid unnecessary antibiotic prescription, particularly when patients were still in the ER, considering that when the results were generated after the patients had been discharged home, they were not necessarily informed that antibiotics could be discontinued. The potential benefits of rapid result availability were thus lost. Globally, considering the patients with a negative test result, hospitalization could have been avoided in up to 1.5% of patients, additional investigations in up to 7.5% of cases and antibiotic prescriptions in up to 9.4% of cases, partially due to the test results. Oseltamivir was administered in 9.4% of patients thanks to the test results. The prevalence of influenza in the studied population was 25.8% and likely the higher the prevalence, the more profound the impact.

As previously discussed, patient isolation was instituted based on the positive test results for 11 of 30 (36.7%) already hospitalized patients. Although it is recommended to isolate and administer empirical oseltamivir therapy to patients with ILI fulfilling therapeutic indications and to not depend on the rapid test result, in practice this recommendation is not always followed because in some cases, such therapy might simply have been overlooked or another diagnosis might have obfuscated the initial influenza suspicion. Some elderly patients might not develop fever, thereby not fulfilling the ILI definition and hampering proper management. Finally, during epidemics, some patients, due to lack of capacity in medical units, are sometimes placed in surgical units, whose staff are usually less trained in respiratory isolation procedures and influenza management. When a positive influenza result is communicated, antiviral therapy and isolation are less likely to be overlooked.

Notably, of the 4 suspected nosocomial infection, all of the 3 influenza A cases were diagnosed with the Alere i but not with the Sofia. Although the number of patients is low, this finding might suggest a potential usefulness of the Alere i in the early management of nosocomial outbreaks in comparison to less sensitive rapid tests.

Whereas the economic impact could hardly be evaluated in the present study, the likely sparing of hospitalization days for some patients and the decrease in additional investigations and antibiotic prescriptions might cover the cost of the tests performed. The appraised total Alere i cost for the 267 samples was 13,350 euros compared to 5340 euros for Sofia. Because we had 14 samples positives with Alere i but negative with Sofia, one extra positive sample was detected with Alere i for every 19.07 patients tested (considering a disease prevalence of 25.8% in our population). This finding represents an additional cost of 572 euros to obtain one additional positive sample. Nevertheless, it would be impossible to perform Alere i influenza A&B for all patients in our settings (large public hospitals) due to technical (only one sample could be analyzed at a time) and financial considerations (the cost would be billed to the patient because no reimbursement by the social welfare is available in Belgium). Critical populations should be targeted because rapid diagnosis and intervention are more likely to prevent complications in such populations.

5. Conclusion

In this study, Alere i Influenza A&B testing showed better sensitivity than Sofia influenza A + B testing for the diagnosis of influenza A. For influenza B, although the sample number was low, performance appeared equally unsatisfactory for both tests. This was a pilot study to appraise the usefulness of rapid influenza diagnostic testing for patient management. It highlights that these tests contributed to the improved application of guidelines concerning oseltamivir prescription and patient isolation, particularly when the results were delivered for patients who remained in the ER. The same observation held for the sparing of antibiotics, additional investigations and hospitalization, which supports the contention of 24/7 availability of these rapid diagnostic tests. The use of more sensitive techniques could also permit the evaluation of the contribution of a negative result to patient management because such a result would confidently exclude a viral infection. Moreover, should broader panels of pathogens be detected, the impact could be even greater. This latter scenario might be possible with the recent boom in multiplex point-of-care PCR techniques, which will likely revolutionize diagnostic possibilities and will require integration in patient-management algorithms.

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Another technique available for viral diagnostic is the use of microarrays or microchips for nucleic acid detection. Their principle relies in the fixation of oligonucleotides specific from conserved sequences of viruses on a solid support matrix. These oligonucleotides will capture amplified nucleic acid sequences from clinical specimens with binding of the sequences then detected by laser scanning. [8]

The advantages of molecular techniques over non-molecular ones is that they are nowadays the most sensitive techniques. They can be used to detect non-culturable viruses or inactivated viruses due to inadequate transport or storage of the specimens. When needed, they can provide a quantitative result which can be useful for the monitoring of certain viral infection such as HIV, HBV or HCV. [25] [26] [27] However, data correlating viral load and symptoms are sometimes conflicting namely for respiratory viruses, probably due to the different sampling and analyzing methods used. [28, 29, 30, 31, 32, 33] Utilization of new quantification techniques such as digital PCR might improve the interpretation of the results. [34] [35] [36] Digital PCR differs from real-time methods in the way the sample target is measured, by dividing the reaction into multiple, smaller endpoint reactions which allows direct absolute quantification of the target. The main advantages of digital PCR in comparison to real time PCR are that it doesn't depend on calibration curves and it is less susceptible to enzyme inhibition. [37] Some authors advocate for the quantification of housekeeping genes (genes present in a constant proportion in human cells) as a marker of cellularity. They can thus express the quantification of viruses in number of copies per cell which could help compare results between studies and establish thresholds of infection. [29] [38] Moreover, most recent techniques can deliver a result in a timeframe suitable for impacting patients' management. The disadvantages of these molecular tests are that sometimes the PCR can be inhibited by factors contained in the samples. Their capacity to detect very low quantities of viruses expose them to contaminations by DNA or RNA not originally contained in patients' samples. For the same reason, they can detect latent or traces of viruses which are not related to the actual condition of the patient. And finally, when prescribing a molecular test, the targets to amplify should be chosen carefully such as not to miss a diagnosis. The development of multiplex molecular tests detecting a broad range of pathogens causing similar clinical conditions make this situation less likely to occur. [8]

3.5 State of play on direct viral detection tests

The developments of direct viral diagnostic tests over years have aimed towards a boost of speed and sensitivity. Considerable efforts have also been made in order to provide user friendly tests with short hands-on time and easy or automated interpretation of results.

Numerous lateral flow chromatography tests and nucleic acid amplification tests are approved or cleared by the Food and Drug Administration (FDA), a United States organization granting the authorization of marketing to manufacturers. [9] For products sold within the European Economic Area (EEA), the Conformité Européenne (CE) marking is necessary. [39] These labels also warrant the quality of the diagnostic tests to consumers. For immunofluorescence tests and cell cultures, as each laboratory uses its own cell lines and reagents, there is usually a FDA approval or a CE marking for each individual reagent of the test procedure.

Tables 3 sums up the sensitivity and specificity reported in literature of the various viral diagnostic test for influenza viruses and RSV. A Pubmed search was conducted using the terms "influenza immunofluorescence", "influenza shell vial", "influenza cell culture", "influenza antigen detection" and "influenza PCR" from 1998 to 2018. The same was performed for RSV. To be eligible, studies had to be prospective, tests had to be performed on fresh samples, and population had to be well defined and not immunocompromised. The comparison method had to be a molecular technique.

Virus	Population	IF		LFC (colloidal gold)		LFC (fluorescence)		Shell vial		cell culture		isothermal amplification		PCR		References
		Se	Sp	Se	Sp	Se	Sp	Se	Sp	Se	Sp	Se	Sp	Se	Sp	
Influenza A	Paediatric	69-78.6	76.6-99.7	55.6-93.8	95.3-99.5	82.9-95.8	91.1-100	60.3	100			97.1	100	93.7-98.4	98.8-100	[23] [40] [41] [42] [43] [44] [45]
Influenza A	Adult			44.4-60.3	99.9-100	56.5-71.4	98.2-98.5	65.1-73.3	100	68.6-74.4	100	55.2-91.3	98.3-100	96.4-100	98.3-99.6	[23] [46] [47] [48] [49] [50]
Influenza A	Both	92.5	99.2	52.2-84	96.5-100	75.3-76.8	98.3-98.6	77.3-83.9	100	55.8-87.5	99.6-100			96.4-100	99.6	[22] [51] [52] [53] [54] [55] [56] [57]
Influenza B	Paediatric	63.2-78.4	99.8	51.4-94.2	99.5-100	66.7-98.1	70.7-98.8	75-88.9	99.5-100			66.7	100	64.9-100	100	[23] [41] [44] [45] [58]
Influenza B	Adults			25-37.6	99.9-100	33.3-40	97.6-99.5					40 -75	97.1-99	93.3-94.4	99.6-100	[23] [46] [47] [48] [49]
Influenza B	Both	100	99.2	36.8-81	100	48.3-50	92.4-98.3			33.3-86.3	99.3			91.4	100	[22] [51] [52] [53] [54] [55] [56]
RSV	Paediatric	69.4-93.5	99.6-99.7	79.4-80	98-98.4			86.5-91.3	100	58.8	100	98.5	97.8	84-100	97.7-100	[40] [44] [59] [58] [60] [61]
RSV	Adults	24	98.5	12	100							100	100			[59] [62]
RSV	Both			63.6-84	98.7-100	92.1	91.8			54.5-56.9	100	98.6	98	97.1-100	95.8-98.6	[51] [52] [54] [59] [63] [64]

Table 3: Sensitivity (Se) and specificity (Sp) of the different viral diagnostic tests for influenza A, influenza B and RSV in percentage. IF: immunofluorescence, LFC: lateral flow chromatography, PCR: polymerase chain reaction, RSV: respiratory syncytial virus

The sensitivity of a technique besides depending on the technique itself also depends on the circulating strains [65], on the studied population (children or adults) [66] [67] and the reference method to which it is compared. [68]

The following article compares several techniques (antigen detection tests, immunofluorescence, cell culture, microarray and PCR) for the diagnosis of respiratory viruses. It explains the pros and cons of each techniques and details various influencing factors. [51]

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Prospective evaluation of diagnostic tools for respiratory viruses in children and adults

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ABSTRACT

Aim: To compare the performances of molecular and non-molecular tests to diagnose respiratory viral infections and to evaluate the pros and contras of each technique.
Methods: Two hundred ninety-nine respiratory samples were prospectively explored using multiplex molecular techniques (FilmArray Respiratory Panel, Clart Pneumovir), immunological techniques (direct fluorescent assay, lateral flow chromatography) and cell cultures.
Findings: Molecular techniques permitted the recovery of up to 50% more respiratory pathogens in comparison to non-molecular methods. FilmArray detected at least 30% more pathogens than Clart Pneumovir which could be explained by the differences in their technical designs. The turnaround time under 2 hours for the FilmArray permitted delivery of results when patients were still in the emergency room.

1. Introduction

Since the discovery of viruses in the twentieth century, considerable efforts have been made to improve the technics to detect and identify them. Cell cultures were the first diagnostic tool to be used in the mid-1950s, and since then, new techniques have been developed to decrease the time to a result (immunofluorescence, lateral flow chromatography) or to boost the sensitivity (molecular techniques) (Levine, 1996; Ginocchio and Harris, 2011). Many improvements have been made, and techniques combining speed and sensitivity are currently available, such as fully automated 'sample-in, result-out' multiplexed syndromic molecular tools (Bluchan and Ledeboer, 2014). Aside from being effective in terms of sensitivity and specificity, these latter diagnostic tools are able to recover non-cultivable viruses. As a consequence, questions regarding the usefulness of 'older' diagnostic methods regularly arise (Leland and Ginocchio, 2007; Hodinka and Kaiser, 2013). Meanwhile, important questions concerning these 'new' expensive rapid molecular techniques remain unanswered, such as their cost-effectiveness in terms of patient's management, or the clinical significance of detecting nucleic acids of micro-organisms that could be non-infectious at the time the sample is collected.

The objective of this work was to compare the performances of antigen detection and cell cultures techniques routinely used since years for the diagnosis of respiratory viral infections in the setting of a tertiary care hospital to those of newer molecular techniques (Clart Pneumovir, Genomica, Coslada, Spain and FilmArray Respiratory Panel, Biofire, Biomérieux, Marcy L'Etoile, France).

2. Materials and methods

2.1. Population and inclusion criteria

The study was initiated on the 1st of February (week 5) and ended on the 15th of March 2016 (week 11) in the Saint-Pierre University Hospital, a tertiary general hospital with 626 beds located in downtown Brussels. This was during the peak of the 2015–2016 influenza season which was moderate in Belgium and lasted from week 4 to week 13. More than 90% of influenza A isolates collected in Belgium were

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A(H1N1)pdm2009. Regarding influenza B, circulating strains were almost exclusively from the Victoria lineage according to the Belgian Scientific Institute of Public Health (Belgian Public Health Institute, 2019). The enrolment period was chosen in order to be sure to gather positive samples for influenza as it is intended to evaluate, in another article, the impact of the results on antiviral prescription. This choice obviously affects the prevalence of other viruses. Adults and children attending the emergency room (ER) and presenting with upper or lower respiratory symptoms were prospectively included if either intended to be maintained in the hospital or had any of the following conditions known to expose to a higher rate of complications of viral respiratory infections: chronic respiratory diseases such as cystic fibrosis or asthma, sickle-cell disease, asplenia, neuromuscular diseases, severe neurological affections, hereditary metabolic disorders including diabetes, congenital or acquired immunosuppression, heart defects, chronic nephropathies, chronic liver diseases and pregnancy. Children under 3 months of age with a fever without focus of infection were also included.

