Surveillance of upper respiratory infections using a new multiplex PCR assay compared to conventional methods during the influenza season in Taiwan

Shu-Chun Chiu[a,1], Yung-Cheng Lin[b,1], Hsiao-Chi Wang[a,c], Jen-Jen Hsu[a], Ting-Kai Yeh[a], Hsin-Fu Liu[b,d,e,**], Jih-Hui Lin[a,c,*]

[a] Center of Diagnostics and Vaccine Development, Centers for Disease Control, Taiwan, Taipei 11561, Taiwan
[b] Department of Medical Research, Mackay Memorial Hospital, New Taipei City 25160, Taiwan
[c] National Influenza Center, Centers for Disease Control, Taipei 11561, Taiwan
[d] Department of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung 20224, Taiwan
[e] Department of Nursing, National Taipei University of Nursing and Health Sciences, Taipei 11219, Taiwan

A R T I C L E   I N F O

A B S T R A C T

Objectives: To improve diagnosis as part of laboratory surveillance in Taiwan, influenza-like illness (ILI) surveillance was conducted using a new multiplex PCR assay (FilmArray) and the results compared to those of conventional methods The study was performed during the winter months.
Methods: Throat swabs from patients with an ILI presenting to physicians in sentinel practices were collected during the 2016–2017 influenza season.
Results: A total of 52 samples tested positive by FilmArray Respiratory Panel. Forty percent were influenza A virus, and subtype H3N2 virus was the major epidemic strain. However, nearly 60% of ILI cases seen at sentinel sites were caused by non-influenza pathogens. The results of the FilmArray assay and cell culture were identical, and this assay was more sensitive than a rapid influenza diagnostic test. Genetic analyses revealed new influenza A H3N2 variants belonging to a novel subclade 3C.2a2.
Conclusions: The FilmArray assay facilitates urgent testing and laboratory surveillance for common viral and bacterial respiratory pathogens. This study demonstrated the use of a highly sensitive assay using clinical samples that is feasible for application worldwide. This may lead to an increased rate of diagnosis of viral infections and to improved patient outcomes, and in particular to a reduction in the overuse of antibiotics and antivirals.

Introduction

Acute respiratory infections are often caused by viruses and are an important cause of morbidity and mortality in Taiwan (Chang et al., 2016; Chiu et al., 2014; Kim et al., 2003; Tsai et al., 2001). During past influenza seasons, many severe cases and deaths were reported among the middle-aged population group, which had a major socioeconomic impact. Therefore, the Taiwan Centers for Disease Control (CDC) expanded their scope to provide free influenza vaccinations for children aged 6 months to <18 years, pregnant women, women within 6 months of giving birth, adults aged >50 years, at-risk individuals with chronic medical conditions, individuals with rare diseases or major illnesses/injury, health care and public health personnel, poultry farmers and animal health inspectors, individuals with a body mass index (BMI) of $\geq 30 \text{ kg/m}^2$, and residents and personnel at nursing institutes. This campaign increased the flu vaccination coverage from 13% to 25% of the total population of Taiwan.

However, clinical signs and symptoms of upper respiratory infections are similar for many different viruses, making an etiological diagnosis based on clinical presentation alone difficult and sometimes leading to delays in therapeutic management. The ability to detect viruses has improved with the availability of PCR,

* Corresponding author at: Center of Diagnostics and Vaccine Development, Centers for Disease Control, Taiwan. Tel: +886 2 27850513 ext. 886; Fax: +886 2 27853944.
** Corresponding author at: Department of Medical Research, Mackay Memorial Hospital, Taiwan. Tel: +886 2 28094661 ext. 2073; Fax: +886 2 28085952.
E-mail addresses: hsinfu@ms1.mmh.org.tw (H.-F. Liu), jeffy320@cdc.gov.tw (J.-H. Lin).
[1] Shu-Chun Chiu and Yung-Cheng Lin contributed equally to this work.

and several new viruses have been identified (Poutanen et al., 2003; van den Hoogen et al., 2001; Zaki et al., 2012). The use of more sensitive methods has led to an increased rate of diagnosis for viral infections and to improvements in patient outcomes. With an automated multiplex PCR device, the FilmArray Respiratory Panel, 17 viral pathogens and 3 bacterial pathogens can be detected in a closed system that requires only 3 min of hands-on time and approximately 1 h of instrumentation time.

The aim of this study was to determine whether the results provided by this new multiplex PCR technique would be useful for application in public health laboratories for the surveillance of influenza-like illness (ILI).

Materials and methods

Samples

Throat swabs were collected from patients with an ILI during the influenza season November 2016 to January 2017 in Taipei, Taiwan. Taipei City is the capital city and accounts for approximately one tenth of the total population of Taiwan. ILI was defined as the sudden onset of fever ($\geq 38\,^\circ$C) and a cough and/or a sore throat without a known cause other than influenza. The biological materials obtained in this study were used for standard diagnostic procedures as requested by the patient's physician; no specific sampling method was used and no modifications were made to the sampling protocols. In accordance with local regulations, the procedure did not require specific patient consent.

Conventional culture and PCR

Conventional methods were used for the clinical samples. Samples were inoculated in MDCK, MK2, HEp-2, RD, MRC-5, Vero, A549, and HeLa cell lines, and cell growth was evaluated in MEM (Minimum Essential Medium) containing 2% (v/v) fetal bovine serum and antibiotics in a 5% CO_2 humidified incubator for 7–10 days, or until a cytopathic effect (CPE) was observed. An indirect immunofluorescence assay (IFA) using monoclonal antibodies (Merck Millipore Cat. Nos. 3105 and 3360) to influenza A and B viruses, parainfluenza virus types 1–3, respiratory syncytial virus (RSV), adenovirus, and enterovirus was also performed. Furthermore, conventional PCR was used to detect rhinovirus, enterovirus, coronavirus, Chlamydia pneumoniae, and Mycoplasma pneumoniae, as reported in previous studies (Freymuth et al., 1999; Rose et al., 2003).

Multiplex PCR

Clinical samples were analyzed using the BioFire FilmArray Respiratory Panel (BioFire Diagnostics, Salt Lake City, UT, USA). This panel was used in a multiplex PCR assay that detects 20 respiratory pathogens, including RSV, influenza A virus H1, influenza A virus H1 2009, influenza A virus H3, influenza B virus, adenovirus, parainfluenza virus types 1–4, human rhinovirus (HRV)/human enterovirus (HEV), human metapneumovirus, human bocavirus, human coronavirus types OC43, 229E, NL63, and HKU1, Bordetella pertussis, Chlamydia pneumoniae, and Mycoplasma pneumoniae (BioFire Diagnostics, 2013; Poritz et al., 2011).

Phylogenetic characterization

Pairwise alignment was performed using BioEdit 7.2.5 (Hall, 1999), while multiple sequence alignment was performed using MUSCLE 3.8 (Edgar, 2004), where the aligned sequences were further manually inspected and edited. Phylogeny reconstruction and evaluation were inferred with the maximum likelihood method in MEGA 6.0.6 (Tamura et al., 2013), using the transition/transversion ratio and alpha parameter of the gamma distribution estimated by maximum likelihood with TREE-PUZZLE software (Schmidt et al., 2002). The robustness of the maximum likelihood trees