2.2. Study workflow

Upon inclusion, a respiratory sample was collected. Nasopharyngeal aspirate (NPA) samples were typically collected from children under 2 years old, and nasopharyngeal swabs (NPS) (flocked swab + UTM 3 mL, Copan, Brescia, Italy) were collected from older children and adults. The samples were immediately sent to the microbiology laboratory for testing. Prior to testing, NPA were diluted with 3 mL of viral transport medium composed of veal infusion broth (Difco, Becton Dickinson, Sparks, MD, USA) supplemented with bovine albumin (Sigma Aldrich, St. Louis, MO, USA). Lateral flow chromatography (LFC) tests and the FilmArray Respiratory Panel were used to test samples 24/7, whereas direct fluorescent assays (DFA) and cell cultures were performed during working hours (8:00 am to 5:00 pm) from Monday to Saturday. The Clart Pneumovir test was performed once a week. Lab results as well as clinical data from patients' chart were recorded and analyzed. Antigen detection tests and cell cultures are routine tests performed for patients attending the emergency departments. FilmArray and Clart Pneumovir were performed for the study.

2.3. Antigen detection tests

Because only 3 tests per day are reimbursed by the social welfare, the combination of LFC and DFA tests performed varied during the evaluation based on the most prevalent circulating viruses. From February 1st to the 10th, influenza (Sofia influenza A + B, Quidel, San Diego, CA, USA), RSV (BinaxNOW RSV, Alere, Waltham, MA, USA) and human metapneumovirus (hMPV) DFA (Argene, Biomérieux, Marcy L'Etoile, France) tests were performed. Fifty-nine samples were analyzed with this combination. From February 10th to March 15th 2016, metapneumovirus detection was replaced by an adenovirus detection test (Adenorespi K-set, Coris Bioconcept, Gembloux, Belgium).

2.4. Cell cultures

Cell cultures were performed as follows: an aliquot of the sample was inoculated on confluent Vero (African green monkey kidney), MRC5 (human lung) and LLC-MK2 (rhesus monkey kidney) cell cultures (Vircell, Santa-Fé, Spain) in 24-well or 6-well tissue culture plates (Greiner-Bio One, Frickenhausen, Germany). Cultures were incubated at 36 °C in a 5% CO_2 atmosphere for 2 weeks for the Vero cultures plates and LLC-MK2 cells and 3 weeks for the MRC5 cells. The culture media were replaced weekly. Cultures were examined every two to three days using an inverted microscope. Hemadsorption was performed on the LLC-MK2 cells at the end of the second week of incubation.

2.5. Molecular techniques

The FilmArray Respiratory Panel 1.7 is a closed 'sample-in, result-out' multiplex PCR system that integrates sample preparation, amplification, detection and analysis of results in approximately an hour. The panel detects the most common respiratory viruses: adenovirus, coronavirus (229E, HKU1, NL63 and OC43), human metapneumovirus, human rhinovirus/enterovirus (without distinction between the two), influenza A (with differentiations of H1, H1-pdm2009 and H3 strains), influenza B, parainfluenza 1–4 and respiratory syncytial virus (RSV). The panel also detects 3 bacteria; *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae* and *Bordetella pertussis* which were not evaluated in this study. Tests were performed according to the manufacturer's instructions.

The Clart Pneumovir test is a microarray technique that targets the same pathogens as the FilmArray Respiratory Panel, with the exception of coronaviruses HKU1, NL63 and OC43 and the 3 aforementioned bacteria. Among enteroviruses, this technique only detects echoviruses, but it can differentiate them from rhinoviruses. The extraction of nucleic acids was carried out with the QiaSymphony system SP (Qiagen) using the QiaSymphony Virus/Bacteria Midi kit (input volume 1500 µL, output volume 110 µL). The Pneumovir assay was performed according to manufacturer's instructions, and detection and interpretation of the results were conducted by a CARreader (Genomica).

2.6. Establishment of "composite" reference standard

As the molecular tests used were presumably more sensitive than the reference standard (viral culture), we constructed a "composite" reference standard to avoid bias in establishing the specificity of the evaluated tests. This "composite" reference standard was constructed as follows: when discrepant results between the 2 molecular techniques were observed, a third molecular technique was performed (Argene, Biomérieux, Marcy L'Etoile, France). A sample was considered truly positive for a pathogen if at least 2 molecular techniques were positive for this pathogen. For the targets included in the FilmArray test but not in the Clart Pneumovir panel (coronaviruses HKU1, NL63 and OC43), only the specificity of the positive results were evaluated using the Argene or laboratory developed tests.

2.7. Statistical analyses

Data were analyzed using two sided exact chi-square Fisher's tests followed in case of statistical significance by chi-square trend tests. The statistical software used were Medcalc V14.12.0 and IBM SPSS V24.0.

3. Results

A total of 299 samples (93 NPA and 206 NPS) from 291 patients were analyzed: 149 samples were obtained from 142 children, of whom 62 were female and 80 were male (mean age: 1 years and 10 months old; median: 7 months old); and 150 samples were obtained from 149 adults, of whom 73 were female and 76 were male (mean age: 53 years old; median: 52). For children, 80% (119/149) of samples were positive for at least one pathogen. The pathogen detected, ranging from most to least, was rhino/enteroviruses (49), influenza B (32), influenza A (25), coronaviruses (22), adenovirus (18), metapneumovirus (10), RSV (7) and parainfluenza (2). For adults, 63% (94/150) of samples were positive, and the detected viruses were influenza A (32), influenza B (26), rhino/enteroviruses (13), coronavirus (12), adenovirus (8), metapneumovirus (6) and RSV (4). No parainfluenza viruses were detected in the adult patients. All influenza A isolates were A(H1N1)pdm09 with the exception of one A(H3N2) from one adult patient. Influenza B strains were not subtyped. Co-detection was more common in children than in adults (35.3% vs 8.5%; $p < 0.001$).

Table 1 details the sensitivity and specificity observed for the

Table 1
Sensitivity and specificity of the techniques for the different pathogens.

	n° positive (n° co-detection)	Antigen detection		Cell cultures		Clart Pneumovir		FilmArray	
		Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Adenovirus	26 (20)	2/22 (9.1%)	100%	4 (15.4%)	100%	13 (50%)	100%	24 (92.3%)	98.5%
Influenza A	56 (10)	43 (76.8%)	98.3%	49 (87.5%)	99.6%	55 (98.2%)	99.6%	54 (96.4%)	99.6%
Influenza B	58 (23)	28 (48.3%)	98.3%	43 (73.1%)	99.3%	56 (96.5%)	98.7%	53 (91.4%)	100%
Metapneumovirus	16 (4)	2/3 (66.6%)	100%	0 (0%)	100%	9 (56.2%)	98.9%	14 (87.5%)	100%
RSV	11 (3)	7 (63.6%)	100%	6 (54.5%)	100%	8 (72.7%)	98.6%	11 (100%)	98.6%
Parainfluenza	2 (2)	NA	NA	1 (50%)	100%	2 (100%)	99.7%	2 (100%)	98%
Rhino/enterovirus	62 (30)	NA	NA	16 (25.8%)	100%	32 (51.6%)	99.6%	61 (98.4%)	96.6%
Coronavirus 229E	1 (1)	NA	NA	NA	NA	1 (100%)	100%	1 (100%)	100%
Coronavirus HKU1	20 (10)	NA	NA	NA	NA	NA	NA	20 (NA)	98.7%
Coronavirus NL63	6 (1)	NA	NA	NA	NA	NA	NA	6 (NA)	99%
Coronavirus OC43	7 (1)	NA	NA	NA	NA	NA	NA	7 (NA)	98.7%

Table 2
Rate of false negative (FN), partial agreement (PA) and total agreement (TA) of the different techniques for the positive samples in comparison to the composite standard.

Non-molecular techniques			Clart Pneumovir			FilmArray		
FN	PA	TA	FN	PA	TA	FN	PA	TA
88	31	94	50	33	130	6	6	201
41.3%	14.5%	44.1%	23.5%	15.5%	61%	2.8%	2.8%	94.4%

different techniques, depending on the pathogens. Table 2 provides the rate of false negative results, partial agreement (meaning at least one but not all the expected pathogens were detected) and complete agreement (meaning all the expected pathogens were detected) of the non-molecular techniques and molecular techniques compared to the established standard. The FilmArray test produced fewer false negative results and partial results compared to non-molecular techniques and the Clart Pneumovir test ($p < 0.001$). False negative results with molecular techniques were significantly more frequent in samples with co-detections compared to those with only one pathogen: 12% vs 3% for the FilmArray test ($p = 0.034$) and 76% vs 11% for the Clart Pneumovir test ($p < 0.001$). Similar result was also observed for cell cultures when limiting the analysis to culturable viruses (100% Vs 37%; $p < 0.001$).

Table 3 reports the mean turnaround time (TAT) of the techniques from the reception of the sample in the laboratory to the introduction of the results into the laboratory information system. The LFC and FilmArray techniques have the shortest TAT, while cell cultures and the Clart Pneumovir test have the longest TAT. The TAT for negative cell cultures is about 3 weeks as they are discarded after this delay.

Tables 4 and 5 provide the detection rates for different techniques for influenza A and B, depending on the duration of the cough or the age of the patients. There is an effect of cough duration for influenza A with the antigen detection test ($p = 0.032$) and cell cultures ($p = 0.010$), due to a decrease in diagnostic sensitivity with cough duration ($p = 0.017$ and $p = 0.010$, respectively). The decrease is observed as soon as the cough duration exceeds 5 days. An age effect was also observed for influenza A with the antigen detection test ($p = 0.006$) and cell cultures ($p = 0.023$), due to a decrease in sensitivity with age ($p = 0.002$ and $p = 0.004$, respectively). The decrease is observed from the first age category with the antigen detection and from 15 years for the cell cultures. No effect of cough duration or age was observed with the detection of influenza A for the FilmArray and Clart Pneumovir tests or on the detection of influenza B with any method.

Table 4
Rate of positive influenza tests depending on the duration of the cough (when reported).

		≤ 1 day	> 1–3 days	> 3–5 days	> 5 days
Influenza A	Antigen detection	11/14	14/16	4/6	2/7
	Cell cultures	13/14	15/16	6/6	3/7
	Clart Pneumovir	14/14	16/16	6/6	6/7
	FilmArray	14/14	16/16	6/6	6/7
Influenza B	Antigen detection	5/15	6/13	4/6	1/3
	Cell cultures	11/15	9/13	6/6	2/3
	Clart Pneumovir	14/15	12/13	6/6	3/3
	FilmArray	14/15	13/13	6/6	3/3

4. Discussion

4.1. Analytical performance and factors influencing molecular tests

As expected, molecular techniques were observed to be the most sensitive.

However, the performances of the two evaluated techniques differed; lower sensitivity for the detection of several targets was observed for the Clart Pneumovir test, notably this technique only detects echoviruses among all enteroviruses. For adenoviruses, metapneumoviruses and RSV, the explanation probably relies more on technical considerations. Indeed, false negative results with the FilmArray and the Clart Pneumovir tests were not always for samples with a low viral

Table 3
Mean turnaround time of the different techniques from the reception of the sample in the laboratory to the introduction of the result in the laboratory information system. *Samples received during working hours and analyzed the same day. **Samples received outside working hours. d: day(s); h: hour(s); hMPV: human metapneumovirus; min: minute(s); RSV: respiratory syncytial virus.

	Antigen detection		Cell cultures						Clart Pneumovir	FilmArray
	Influenza A + B	hMPV	Influenza A	Influenza B	Adenovirus	Enterovirus	Rhinovirus	RSV		
Turnaround time	1h01min	3h22min* 18h17min**	4d22h41min	4d15h27min	5d13h14min	4d21h43min	6d22h01min	7d18h14min	4d16h46min	1h49min

Table 5
Rate of positive influenza tests depending on the age of the patients.

		≤ 2 years	> 2–15 years	> 15–50 years	> 50 years
Influenza A	Antigen detection	18/18	6/7	15/24	4/7
	Cell cultures	18/18	7/7	20/24	4/7
	Clart Pneumovir	18/18	7/7	24/24	6/7
	FilmArray	18/18	7/7	22/24	7/7
Influenza B	Antigen detection	8/19	8/13	9/16	3/10
	Cell cultures	12/19	11/13	13/16	7/10
	Clart Pneumovir	18/19	13/13	15/16	10/10
	FilmArray	17/19	13/13	16/16	8/10

load, as indicated by the cycle threshold (which correlates with the viral load) of the third molecular technique used to elucidate discrepant results between the first two techniques. As an example, the mean Ct value and standard deviation of negative and positive samples for adenoviruses with Clart Pneumovir were 32.7 (1.6) and 31.8 (2.2) respectively ($p = 0.402$). The choice of primers and probes used to design tests can determine whether a certain strain of virus will be detected. Although the FilmArray test had a better sensitivity than the Clart Pneumovir test for the detection of adenoviruses, it may lack sensitivity in detecting certain strains, which could impact the management of immunocompromised patients (Song et al., 2016).

A huge advantage of molecular techniques is that they allow the recovery of those pathogens that are difficult to culture, that will not grow in culture or for which no antigen detection tests are available. In the present study, the rate of complete detection of the pathogens present in the samples was far more important with molecular techniques compared to non-molecular ones. This has already been reported. (Weinberg et al., 2004) Viruses with poor detection using non-molecular techniques primarily include adenoviruses, metapneumoviruses and rhino/enteroviruses. There were also positive samples for coronaviruses (Mahony, 2008) which, in our experience, do not commonly grow with routine cell lines, even if coronavirus NL63 was originally described in LLC-MK2 (van der Hoek et al., 2004).

The rate of co-detection was more important for children, which is a common finding and could be explained by notably increased, longer-lasting viral shedding in children under 3 months of age due to less developed mucosal immunity (Sharma et al., 2012). The co-detection of viruses in children is apparently not linked with a more severe outcome (Comerlato Scotta et al., 2016), and the molecular detection of a virus in a respiratory sample is not always associated with symptoms. The type of detected virus and the age of the patient can be helpful to decide on a care plan (Self et al., 2016).

As previously stated, false negative results with molecular techniques were significantly more frequent in samples with multiple pathogens compared to those with only one pathogen; this finding could be due to possible competition for the reagents when multiple targets are to be detected (Bezerra et al., 2011).

The specificity of all molecular techniques was over 98%, except that for rhino/enteroviruses with the FilmArray test (96.6%). The differences observed between molecular techniques could also be due to primer and probe choices.

Overall, the Clart Pneumovir is less sensitive than the FilmArray except for influenza B and its hands-on time and turnaround time are longer.

4.2. Analytical performances and influencing factors of non-molecular techniques

Only 3 positive samples were analyzed for metapneumovirus using an antigen detection test, as it was substituted for adenovirus test during the evaluation. This test nonetheless appeared more sensitive than the cell culture tests, as no metapneumovirus was recovered from cell cultures. This finding was under the expected sensitivity, which is approximately 50% according to the literature (Tang and Crowe, 2011).

The apparent low sensitivity of non-molecular techniques observed for adenoviruses deserves comment. Indeed, prolonged shedding after the primary infection is classically described for adenovirus. Adenoviruses can also be detected in tonsillar tissue or isolated from a throat sample of up to 11% of healthy children (Song et al., 2016; Kalu et al., 2010). These states of prolonged shedding or "latency" are more likely to be detected with molecular techniques. It is also noteworthy that 85% of adenoviruses detected in this evaluation were associated with one or more other pathogens, supporting the hypothesis that they could be bystanders in some cases.

The sensitivity of cell cultures for rhino/enterovirus was also low and could be partially attributed to the fact that group C rhinoviruses do not grow on standard cell cultures (Jacobs et al., 2013). Enteroviruses grow inconsistently on cell cultures, depending on their type, and no cell line enables the detection of all strains (Stellrecht et al., 2011). Unfortunately, rhinoviruses and enteroviruses in this study were not typed.

The presence of multiple viruses in a sample also influences cultures because the growth of the fitter or more abundant virus can mask the growth of others (George et al., 2002). For example, on the 46 false negative cultures for rhino/enteroviruses, 16 were positive for another virus. Likewise, for adenoviruses, for the 22 false negative cell cultures, 8 recovered another virus. Molecular techniques supposedly do not suffer from this drawback, although as previously discussed, a higher rate of false negatives was observed when multiple pathogens were present in the sample.

Cell cultures, however, can enable the detection of unsuspected viruses. This detection was the case in this evaluation for one measles virus, which was not originally suspected, and 2 herpes simplex viruses and 5 cytomegaloviruses (CMV). In one case, CMV could explain the symptoms exhibited by a 3-month-old patient with a fever without focus of infection. The other cases were more likely to be recurrences or prolonged shedding.

Regarding the sensitivity of antigen detection tests for influenza, influenza A is more easily detected than influenza B, which has already been reported (Busson et al., 2014). The decreased sensitivity for influenza A with antigen detection and cell cultures, which depends on the age of patients, can be explained by the higher viral shedding in children, and also because for younger children, NPA were preferred to NPS. Aspirates usually increase the detection rate for viruses over swabs (Loens et al., 2009). The decreased sensitivity of antigen detection and cell cultures when the duration of the cough increases is expected, because viral shedding is more important in the first days of the disease (Aoki and Boivin, 2009). The reason for the lack of similar findings for influenza B is unclear. The absence of sensitivity loss for molecular techniques, depending on the age of the patients and the duration of the cough, can be attributed to the high sensitivity of the techniques.

Usually, antigen detection tests have a faster turnaround time than cell cultures but a lower sensitivity except for viruses growing poorly, especially metapneumovirus and RSV, for which the sensitivity of antigen detection tests can be better than the one of cell cultures.

The specificity of non-molecular tests was above 98%. False positive

results in cell cultures can occur due to cross-contamination between wells on culture plates.

4.3. Turnaround time

Antigen detection tests based on lateral flow chromatography are usually the easiest and fastest to perform. Techniques based on immunofluorescence are slightly more time-consuming. Currently, totally automated techniques with a short hands-on time, such as FilmArray, can be realized in a time frame comparable to that of LFC tests. However, only one can be performed at one time, and the analyzer remains occupied for slightly more than an hour. The number of required instruments is based on the test volume at each facility and the desired TAT. Cell cultures remain the most time-consuming techniques, and results often arrive too late to impact patient management (Ginocchio, 2007). This was also the case for Clart Pneumovir in our setting, as it was only performed once a week due to the complexity of the corresponding analytical process.

4.4. Cost

In Belgium, non-molecular techniques for the diagnosis of respiratory viruses are reimbursed by the social welfare program, which is not the case for molecular techniques which are charged to the patients. During this evaluation, molecular techniques were free of charge for patients, but they cost approximately 135 euros per patient. The necessity of molecular tests must be seriously considered before prescription. These tests should probably be reserved for the most severely ill patients, for whom rapid and comprehensive microbiological evaluation is most likely to impact patient management. As a comparison, antigen detection tests cost, including workforce, can range between 5.5 and 14 euros depending on the test and cell cultures cost about 15 euros. However, non-molecular tests are reimbursed by the social welfare in Belgium permitting a broader prescription. The possibilities to implement molecular tests can vary between facilities and countries depending on local policies. Using molecular rather than non-molecular techniques could impact patients' isolation strategies (Richardson et al., 2016), antibiotic/antiviral use or reduce of the length of stay (Brendish et al., 2017; Rogers et al., 2015), but cost-benefit analyses are difficult to appraise because of many intertwined elements.

5. Conclusions

Molecular techniques have considerably increased our capacity to detect respiratory viruses in a timely manner. The main factors limiting a wide utilization of these techniques are cost and the difficulty to absorb the workload in large facilities with numerous samples to analyze per day. Another drawback is that latent viruses or traces of genetic material may be detected. The interpretation of a positive result might be difficult, and it must be carefully correlated to the clinical history of the patient as the presence of a virus in the respiratory tract is a factor exposing to bacterial superinfection (Vareille et al., 2011). It is possible to quantify the viral load in a respiratory sample, but there are conflicting reports regarding correlations between viral load and outcome (Granados et al., 2017; Wishaupt et al., 2017). Technical improvements should be made to prevent variation in quantification caused by sample dilution with saline instilled during aspirates or by the variable quantity of sampled material with swabs. These improvements could render comparisons between studies more reliable and permit the establishment of a threshold, above which detected viruses are indeed involved in an ongoing infectious process. In addition, a positive antigen detection test usually correlates with a high viral load in a sample, and a positive cell culture can only be achieved with infective viral particles. Whatever technique is used, fully understanding the benefits and limitations of each is crucial for interpretation. The choice of technique should depend, besides financial considerations, on the necessary sensitivity and speed based on symptoms, comorbidity and risk of a detrimental outcome for each patient. A tertiary care hospital should be able to offer diagnostic tools suitable for each individual case; if testing all patients with molecular techniques is not possible, the use of non-molecular techniques is preferable over nothing at all.

Conflict of interests

None declared.

Ethical approval

This study was approved by the ethics committee of University Hospital Saint Pierre (Brussels).

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4 Clinical input of NAAT for viral diagnostic

As described in the aforementioned article, molecular techniques can provide a sensitive and exhaustive result in a timely manner. However, the cost-effectiveness of such techniques has not been clearly proven yet.

Many studies have been led over the last decade in order to address this issue, but they differ in terms of design (included populations, control groups, gold standard for the tests), making the comparison of findings complicated. Moreover, the results of the molecular technique has to be provided as early as possible in the course of patients' management to impact antibiotics and antiviral prescription, realization of ancillary tests, admission and isolation [23] [69] [70] which is not the case for many of these studies.

Table 4 gathers results from recent studies over the impact of multiplex PCR techniques on the management of patients. Articles published from 2008 to 2019 were selected using the following entries "respiratory virus", "PCR", "management", "impact" from Pubmed. [69] [70] [71] [72] [73] [74] [75] [76] [77] [78] [79] [80] [81] [82]

The article from Britain-Long et al compares antibiotics prescription in a group of adult patients receiving a result of homemade molecular panel in less than 24 hours to the one receiving the results after 24 hours to 12 days. [71] The group with early result had less antibiotics prescription however there was not difference between the two groups on the follow-up visit.

Hernes et al [72] evaluated antibiotics prescription and length of stay in hospital between three groups of adult patients; symptomatic patients with positive respiratory virus PCR, symptomatic patients with negative PCR and non-symptomatic patients with negative PCR. They did not observe a difference in antibiotics prescription neither for length of stay between groups of symptomatic patients.

Schulert et al [73] compared length of stay, duration of antibiotic treatment and ancillary tests prescription in two groups of children having a multiplex PCR viral respiratory panel performed within 24 hours of admission. They observed a shorter antibiotics duration and a shorter length of stay in the group with a positive PCR in comparison to the group with a negative PCR.

Several studies evaluated the use of non-molecular techniques in comparison to multiplex molecular techniques on consecutive winter seasons. Subramony et al found shorter antibiotics duration for children tested with multiplex PCR whatever the result and also whatever the technique used if there was a positive result. There also were less chest radiographs in the first day for the group tested with multiplex PCR but more chest radiographs whatever the technique if there was a positive result. Patients tested with multiplex PCR stayed longer in isolation. [74] Rappo et al. reported a lower and shorter antimicrobial use, less chest radiographs and a shorter length of stay for adult patients tested with multiplex PCR only if it was positive for influenza. [70] Echevarria et al observed a decrease antibiotics use for children, a decrease in antiviral prescription in adults and a decrease in ancillary tests prescription for children when tested with multiplex PCR. [81]

All the studies having antiviral prescription as outcome reported a faster and more appropriate prescription of oseltamivir when a rapid multiplex PCR is used. [69] [78] [82]

The article following table 4, and also included in it, reports our contribution in order to try to determine the cost-effectiveness of a rapid molecular assay detecting respiratory viruses in the

management of paediatric and adult patients visiting the emergency department during the 2015-2016 influenza epidemics. For this article, we chose to test all patients with the same standard of care (FilmArray Respiratory Panel) as molecular techniques already demonstrated better performances in terms of sensitivity and versatility in comparison to non-molecular techniques. In our opinion, it would not have been ethical to test the included critical patients with suboptimal techniques. We afterward took several parameters into consideration, including test results, in order to determine which of them were involved in the different outcomes evaluated. [82]

Authors	Years	Period	Country	Population	N	Type	Inclusion	Outcome
Brittain-Long et al. 2011	2006-2009	Oct 2006 to Apr 2009	Sweden	Adults ≥18 year-old attending 12 outpatients units	406	Prospective	Acute respiratory tract infection	Antibiotic prescription at initial visit and total antibiotic prescription during the study period
Hernes et al. 2014	2008-2009	Feb 2008 to Feb 2009	Norway	Adults ≥60 year-old hospitalized in the department of internal medecine	203	Prospective	Symptoms of ongoing respiratory tract infection or no symptoms for control group	Antibiotic prescription and length of stay
Schulert et al. 2013	2009-2010	Aug 2009 to Dec 2010	USA (TN)	Children	717	Retrospective	Patient with multiplex PCR-based respiratory viral panel ordered within 24h of admission	Length of stay, duration of antibiotics, ancillary tests prescription
Subramony et al. 2016	2010-2014	June 2010 to June 2012 and Oct 2012 to May 2014	USA (NY)	Children < 18 year-old	4779	Retrospective	Patients < 18 yo tested for respiratory pathogens in the ER before admission	Duration of antibiotics use, chest radiograph use, admission, time in isolation
Rappo et al. 2016	2010-2012	2 consecutive winters	USA (NY)	Adults ≥18 year-old	337	Retrospective	Having a positive respiratory specimen received in the Microbiology laboratory within 48h of hospital arrival	Admission, turnaround time, LOS, duration of antimicrobial use, time to oseltamivir, chest radiographs
Rogers et al. 2015	2011-2013	Nov 2011 to Jan 2012 and Nov 2012 to Jan 2013	USA (GA)	Children ≥3 months to 21 year-old	1136	Retrospective	Children admitted with acute respiratory illness	Time to result, LOS in ER, LOS in the hospital, antibiotics prescription, duration of antibiotics use, time in isolation
Xu et al. 2013	2011-2012	Dec 2011 to Apr 2012	USA (WA)	Children up to 21 year-old	3936	Prospective	Patients who underwent respiratory viral testing	Turnaround time, antiviral prescription
Semret et al. 2017	2012-2015	3 consecutive winters	Canada	Adults	800	Prospective	Acute respiratory tract infection, exacerbation of COPD or asthma, unexplained sepsis or ILI and hospitalized since more than 24h	Antibiotics and antiviral prescription
Mayer et al. 2017	2012-2014	Sept 2012 to Nov 2014	Switzerland	Children and adults	254	Retrospective	All children and adults whose respiratory samples were tested for respiratory viruses with a 16-plex rtPCR test	Antibiotics prescription, LOS
Green et al. 2016	2014-2015	Dec 2014 to April 2015	USA (CT)	Adults	408 (295 outpatients)	Retrospective	Patients sampled with a posterior nasopharyngeal swab in ER, outpatients clinics or urgent care clinics.	Antibiotics and antiviral prescription for outpatients, admission
Andrews et al. 2017	2015	January to July 2015	UK	Adult patients (≥16 year old) in ER	545	Prospective	URTI, ILI +/- LRTI	Length of stay, antimicrobials use, readmissions, all-cause mortality, length of ward stay, turnaround time
Keske et al. 2018	2015-2016	Jan 2015 to Dec 2016	Turkey	Children and adults	1317	Retrospective	Children and adults with ILI (WHO definition)	Inappropriate antibiotics use
Echavarria et al. 2018	2016-2017	April-Nov 2016 and April-Oct 2017	Argentina	Children and adults	432	Prospective	Children and adults with acute lower respiratory tract infection attending ER (exclusion of patients with congenital cardiac disease, neurological or genetic disorder, cancer, HIV, immunosuppression or solid organ or hematopoietic stem cell transplantation)	Antibiotic and antiviral prescription, ancillary tests, admission rate, LOS
Busson et al. 2019	2016	Feb-March 2016	Belgium	Children and adults	299	Prospective	Adults and children with respiratory symptoms attending the ER and either hospitalized or with a comorbidity or condition exposing to respiratory complications. Children < 3month-old with fever without focus.	Antibiotic and antiviral prescription, ancillary tests, admission rate, LOS, isolation

Table 4 (part 1): Selection of articles over the impact of multiplex molecular tests for respiratory viruses on the management of patients (AB: antibiotic, CoV: coronavirus, DFA: direct fluorescent assay, ER: emergency room, IFA: immunofluorescent assay, ILI: influenza-like illness, IQR: interquartile range, LOS: length of stay, LRTI: lower respiratory tract infection, PCR: polymerase chain reaction, SD: standard deviation, TAT: turnaround time, URTI: upper respiratory tract infection, vs: versus)

Control	Viruses	Standard of care	TAT	AB use	AB duration	Antiviral	Ancillary tests	Admission	Length of stay	Isolation	Mortality
Early (one day) vs delayed (8 to 12 days) result	Respiratory panel (except CoV HKu1)	Homemade PCR respiratory panel	1 day to 12 days	Less prescription on the initial visit. Same rate on the follow-up visit.	Not evaluated	Not evaluated	Not evaluated	Not evaluated	Not evaluated	Not evaluated	Not evaluated
Symptomatic with positive PCR vs symptomatic with negative PCR vs non-symptomatic with negative PCR	Influenza, RSV, hMPV, parainfluenza, adenovirus	Homemade PCR respiratory panel	24-48h	No difference	Not evaluated	Not evaluated	Not evaluated	Not evaluated	No difference between symptomatic groups	Not evaluated	Not evaluated
Positive respiratory panel vs negative respiratory panel	Respiratory panel (except adenoviruses and including bocaviruses)	Multiplex PCR-based viral respiratory panel	Not reported	Not evaluated	Shorter for patient with positive respiratory panel (depending on admission service)	Not evaluated	No difference	Not evaluated	Shorter LOS if positive respiratory panel (depending on underlying diseases)	Not evaluated	Not evaluated
Non-PCR methods vs multiplex PCR (MPCR)	Respiratory panel	Non-MPCR then MPCR	Not reported	Not evaluated	Shorter antiotics use in MPCR group and in both groups if positive result	Not evaluated	Less chest radiographs in the first 2 days in MPCR group. More chest radiographs in both groups if positive result	Not evaluated	Not evaluated	More days in isolation for MPCR group	Not evaluated
Conventional methods in season 1 vs FilmArray RP in season 2	Respiratory panel (except CoV HKU1, OC43 et 229E)	Rapid antigen testing, Prodess ProFlu+ PCR, Luminex PCR, DFA, viral culture, FilmArray	For influenza: 7,7h in season 1 (rapid antigen) vs 1,5h in season 2 (FilmArray)	Lower antimicrobial use for influenza positive with FilmArray	Shorter duration for influenza positive with FilmArray	No effect on the rate of prescription. Diminution of time to first dose in comparison to false negative with Ag detection tests	Less chest radiographs for influenza positive with FilmArray	Trend toward lower rate of admission for influenza with FilmArray when tested in ER	Lower LOS for influenza positive with FilmArray	Not evaluated	Not evaluated
Group pre-RRP vs RRP (rapid rspiratory panel) group	Respiratory panel (except CoV HKU1, OC43 et 229E)	Conventionnal PCR vs Rapid respiratory panel	Pre-RRP: 18,6h (SD: 8,2) RRP: 6,4h (SD: 4,9) 51,6% of results received in ER for RRP group vs 13,4% in pre-RRP group	No difference	Shorter in RRP group, especially if time to result <4h	Not evaluated	Not evaluated	Not evaluated	Shorter LOS in the ER	Shorter in RRP group when result is positive	Not evaluated
DFA group vs FilmArray group	Respiratory panel except CoV OC43	DFA on NPA vs FilmArray on midturbinate nasal swab	7h for DFA and 1,6h for FilmArray	Not evaluated	Not evaluated	81% of positive patients received oseltamivir in a timely manner	Not evaluated	Not evaluated	Not evaluated	Easier cohorting	Not evaluated
Influenza positive vs other than influenza virus vs virus negative	Respiratory panel (except CoV HKu1 and NL63)	Homemade PCR respiratory panel	6-24h	No effect	Not evaluated	Trigger oseltamivir if influenza positive	Not evaluated	Not evaluated	Not evaluated	Not evaluated	Not evaluated
Retrospective evaluation of appropriate antibiotics prescription depending on the diagnosis: viral respiratory infection, bacterial respiratory infection, mixed respiratory infection and patients with no detected pathogen	Respiratory panel (except CoV HKU1)	Anyplex II RV16	Within 24h	Children were more frequently correctly managed without antibiotics after virus detection than adults	Not evaluated	Not evaluated	Not evaluated	Not evaluated	Longer LOS for patients with bacterial respiratory infection vs patients with viral infection	Not evaluated	Not evaluated
Group positive for influenza vs group positive for a virus other thant influenza vs group with negative multiplex PCR	Respiratory panel	FilmArray	2,0h	Less antibiotics prescription when influenza positive	Not evaluated	More antiviral prescription when influenza positive	Not evaluated	Admitted patients had less often a positive PCR but were older	Not evaluated	Not evaluated	Not evaluated
FilmArray RP vs routine PCR	Respiratory panel	FilmArray RP for intervention arm, routine PCR for control arm	19h (IQR: 8,1-31,7)	No difference	Not evaluated	Time to first dose shorter in intervention arm for influenza positive patients	Not evaluated	No effect on readmission	No effect on LOS and length of ward stay	Not evaluated	No effect
Patients tested with FilmArray in 2015 vs those tested in 2016 after training in antimicrobial stewardship	Respiratory panel	FilmArray	"A few hours"	Decrease of inappropriate antibiotics use for children	Decrease in antibiotics duration for children and adults	Not evaluated	Not evaluated	Not evaluated	Not evaluated	Not evaluated	Not evaluated
IFA vs FilmArray group	Respiratory panel	Randomized blind trial (IFA or FilmArray)	26h40min for IFA and 1,9h for FilmArray	Decrease antibiotics prescription for adults and children	Not evaluated	Decrease antiviral prescription for adults	Decrease ancillary test prescription for children	No difference	No difference	Not evaluated	Not evaluated
Hospitalized patients vs non-hospitalized patients / Patients with antibiotics vs patients without antibiotics	Respiratory panel	FilmArray	1,8h	No difference between patients with a positive result and patients with a negative result	Not evaluated	More appropriate prescription	No reduction	Less admission for children positive for influenza B	No effect on hospitalization LOS. Possible shorter LOS in ward for children.	Easier cohorting	Not evaluated

Table 4 (part 2): A green box in the turnaround time (TAT) column means the result of the molecular test was provided in 2 hours or less. For the outcomes, a green box means the author found a positive effect of the molecular technique on this outcome, a red box means there was no effect and an orange box means the finding is uncertain. For the column 'viruses', respiratory panel refers to the following viruses: adenovirus, coronaviruses (229E, HKU1, NL63, OC43), enterovirus, influenza virus A & B, metapneumovirus, parainfluenza virus 1-4, respiratory syncytial virus, and rhinovirus.

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Contribution of the FilmArray Respiratory Panel in the management of adult and pediatric patients attending the emergency room during 2015–2016 influenza epidemics: An interventional study

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ABSTRACT

Aim: To evaluate the contribution of a multiplex PCR for respiratory viruses on antibiotic and antiviral prescription, ancillary test prescription, admission and length of stay of patients.
Methods: Two hundred ninety-one adult and pediatric patients visiting the emergency department during the 2015–2016 influenza epidemic were prospectively included and immediately tested 24/7 using the FilmArray Respiratory Panel. The results were communicated to the practitioner in charge as soon as they became available. Clinical and biological data were gathered and analyzed.
Findings: Results from the FilmArray Respiratory Panel do not appear to impact admission or antibiotic prescription, with the exception of a lower admission rate for children who tested positive for influenza B. Parameters that account for the clinical decisions evaluated are CRP level, white blood cell count, suspected or proven bacterial infection and, for adult patients only, signs of respiratory distress. Length of stay is also not significantly different between patients with a positive and a negative result. A rapid influenza test result permits a more appropriate prescription of oseltamivir.

Introduction

Multiplex molecular techniques for respiratory virus detection have already shown benefits in terms of sensitivity gained and a greater range of detected pathogens in comparison to conventional techniques. Recent progress has made it possible to shorten turnaround time (TAT) and to allow delivery of results in a timely manner, especially in comparison to cell culture and direct fluorescence assays (DFA) (Hodinka and Kaiser, 2013; Xu et al., 2013; Zumla et al., 2014). However, molecular techniques have not clearly shown cost-effectiveness. Studies report conflicting results, possibly due to differences in study design. What seems to be agreed upon is that results of molecular tests should be delivered rapidly in the course of patient management. The availability of results in the emergency room (ER) would most likely help avoid antibiotic use and ancillary test prescription, improve antiviral prescription and shorten length of stay in the ward by facilitating discharge of patients or cohorting of hospitalized patients, namely for influenza viruses (Xu et al., 2013; Rappo et al., 2016; Busson et al., 2017). Selecting the population for which the test should be applied also seems of great importance to increase cost-effectiveness (Boeckh, 2008; Vallières and Renaud, 2013). To shed some light on this important issue, we report the results of a prospective interventional study including selected adult and pediatric patients visiting the emergency departments of the tertiary care

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hospital Saint-Pierre in Brussels during the 2015–2016 influenza epidemic. All patients were immediately tested 24/7 with the same standard of care, the FilmArray (FA) Respiratory Panel (bioMérieux, Marcy L'Etoile, France). The evaluated outcomes were antibiotic and antiviral prescriptions, admission, length of stay of hospitalized patients, prescription of ancillary tests and patient isolation.

Materials and methods

Population and inclusion criteria

The study took place from the 1st of February (week 5) to the 15th of March (week 11) 2016 in Saint-Pierre University Hospital, a 626-bed tertiary care hospital in Brussels. The 2015–2016 influenza season in Belgium was moderate and lasted from week 4 to week 13. More than 90% of influenza A isolates collected in Belgium were A(H1N1)pdm2009. Regarding influenza B, circulating strains were almost exclusively from the Victoria lineage, according to the Belgian Public Health Institute (2016). Adults and children visiting the emergency room and presenting with upper or lower respiratory symptoms were prospectively included if either they were expected to be hospitalized or if they had any of the following conditions: chronic respiratory diseases (such as cystic fibrosis, asthma or chronic obstructive pulmonary disease), sickle-cell disease, asplenia, neuromuscular diseases, severe neurological impairments, hereditary metabolic disorders including diabetes, congenital or acquired immunosuppression, heart defects, chronic nephropathies, chronic liver diseases and pregnancy. Children under 3 months of age with a fever without focus (FWF) were also included.

Study workflow

Upon inclusion, patients had a respiratory sample collected, usually nasopharyngeal aspirates (NPA) for children <2 years and nasopharyngeal swabs (NPS) (flocked swab + UTM 3 mL, Copan, Brescia, Italy) for older children and adults. The samples were sent to the microbiology laboratory accompanied by a form on which the practitioner noted his intention concerning the management of the patient (hospitalization, isolation, prescription of antibiotics, antiviral treatment and ancillary tests). Samples were immediately analyzed with the FilmArray Respiratory Panel 24/7. The results were communicated as soon as they were available to the practitioner in charge of the patient. Data were collected concerning the changes in the management of the patient for the parameters noted on the form previously sent to the lab with the sample. Other parameters were collected from patients' files.

FilmArray Respiratory Panel

FilmArray Respiratory Panel v1.7 is a fully automated multiplexed PCR technique with short hands-on time (<5 min). It detects 14 viral targets: adenovirus, coronaviruses (OC43, NL63, 229E, HKU1), influenza A (with distinction between H1, H1-pdm2009 and H3), influenza B, human metapneumovirus, parainfluenza 1–4, human rhinovirus/enterovirus (without distinction between the two), respiratory syncytial virus (RSV), and 3 bacterial targets; *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae* and *Bordetella pertussis*. Tests are performed, one at a time, on an analyzer (FilmArray 2.0 system) in approximately one hour. Before testing, NPA were diluted with 3 mL viral transport medium composed of veal infusion broth (Difco, Becton Dickinson, Sparks, MD, USA) supplemented with bovine albumin (Sigma-Aldrich, St. Louis, MO, USA). Testing was performed using 300 μL of diluted sample.

Statistical analysis

Continuous variables were compared between groups with Student's t-test and nominal variables with Pearson's chi-squared test. Correction for age was performed using binary logistic regression where age was significantly different between compared groups. The software used were IBM-SPSS v24.0 and NCSS v10. For adults, statistical analyses for viruses were only performed on the 3 most prevalent viruses, namely, influenza A, influenza B and rhino/enteroviruses. The p-Value was considered significant if <0.05.

Results

A total of 299 samples from 291 patients were analyzed; 149 samples were obtained from 142 children (<15 years old), 62 females and 80 males (mean age: 1.8 years old; median age: 7 months), and 150 samples were obtained from 149 adults (≥15 years old), 73 females and 76 males (mean age: 53 years old; median age: 52.1). The characteristics of the population are detailed in Table 1. Samples were composed of 93 NPA and 206 NPS. Detected pathogens are listed in Table 2. One hundred and twenty-five out of the 149 samples from children (83.9%) and 85 out of the 150 samples from adults (56.7%) were positive according to FA. Mean turnaround time for delivering the result was 1.8 h.

Results for the pediatric population

Hospitalization

Of the 149 visits to the pediatric ER, 45 were discharged. Hospitalized children (104) were significantly younger than nonhospitalized children (1.4 vs 2.7 years; p = 0.019). Due to a significant difference in median age in the different groups, a correction for age was performed using binary logistic regression in order to mitigate the influence of age, if any, on the calculations. Results of the statistical analyses are in Table 3. Significant p-Values are noted in bold characters. Results at the limit of significance are underlined.

To focus on the most critical population, patients who were not hospitalized after examination and for whom no intention of hospitalization was reported on the form attached to the sample were excluded from the analysis. The remaining subset (n = 125) was split up into patients effectively hospitalized (n = 104) and those discharged from ER after examination (n = 21). The above-mentioned parameters were then compared between the two groups to determine which could have been utilized in the decision to discharge or admit. The mean age was significantly lower for hospitalized patients (1.4 vs 2.8 years; p = 0.032); a correction for age was performed (Table 3).

Table 1
Characteristics of enrolled patients. Mths = months, NA = not applicable, y = years.

	Children	Adults
Age (mean/median)	1.8 y/7 mths	53 y/52.1 y
Gender (male/female)	80 (56.3%)/62 (43.7%)	76 (51%)/73 (49%)
Number of samples/patients	149/142	150/149
Chronic respiratory disease	14 (9.9%)	60 (40.3%)
Heart defect	3 (2.1%)	39 (26.2%)
Sickle-cell disease	6 (4.2%)	3 (2%)
Neuromuscular disease/severe neurological affection	10 (7%)	9 (6%)
Immunosuppression	2 (1.4%)	22 (14.8%)
Chronic nephropathy	5 (3.5%)	0 (0%)
Diabetes	0 (0%)	31 (20.8%)
Pregnancy	0 (0%)	11 (7.4%)
<3 months old	55 (38.7%)	NA

Table 2
Detected pathogens; total number of positive samples and number of samples with co-detection. RSV = respiratory syncytial virus.

	Children		Adults		Total	
	Detected	Co-detected	Detected	Co-detected	Detected	Co-detected
Rhino/enterovirus	56 (28.4%)	33/56 (58.9%)	13 (13.1%)	5/13 (38.5%)	69 (23.3%)	38/69 (55.1%)
Influenza A	25 (12.7%)	12/25 (48%)	30 (30.3%)	0/30 (0%)	55 (18.6%)	12/55 (21.8%)
Influenza B	30 (15.2%)	16/30 (53.3%)	23 (23.2%)	6/23 (26.1%)	53 (18%)	22/53 (41.5%)
Adenovirus	21 (10.7%)	17/21 (80.9%)	6 (6.1%)	3/6 (50%)	27 (9.1%)	20/27 (74.1%)
RSV	10 (5.1%)	8/10 (80%)	5 (5%)	0/5 (0%)	15 (5.1%)	8/15 (53.3%)
Metapneumovirus	10 (5.1%)	4/10 (40%)	5 (5%)	0/5 (0%)	15 (5.1%)	4/15 (26.7%)
Coronavirus HKU1	17 (8.6%)	15/17 (88.2%)	7 (7.1%)	0/7 (0%)	24 (8.1%)	15/24 (62.5)
Coronavirus OC43	6 (3%)	3/6 (50%)	5 (5%)	1/5 (20%)	11 (3.7%)	4/11 (36.4%)
Coronavirus NL63	8 (4.1%)	4/8 (50%)	1 (1%)	0/1 (0%)	9 (3%)	4/9 (44.4%)
Coronavirus 229E	0	0	1 (1%)	0/1 (0%)	1 (0.3%)	0/1 (0%)
Parainfluenza	7 (3.6%)	6/7 (85.7%)	1 (1%)	1/1 (100%)	8 (2.7%)	7/8 (87.5%)
M. pneumoniae	3 (1.5%)	2/3 (66.7%)	1 (1%)	0/1 (0%)	4 (1.3%)	2/4 (50%)
C. pneumoniae	4 (2%)	4/4 (100%)	1 (1%)	0/1 (0%)	5 (1.7%)	4/5 (80%)
B. pertussis	0	0	0	0	0	0
Total	197	124/197 (62.9%)	99	16/99 (16.2%)	296	140/296 (47.3%)

When data were available, length of stay of hospitalized children was compared between the group of patients with a positive FA result (n = 82) and the group with a negative result (n = 19). The hypothesis was that having a positive result early in the course of patient management could shorten length of stay. The mean age of the two groups was not significantly different (1 year for the group with a positive result vs 1.8 years for the group with a negative result; p = 0.323). The mean length of stay was not significantly different between the two groups (3.9 days for the group with a positive FA result vs 5.2 days for the group with a negative FA result; p = 0.286).

Antibiotic prescription

The same above-mentioned parameters were compared between groups of children receiving antibiotics (n = 72) and those not receiving antibiotics (n = 77).

Patients for whom there was no intention of antibiotic prescription and who did not receive antibiotics after availability of test results were excluded. The remaining subset was separated into two groups depending on whether the patients indeed received antibiotics (n = 66) or not (n = 13). The same parameters as above were compared between the two groups (Table 3).

Patient isolation

The patient isolation policy regarding children implies that every child with a suspected or confirmed infectious disease, respiratory or not, should be isolated. As all included children were suspected of having an infectious disease, a positive FA result did not change the decision to isolate patients. Moreover, isolation was not avoided based on negative results as a negative FA result does not rule out every infectious cause. On the 24 children with a negative FA result, 12 patients had a fever of undetermined origin, 2 patients had a urinary tract infection, one patient had measles, one patient had scarlet fever, one patient had rotavirus infection, one patient had a bacterial bronchopneumonia, one patient had a cutaneous infection and 5 patients had a diagnosis of a non-infectious disease. Some of these conditions require isolation and it is usually maintained until confirmation is obtained that it is no longer necessary.

Results for adults

Hospitalization

When comparing the groups of hospitalized patients (n = 93) and discharged patients (n = 57), mean age was significantly higher for hospitalized patients. A correction for age was performed and statistical analyses results are in Table 4. Significant p-Values are in bold characters and p-Values at the limit of significance are underlined.

Again, the subset of patients for whom hospitalization was not intended and not instituted after test results were received was excluded to focus on the most critical population. The remaining subset (n = 106) was then split up into the group of patients who were indeed hospitalized (n = 93) and those who were discharged from the ER (n = 13). The groups were significantly different in age, as hospitalized patients were older (60.4 vs 36.8 years; p < 0.001) and a correction for age was performed for statistical analyses (Table 4).

When data were available, length of stay of hospitalized patients was compared between the group with a positive FilmArrray result (n = 49) and the group with a negative result (n = 43). As the mean age was different between the two groups (55.8 years for the group with a positive result vs 65.7 years for the group with a negative result; p = 0.010), a correction for age was applied. Even though the length of stay was longer when the FA result was negative (15.7 vs 9.3 days), the apparent difference was at the limit of significance after correction for age (p = 0.056).

Antibiotic prescription

The group of patients receiving antibiotics (n = 70) was compared to the group that did not receive antibiotics (n = 80). As patients receiving antibiotics were significantly older (61 vs 46.2 years; p < 0.001), a correction for age was applied.

The group of patients with no intention for antibiotic treatment and who did not receive antibiotics after results were received was then excluded from the analysis. The remaining subset was split up into the group of patients receiving antibiotics (n = 57) and those who did not (n = 21). As patients receiving antibiotics were significantly older (60.2 vs 44.4 years; p = 0.001), a correction for age was applied (Table 4).

Patient isolation

Local isolation procedures for respiratory pathogens in adults recommend isolation of patients infected with viruses, atypical bacteria (*M. pneumoniae* and *C. pneumoniae*), *Bordetella pertussis* or *Mycobacterium tuberculosis* complex. If we consider isolation

Table 3
Statistical analysis of clinical and biological parameters for hospitalization and antibiotic prescription for children. Selected population for hospitalization excludes the patients for whom hospitalization was not intended and not instituted after test results were received. Selected population for antibiotics prescription excludes the patients for whom antibiotics prescription was not intended and not prescribed after test results were received.

Children	All children			Selected population			All children			Selected population		
	Hospitalized	Discharged	p-Value after correction for age	Hospitalized	Discharged	p-Value after correction for age	Antibiotics	No antibiotics	p-Value	Antibiotics	No antibiotics	p-Value
N°	104	45		104	21		72	77		66	13	
Age (years)	1.4	2.7	NA	1.4	2.8	NA	1.71	1.88	0.725	1.6	1.4	0.745
Gender (F/M)	51/53	16/29	0.217	51/53	10/11	0.843	40/32	27/50	**0.014**	38/28	4/9	0.127
CRP (mg/L)	29.5	14	**0.004**	29.5	15.8	**0.024**	34.7	16.1	**0.007**	36.1	7.6	<**0.001**
WBC count (.10^3/µL)	12.5	10.6	0.141	12.5	10.1	0.144	13.3	10.6	**0.004**	13.4	5.3	<**0.001**
Chronic respiratory disease	7.6%	20%	0.085	7.6%	9.5%	0.842	11.1%	11.7%	1.000	9%	15.4%	0.612
SpO2 in ambient air (%)	97.6	98.5	0.344	97.6	98.4	0.590	96.6	98.9	**0.017**	97.4	98.4	0.466
O2 supplementation	17.3%	0%	0.998	17.3%	0%	0.998	19.4%	9.1%	0.098	16.7%	0%	0.195
Positive FilmArray RP	80.8%	91.1%	0.053	80.8%	100%	0.997	84.7%	83.1%	0.827	83.3%	100%	0.195
Mean detected pathogens	1.3	1.4	0.225	1.3	1.6	0.069	1.4	1.3	0.421	1.4	1.4	0.985
Influenza A	20.2%	8.9%	0.126	20.2%	14.3%	0.593	16.7%	16.9%	1.000	18.2%	38.5%	0.139
Influenza B	12.5%	56.7%	**0.001**	12.5%	38.1%	**0.012**	18.1%	22.1%	0.683	18.2%	30.8%	0.449
Adenovirus	14.4%	13.3%	0.935	14.4%	14.3%	0.976	16.7%	11.7%	0.481	18.2%	0%	0.199
Metapneumovirus	8.6%	2.2%	0.252	8.6%	4.8%	0.685	4.2%	9.1%	0.330	4.5%	0%	1.000
Parainfluenza	4.8%	4.4%	0.720	4.8%	4.8%	0.717	5.6%	2.6%	0.430	6%	7.7%	1.000
Rhino/enterovirus	37.5%	37.7%	0.580	37.5%	47.6%	0.207	40.3%	33.8%	0.497	39.4%	30.8%	0.756
RSV	6.7%	6.6%	0.891	6.7%	9.5%	0.545	8.3%	5.2%	0.523	7.6%	0%	0.584
Coronavirus HKU1	11.5%	11.1%	0.813	11.5%	14.3%	0.537	13.9%	9.1%	0.443	13.6%	15.4%	1.000
Coronavirus NL63	4.8%	6.6%	0.427	4.8%	4.8%	0.847	2.8%	7.8%	0.278	3%	7.7%	0.421
Coronavirus OC43	2.9%	6.7%	0.466	2.9%	4.8%	0.686	4.2%	3.9%	1.000	3%	7.7%	0.421
Chlamydophila pneumoniae	1.9%	4.4%	0.558	1.9%	4.8%	0.614	2.8%	2.6%	1.000	1.5%	0%	1.000
Mycoplasma pneumoniae	1.9%	2.2%	0.999	1.9%	0%	0.999	2.8%	1.3%	0.610	6%	0%	1.000
Lumbar puncture	21.1%	0%	0.998	21.1%	0%	0.998	27.8%	2.6%	<**0.001**	27.3%	7.7%	0.171
Urinalysis	67.3%	19.5%	**0.004**	67.3%	28.6%	**0.004**	57%	59.7%	0.742	57.6%	61.5%	1.000
Blood culture	84.6%	60%	**0.005**	84.6%	76.2%	0.637	84.7%	70.1%	0.050	87.9%	84.6%	1.000
Bacterial respiratory infection	15.4%	15.6%	0.749	15.4%	14.3%	0.947	27.8%	3.9%	<**0.001**	28.8%	0%	**0.031**
Urinary tract infection	7.7%	0%	0.999	7.7%	0%	0.999	11.1%	0%	**0.002**	12.1%	0%	0.340
Otitis media	3.8%	4.4%	0.812	3.8%	9.2%	0.254	4.2%	3.9%	1.000	4.5%	0%	1.000
Antibiotics	56.7%	28.9%	**0.002**	56.7%	28.6%	**0.039**	NA	NA	NA	NA	NA	NA

Table 4
Statistical analysis of clinical and biological parameters for hospitalization and antibiotic prescription for adults. Selected population for hospitalization excludes the patients for whom hospitalization was not intended and not instituted after test results were received. Selected population for antibiotics prescription excludes the patients for whom antibiotics prescription was not intended and not prescribed after test results were received.

Adults	All adults			Selected population			All adults			Selected population		
	Hospitalized	Discharged	p-Value after correction for age	Hospitalized	Discharged	p-Value after correction for age	Antibiotics	No antibiotics	p-Value after correction for age	Antibiotics	No antibiotics	p-Value after correction for age
N°	93	57		93	13		70	80		57	21	
Age (years)	60.4	41.1		60.4	36.8		61	46.2		60.2	44.4	
Gender (F/M)	43/50	30/27	0.654	43/50	6/7	0.460	30/40	43/37	0.591	26/31	13/8	0.288
CRP (mg/L)	73.9	44	**0.039**	73.9	26.3	0.066	97	31.4	**<0.001**	94.9	29.4	**0.015**
WBC count ($.10^3/\mu L$)	11.2	8	**0.005**	11.2	7.3	**0.028**	11.4	8.8	**0.014**	11.5	9.5	0.224
Chronic respiratory disease	49.5%	24.6%	0.114	49.5%	23.1%	0.542	48.6%	32.5%	0.403	54.4%	38.1%	0.653
Heart insufficiency	36.6%	8.8%	0.051	36.6%	7.7%	0.260	32.9%	20%	0.988	29.8%	28.6%	0.284
Immunosuppression	14%	15.8%	0.728	14%	15.4%	0.808	15.7%	13.7%	0.345	17.5%	9.5%	0.293
Diabetes	29%	7%	0.088	29%	0%	**<0.001**	25.7%	16.3%	0.953	22.8%	33.3%	**0.025**
SpO2 in ambient air (%)	90.7	97.5	**0.009**	90.7	97.5	**0.006**	90.7	95.8	0.055	89.9	93.3	**0.005**
O2 supplementation	51.6%	1.8%	**<0.001**	51.6%	0%	**<0.001**	50%	17.5%	**0.005**	54.4%	28.6%	0.370
Positive FilmArray RP	52.7%	73.7%	0.682	52.7%	69.2%	0.708	50%	70%	0.380	47.4%	71.42	0.350
Influenza A	15%	28%	0.480	15%	30.8%	0.584	15.7%	23.7%	0.818	15.8%	28.6%	0.615
Influenza B	9.7%	24.6%	0.148	9.7%	15.4%	0.875	4.3%	25%	**0.005**	5.3%	14.3%	0.200
Rhino/enterovirus	9.7%	7%	**0.046**	9.7%	7.7%	0.167	10%	7.5%	0.095	8.8%	4.8%	0.283
Urinalysis	50.5%	31.6%	**0.018**	50.5%	38.5%	**<0.001**	51.4%	36.2%	0.071	52.6%	23.8%	**0.024**
Blood culture	82.8%	50.9%	**<0.001**	82.8%	12.5%	**<0.001**	87.1%	56.3%	**<0.001**	89.5%	66.7%	0.123
Bacterial respiratory infection	46.2%	7%	**0.001**	46.2%	0%	**<0.001**	57.1%	8.8%	**<0.001**	52.6%	14.3%	**0.024**
Urinary tract infection	5.4%	1.8%	**<0.001**	5.4%	0%	**<0.001**	5.7%	2.5%	0.280	5.3%	4.8%	0.687
Antibiotics	66,7%	14%	**<0.001**	66,7%	7.7%	**0.009**	NA	NA	NA	NA	NA	NA

Table 5
Prescription of oseltamivir for adults and children depending on the FilmArray result and medical indication.

	Oseltamivir	Children	Adults	Total
Influenza negative	Indicated and avoided	23 (15.4%)	86 (57.3%)	109 (36.5%)
	Not indicated	74 (49.7%)	11 (7.3%)	85 (28.4%)
Influenza positive	Instituted after result	9 (6%)	31 (20.7%)	40 (13.4%)
	Already instituted	0 (0%)	7 (4.7%)	7 (2.3%)
	Symptoms >48 h	1 (0.7%)	6 (4%)	7 (2.3%)
	Not indicated	42 (28.2%)	9 (6%)	51 (17.1%)
		149	150	299

procedures were adequately applied for the 93 hospitalized adults, 37 were appropriately placed in isolation after the FA result was delivered, 6 patients for whom isolations were initially planned were not isolated based on the results, 7 patients for whom isolation was already planned were properly isolated, and 34 patients for whom isolation was not planned were appropriately not isolated after the results were received. Data concerning isolation intentions were missing for 9 patients.

Oseltamivir prescription

In our institution, oseltamivir is prescribed to influenza positive patients presenting symptoms since less than 48 h and either having one or more of the co-morbidities mentioned in the inclusion criteria, or, regardless of co-morbidities, to children born prematurely until they are aged of 6 months, to hospitalized adults and to pregnant women. The impact of positive FA results for influenza A and B viruses on the prescription of oseltamivir is reported in Table 5. Oseltamivir was instituted in 40/105 (38.1%) of patients after a positive influenza test result; it was indicated, had the influenza test been positive, yet avoided in 109/194 (56.2%) of patients after a negative influenza test result. The total estimated avoided financial waste was 3 000 euros (3 545 US dollars).

Prescription of ancillary tests

For children, the subset of patients for whom a lumbar puncture (LP) was intended prior to reception of results was split into those who underwent the test (n = 22) and those who did not (n = 8). The two groups were then compared. A significant difference was only observed concerning the prescription of antibiotics, where children who underwent LP more often had antibiotics prescribed (82% vs 0%; $p < 0.001$). No significant difference was observed for other parameters, notably including those who had a positive FA result; indeed, all patients who did not undergo LP had a positive FA result vs 77.3% of those that did undergo LP ($p = 0.287$). For adult patients, no statistical analysis could be performed because there were only two intended LPs, one of which was not performed.

Of the 299 emergency room tests ordered, avoidance of ancillary tests other than LP was minimal; 8 urinalysis of 160 intended, 1 blood culture of 222 intended and 3 chest radiographs of 197 intended were also avoided. To evaluate the impact on ancillary test prescription for patients admitted after the ER, we listed the number of blood samples, urinalysis, blood cultures, respiratory samples, other microbiological samples (other than urinalysis, blood cultures and respiratory samples), chest radiographs and ancillary tests other than aforementioned per 1000 days of hospitalization when data were available. We then compared the numbers between patients with positive and negative FA results. For adults, the only significant differences

were that patients with a positive FA result (n = 48) had more blood samples (507 per 1000 day of hospitalization vs 381; p = 0.012) and more blood cultures taken (144 vs 61.8; p = 0.033) in comparison to patients with a negative FA result (n = 43). The proportion of patients in intensive care was not significantly different between the two groups (25% vs 25.6%; p = 0.867). For children, patients with a positive FA result (n = 79) had less blood samples (108.4 per 1000 days of hospitalization vs 265.4), less blood cultures (7.4 vs 44.7; p = 0.015) and less other microbiological samples taken (39.9 vs 111.7: p = 0.029) in comparison to patients with a negative FA result (n = 19). The 5 children admitted in intensive care from the ER were excluded from this analysis as they were transferred to another hospital and that access to clinical data was not available.

Discussion

Multiplex molecular techniques for detection of respiratory viruses allow the delivery of test results in a timely manner; however, these techniques have not yet clearly shown their cost-effectiveness. We report here the results of a prospective interventional study including selected adult and pediatric patients attending the emergency room in a tertiary care hospital during the 2015–2016 influenza epidemic. The goal was to analyze whether FA results influenced patient management in terms of antibiotic or antiviral prescription, ancillary test prescription, admission, length of stay and isolation.

We found that parameters significantly associated with hospitalization and antibiotic prescription were mainly high white blood cell count or CRP level, having blood cultures or urinalysis performed in search of a bacterial infection or having a diagnosis of such an infection. Signs of respiratory distress were also associated with hospitalization and antibiotic prescription for adults but not for children. This difference could be explained by the fact that respiratory distress in adults occurred mainly in patients with decompensated chronic obstructive pulmonary disease, which is often caused by an infection. For children, respiratory distress was mainly encountered during bronchiolitis or decompensated asthma; these conditions do not systematically imply antibiotic prescription or hospitalization if the symptoms improve following aerosol treatment in the emergency room. A correction for age had to be applied for children concerning admission and for adults concerning admission and antibiotics prescription. This finding indicates that younger age for children is associated with a higher admission rate and older age for adults with higher admission rates and antibiotic prescriptions. This can be explained as children <1 month of age attending the emergency room are consistently hospitalized according to the local management algorithm. They represented 15 of the 149 included children (10%). However, hospitalized patients are still significantly younger than nonhospitalized ones even after removing the subset of patients aged <1 month from the calculation (1.6 years old vs 2.8; p = 0.032).

Statistical analyses comparing hospitalization status and prescription of antibiotics showed no significant difference between patients with a positive FA result and patients with a negative result. This could be explained by the fact that virology results are not crucial in management algorithms. Indeed, guidelines regarding patients' management with community-acquired pneumonia state that the detection of a virus in a respiratory sample makes a bacterial infection less likely, provided there are no other clinical, biologic or radiographic signs of such an infection (Bradley et al., 2011; Woodhead et al., 2011). When antibiotic prescription is necessary in our institution, it relies on the association of intravenous ampicillin and cefotaxime for children <3 months old. For children >3 months old, cefotaxime alone is prescribed for systemic infections or cefuroxime for respiratory infections. For adults, respiratory infections are treated with cefuroxime or amoxicillin/clavulanic acid. The administration can be intravenous or oral depending on the severity of the symptoms. Antibiotics are maintained at least 48 h for hospitalized patients and reevaluated based on the evolution of the symptoms and the results of the microbiological and blood analysis. The impact of the FA result on antibiotic discontinuation for hospitalized patients would need further evaluation. Keske et al. (2018) observed that in addition to providing a rapid molecular test result, offering training sessions for physicians about the diagnosis and the management of respiratory tract infections could decrease antibiotic use, at least for children. Other studies did not find a difference in antibiotic use when testing adult or pediatric patients with molecular techniques (Hernes et al., 2014; Rogers et al., 2015; Andrews et al., 2017; Semret et al., 2017; Trabattoni et al., 2018); however, some pediatric studies report a shorter antimicrobial treatment duration (Rogers et al., 2015; Schulert et al., 2013). In these studies, the results of molecular tests were not delivered in a timely manner, which can explain the lack of impact on the initial prescription of antimicrobial treatment even though there was an impact on the duration. Duration of antimicrobial treatment was not recorded in our study.

Children discharged from the hospital significantly more often had a positive FA result for influenza B than children admitted to the hospital. The only significant difference was that children with a positive influenza B result less often had urinalysis performed than children with a negative influenza B result (33.3% vs 64.7%; p = 0.004), meaning they were possibly less suspected of having a urinary tract infection. Some studies also report a trend toward a lower rate of admission of adult patients when the influenza test is positive with a molecular technique in comparison to conventional methods (Rappo et al., 2016; Trabattoni et al., 2018).

The contribution of the FA result to patient isolation depends on management algorithms. As previously described, FA results had no impact on the isolation of children, as all children with a proven or suspected contagious infectious process are to be kept in isolation. Nevertheless, rapid delivery of the FA result allowed better management of hospitalized children by cohorting, as individual rooms are not available for every patient. The delay in the emergency room before admission was not recorded but was shortened according to the pediatricians involved in this study. For adults, screening patients with a molecular technique having a broad panel of detected pathogens triggers more isolations and avoids few. This approach, albeit expensive, permits better application of isolation procedures and likely diminishes nosocomial spread of respiratory viral pathogens, which has been shown to be an underappreciated cause of morbidity and mortality in hospitalized patients (Gilca et al., 2014; Chow and Mermel, 2017).

More appropriate prescription of oseltamivir was already reported by other authors (Xu et al., 2013; Mitchell et al., 2018). In our study, oseltamivir could be confidently avoided in cases in which FA was negative for influenza, resulting in savings of approximately 3000 euros. The result was also communicated before oseltamivir was prescribed in 40 cases, reminding its institution. However, the total expense for the utilization of FA tests was approximately 40 000 euros. The use of a sensitive and specific molecular technique targeting only influenza A and B might be a more cost-effective option in adult populations (Trabattoni et al., 2018; You et al., 2017). Moreover, the detection of viruses other than influenza seems to have a low impact on hospitalized adult patients' management (Semret et al., 2017). Techniques detecting a broader panel of pathogens might be more suitable for immunosuppressed patients, notably hematopoietic stem cell transplant recipients, for whom viruses other than influenza should be treated (Boeckh, 2008; Semret et al., 2017).

Concerning the sparing of ancillary tests in the ER, it was minimal. This could be explained since ER management involves empiric testing in order not to delay the triage. We would expect that a positive FA result could explain clinical symptoms of the patients and thus limit further investigations. For adult patients admitted after the ER, we paradoxically observed more blood samples and blood cultures taken for patients with a positive FA result. For children, it was the opposite; we observed less blood samples, blood cultures and other microbiological samples taken in the group with a positive FA result. A hypothesis would be that admitted adults with a viral infection are more likely to already have complications from the infection. For children, the respiratory viruses might more probably be responsible for the clinical symptoms and complications requiring further analysis could be less frequent. However, these findings would require further evaluation in order to appreciate all possible confounding parameters.

Few studies evaluating the cost-effectiveness of multiplex molecular techniques for respiratory viruses delivered test results in a time frame comparable to our study; three were identified in the last 10 years. Firstly, a study from Rappo et al. (2016) found lower antimicrobial use, fewer chest radiographs ordered and a shorter length of stay for patients positive for influenza according to FA in comparison to conventional methods. This difference was significant, regardless of the virus, after correction for age, immunosuppression status and asthma and intensive care unit admission, reflecting the importance of the target population for the tests. However, Rappo et al. studied the management of adult patients during two consecutive winter seasons. This type of design notably adds bias to the interpretation of the data due to difference in circulating viruses and to possible change in management algorithms between two seasons. Secondly, a study from Trabattoni et al. (2018) evaluating adult patients visiting the ER and tested with a rapid molecular technique for influenza in comparison to conventional methods also reported fewer radiographs and biological tests ordered, fewer admissions and shorter length of stay in the emergency room in the group tested by the molecular technique. However, the group tested with conventional methods was older and showed more comorbidities. Thirdly, Echavarría et al. (2018) prospectively studied children and adults visiting the emergency room with acute respiratory tract infection and compared patients tested with immunofluorescent assay to those tested with FA. They demonstrated a decrease in antibiotic prescription for adults and children, a decrease in antiviral prescription for adults and a decrease in ancillary test prescription for children in the FA group. The advantages of our evaluation in comparison to other studies are prospective design, inclusion of selected children and adults visiting the emergency department during the same epidemic season, use of the same standard of care for every patient and delivery of test results while patients were still in the emergency department. In the majority of studies, only test results were taken into consideration, and confounding factors might have been missed, while in our study, clinical parameters were also taken into account during analysis.

Conclusion

Providing a rapid molecular result with the FilmArray Respiratory Panel does not seem to impact hospitalization decisions, length of stay and initial antibiotic and ancillary tests prescription for selected children and adult patients visiting the emergency room of our hospital. Other parameters appeared more consistently to account for hospitalization decisions and antibiotic prescriptions, such as CRP levels, white blood cell count, suspected or proven bacterial infection and, for adult patients only, signs of respiratory distress. For children, younger age is also associated with a higher admission rate, but this could be explained by local management algorithms. For adult patients, older age is associated with higher admission and antibiotic prescription rates. One exception is having an influenza B-positive result, leading to a significantly higher rate of discharge for children, suggesting that use of a sensitive molecular technique targeting only influenza A and B could be more cost-effective in our setting. The positive impact of the use of the FilmArray Respiratory Panel might be more important in high-risk populations, such as immunosuppressed patients, for whom more than just influenza viruses are to be treated, which would possibly avoid detrimental outcomes. Training sessions for physicians about the diagnosis and management of respiratory tract infections could improve the impact.

Benefits resulting from the use of FA, in addition to a more adequate prescription of oseltamivir, are hard to appraise, making cost-benefit calculations difficult. Such benefits are mainly a faster and better implementation of isolation algorithms for hospitalized patients, probably resulting in a decrease in nosocomial infections. These points, as well as the contribution of molecular test results on the avoidance of ancillary tests and on the discontinuation of antibiotics once patients are hospitalized, need to be further evaluated.

Conflict of interests

None declared

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None.

Ethical approval

This study was approved by the ethics committee of University Hospital Saint Pierre (Brussels) with the reference AK/15-10-109/4563Bis.

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The conclusion of this article is that the result of the molecular test has no impact on admission, length of stay and antimicrobial prescription with the exception of a lower admission rate for children with a positive test for influenza B. A rapid molecular test however permitted a more appropriate prescription of antiviral treatment. It also possibly shortened the length of stay in the ward for hospitalized children by facilitating cohorting of patients.

Parameters accounting for the decision to hospitalize and to prescribe antibiotics were high levels of CRP and white blood cells count, suspected or proven bacterial infection and, for adult patients only, signs of respiratory distress. For children, younger age was associated with higher admission rate and, for adult patients, older age was associated with higher admission and antibiotics prescription rates. In order to improve the impact of molecular test result on patients' management, Keske et al. suggested to provide some training sessions to physicians about the diagnosis and the management of respiratory tract infections. [80]

Due to the differences between studies (designs, management algorithms, detection techniques used, included populations,…), it is difficult to predict if the positive or negative effect observed on an outcome in a study will be the same in another setting even if a similar methodology is employed, as there are too many confounding variables. In a bid to demonstrate clinical input of rapid molecular techniques, comparisons should be realised internally in each hospital setting, with the same population and patient management algorithms.

5 Discussion

As previously detailed, actual routinely used direct viral diagnostic tests each have their advantages and inconveniences. Antigen detection tests, especially lateral flow chromatography tests, have a high specificity, are easy to perform and interpret. They can thus be implemented in practically every diagnostic laboratory even those without a dedicated Virology department. Their sensitivity is however most of the time the lowest for viral diagnosis as demonstrated in our article on the detection of influenza [22] and they are only available for viruses having few different serotypes.

Viral cell cultures usually have better sensitivity than antigen detection tests and can therefore monitor their sensitivity and improve detection rate of viruses. They can also confirm the specificity of positive antigen detection tests outside epidemic periods and can sometime detect an unsuspected pathogen as we observed with the detection of *Chlamydia trachomatis*. [19] Their main inconveniences are that the time to positivity is usually long (depending on the viral load in the sample and on the viability of the virus), they require experimented laboratory technicians as well as dedicated premises, and some viruses are hardly or not culturable (namely group C rhinoviruses or some coronaviruses). The use of the shell vial technique can lower the time to positivity but is usually less sensitive than conventional cell cultures.

Antigen detection tests, cell cultures and shell vial techniques are reimbursed by the social welfare system in Belgium. This is of prime importance, especially in hospital taking care of underprivileged patients who might not be able to sustain their health expenses and would be exposed to higher risk of detrimental medical conditions if the access to healthcare was not affordable.

Molecular techniques are presently the most sensitive techniques for viral diagnosis and some multiplex fully automated tests allow to perform a syndromic diagnosis in a timely manner as reported in our evaluation of the FilmArray Respiratory Panel. [51] The main drawback of molecular tests is the price of the reagents as well as that of the equipment, especially for laboratories with high volume of analyses. The implementation of molecular tests is also a technical challenge in term of workflow. Moreover, molecular tests are only reimbursed in specific indications by the Belgian social welfare system, therefore limiting their use. Further health economics analyses to demonstrate their positive impact on patients' management could lead to an extension of the reimbursement to other conditions.

During this thesis, MALDI-TOF MS (Matrix-Assisted LASER Desorption/ionization – Time Of Flight Mass Spectrometry) was also evaluated as a potential diagnostic tool on viral cell cultures. This fast, robust and inexpensive technique has revolutionized the identification of bacteria since the beginning of the 21st century. [12] The technology is based on the analysis of the protein composition of a bacterial cell (proteomics), with ribosomal proteins comprising most bacterial proteins being detected. It allows the acquisition of mass spectra that can be compared to a databank thus providing bacterial identification. It was our intention to extend this technology to the detection of viral proteins among cell proteins in cell cultures. Shortly, cell cultures were inoculated in multiwell plates with known strains of different viruses. The first part of the work was to determine how to obtain mass spectra from cell cultures. The best procedure we could find is as follow; after various times of incubation, inoculated cells were lysed with 70% formic acid and acetonitrile was added to obtain a precipitate. The precipitate was then analysed on Microflex LT automate (Bruker Daltonic, Bremen, Germany) as per manufacturer's instructions. Distinctive mass spectra were obtained, varying on the inoculated cells and on the virus strains. The capacity of MALDI-TOF to detect HSV in cell cultures was then evaluated in comparison to immunofluorescence. We chose HSV as this virus grows well on cell cultures. We observed additional peaks in spectra obtained from inoculated cell cultures in comparison to non-inoculated one. However, it appeared to be a less sensitive technique than direct fluorescent assay, requiring a well grown cell culture with obvious CPE to spot specific viral proteins among cell

proteins on the mass spectra obtained with MALDI-TOF MS (unpublished data). Moreover, the elaboration of a databank is complicated as the same virus grown on different cell lines will not produce identical spectra. Furthermore, interlaboratory variations between cell lines could hinder the process. The use of MALDI-TOF MS in viral diagnosis didn't appear to be a promising direct viral diagnostic technique in our experience.

Accounting for these different elements, our laboratory proposes a wide array of tests for direct viral diagnosis. As samples analysed originate from 5 university hospitals with different specificities, a broad variety of cases is encountered in matters of age, severity of disease and immunity status. Algorithms have been elaborated in concertation with infectious disease specialists in order to propose the most cost-effective test depending on individual cases.

For respiratory viruses' diagnosis, the first line tests comprise of cell cultures and antigen detection tests. Depending on the time of the year, the 3 most prevalent respiratory viruses based on cell cultures results, are tested with antigen detection methods amongst influenza virus, RSV, parainfluenza virus, respiratory adenovirus and metapneumovirus. Cell cultures improve diagnostic sensitivity as compared to antigen detection tests (except for metapneumovirus which grows poorly in cell cultures) and also extend the range of recovered viruses to rhinovirus and enterovirus. After evaluation in 2016, the FilmArray respiratory panel was introduced as a routine test in our laboratory. [82] It is mainly used for immunosuppressed patients and patients in the intensive care units as the aforementioned article didn't show a clear input on patients' management, it was advised to be reserved for the most critical patients. Next, implementation of a rapid molecular test for the combined diagnosis of influenza virus and RSV for use for all hospitalized patients during epidemics is being considered. This thesis was completed before December 2019, therefore SARS-CoV-2 is not taken into account in the algorithm. In time, this algorithm should be updated but presently, lack of perspective owing to the fact that this disease is relatively new would make it hazardous.

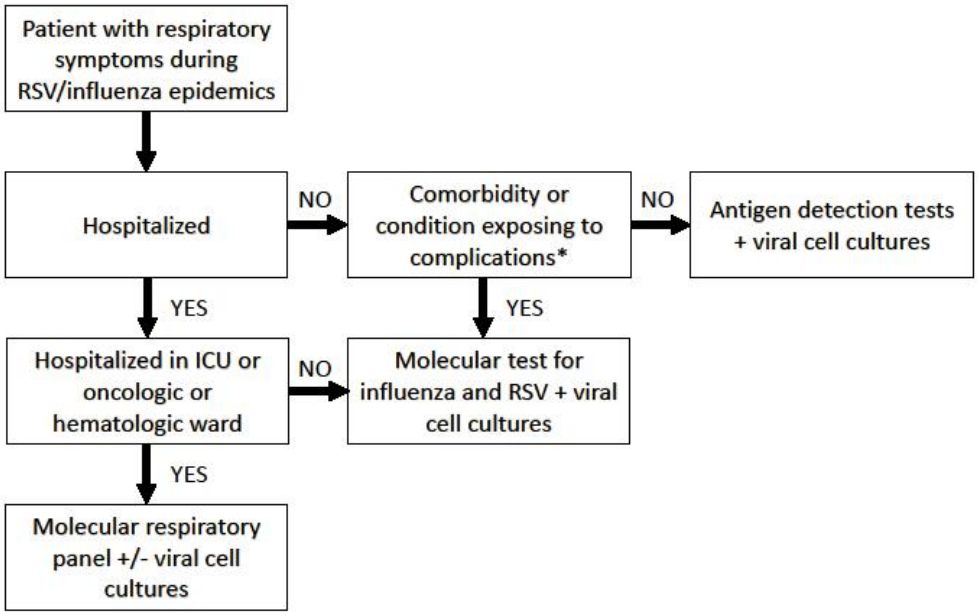

Figure 7: Suggestion of an algorithm for the diagnosis of respiratory viruses. ICU: intensive care unit; RSV: respiratory syncytial virus. *chronic respiratory diseases (such as cystic fibrosis, asthma or chronic obstructive pulmonary disease), sickle-cell disease, asplenia, neuromuscular diseases, severe neurological impairments, hereditary metabolic disorders including diabetes, congenital or acquired immunosuppression, heart defects, chronic nephropathies, chronic liver diseases and pregnancy

Concerning *Herpesviridae*, suspicions of HSV and VZV from skin and mucosa are diagnosed with a combination of shell vial and conventional cell cultures. While shell vial shortens time to response (about 24h for HSV and 96h for VZV) in comparison to cell cultures, the latter offers a higher sensitivity. For HSV and VZV in cerebrospinal fluids or ocular fluids, molecular techniques are favoured mainly for better diagnostic sensitivity. CMV in urine is diagnosed with shell vial technique after 24h of incubation; sampling 3 urines on 3 successive days is recommended to increase sensitivity. For the initial diagnosis of congenital CMV, the use of a molecular technique on a single urine sample is preferred. CMV in cerebrospinal fluids, ocular fluids, tissue biopsies or blood is diagnosed with molecular techniques. Other *Herpesviridae* (mainly EBV and HHV6) are also diagnosed with molecular tools.

Other viruses responsible of meningitis and encephalitis are exclusively detected with molecular techniques in cerebrospinal fluids.

Viruses in blood are also detected with molecular technique as a quantification of the viral load is often required.

Regarding enteric viruses, group F adenoviruses and rotavirus are diagnosed with lateral flow chromatography tests. Their diagnosis is only reimbursed by the Belgian social welfare system for children aged <2 years. Norovirus is also diagnosed with lateral flow chromatography tests but only in epidemic circumstances when stool samples from several patients are sent to the laboratory. This increases the chance to spot the epidemic as lateral chromatography tests are not sufficiently sensitive for the recovery of norovirus. For individual diagnosis of norovirus, the use of a molecular technique is favoured. [83]

Sequencing techniques are not routinely performed in our laboratory for viral diagnosis. They are reserved when expertise is needed for some patients, especially for the detection of mutations conferring resistance toward antivirals (mainly for HSV, CMV or HIV) or for the typing of strains during epidemics.

Diagnostic algorithms mainly depend on available techniques, on their cost, on their ease of use and on their capacity to absorb the workload in a laboratory. This will probably evolve in a near future due to the rapid development of molecular techniques.

6 Perspectives in direct viral diagnosis

Sequencing methods appear to be the most promising techniques for direct viral diagnosis. They are nowadays not used in a routine setting for viral detection but are rather reserved for expertise or characterization of special cases. Their purpose is to determine the nucleic acid sequence in the RNA or DNA of a virus and compare it to sequences stored in a databank to decide which virus it most likely corresponds to. Their principle is based on the original Sanger sequencing technique developed in the early 1990s. [84] Since then, many improvements have been made in order to lower the cost and increase the throughput of the techniques, permitting the sequencing of a whole viral genome within a couple of days. [85] These high throughput sequencing techniques are also referred to as next-generation sequencing (NGS) or whole genome sequencing (WGS). The characterization of genetic information directly from clinical or environmental samples without culturing them is called metagenomics. [86]

NGS challenges the classification of viruses, and experts propose to incorporate viruses that are known only from metagenomics data to the actual taxonomy established by the International Committee on Taxonomy of Viruses. [87] Some authors propose the use of bioinformatics tools to improve viruses' classification. [88] [89]

The sensitivity of sequencing techniques for viral diagnosis seems to be lower in comparison to PCR based tests. [90] [91] Some enrichment methods could improve sensitivity of NGS techniques. [92] However, one of the advantages of NGS over PCR is that it is not necessary to target which virus to detect. This allows diagnosis of unsuspected viruses potentially responsible for various conditions such as acute flaccid paralysis, myocarditis, meningoencephalitis, uveitis or acute liver failure. [93] [94] [95] [96] [97] [98] [99] [100] Another advantage of NGS is that it can detect mutated viruses that would not have been detected by PCR. [101] NGS could also establish a possible viral cause to some cancers. [102] [103] Its use would probably be more helpful for immunocompromised patients for whom uncommon viruses can cause infections. [97] [98] [104] [105] Additionally, the amount of read counts, which corresponds to the number of sequences identified matching each detected virus, correlates to the quantification of viruses with PCR; this could help decide which virus is more involved in the clinical symptoms when several viruses are co-detected. [106] Besides quantification of viruses, the study of the host reaction to viruses could help determine their implication in the infectious process and also predict the severity of the disease. This can be done thanks to transcriptomics (also called RNA sequencing) which analyses the RNA leading to proteins synthesis in cells. [107] [108]

Metagenomics analysis of various body samples has recovered many viruses, mainly bacteriophages, constituting the virome. The virome is the repertoire of all viruses that are found on the surface of and inside our bodies in the absence of clinically significant symptoms or infection. This includes viruses that cause acute, latent, or persistent infections; viruses infecting eukaryotes, bacteria and archea; and endogenous viral elements integrated into host chromosomes. [109] The healthy human virome is present in the gastrointestinal tract, the oral cavity, the respiratory tract, the genitourinary tract, the skin and even in the blood which has often been considered sterile. The virome is part of the microbiome together with the bacteriome and the mycobiome. The imbalance of the microbiome could offer new leads towards the understanding of some conditions such as glucose metabolism disorders or inflammatory bowel diseases. [110] [111] [112]

The exhaustive detection of NGS could be applied to blood transfusion safety for the search of new, emerging and/or unexpected viruses in blood donations [113] [114] as well as to the surveillance of water- or foodborne diseases [115] [116] [117] [118] and vector-borne infections. [119] [120] [121] The challenge will be to determine the pathogenicity for humans of the newly discovered viruses.

Besides detecting viruses, sequencing techniques also enable genotype characterization which can give information concerning virulence or antiviral resistance. [122] [123] [124] Moreover, NGS is a powerful epidemiologic tool allowing to establish the relation between different strains during outbreaks and to identify different clusters in what could have appeared as a single outbreak with conventional diagnostic methods. This can provide evidence for source and patterns of transmission. [125] [126] [127] NGS can also be used as a surveillance epidemiologic tool to characterize circulating strains of a virus at a national level. [128] [129] [130]

Portable real-time DNA/RNA sequencing devices such as MinION are being made available which offers a rapid and relatively cheap sequencing tool. [131] [132] This kind of device will most probably make NGS more affordable in a near future.

7 Conclusion

A long path has been treaded since the development of the first diagnostic tools for viruses in the 1950s'. Nowadays, cell cultures for viral diagnosis are mainly still in use in reference centres or huge laboratories that can maintain the expertise needed for their realization. Antigen detection tests are widely used in diagnostic laboratories as they are easy to perform and cheap. However, non-molecular tests, due to their lower sensitivity, tend to be replaced with time.

The future of viral diagnostic will be molecular for sure. Nucleic acid amplification tests are more and more automatized, rapid, user-friendly and cheap, increasing their utilization in diagnostic laboratories. The simultaneous detection of several viruses lowers the risk of missing an infection. NGS techniques are presently booming but there are still challenges to overcome before they can become routine tests. Technical improvements are still required to make NGS faster and cheaper. Bioinformatics software and databanks are also very important for the treatment of the mass of data to analyse. [133] In a near future, it will be possible to know in a short frame of time the full viral content of a sample; the simultaneous analysis of the viral genome will also give clues on antiviral resistance, virulence and origin or source of the strain.

The risk is that physicians might be overwhelmed by loads of data. Another challenge will be to establish the significance of the results; what is in correlation with an infection? What is part of the commensal virome? And finally, the determination of the cost-effectiveness of NGS techniques in the management of patients will have to be proven. Until then, the use of a mix of non-molecular and molecular tests permits to offer diagnostic algorithms adapted to individual cases.

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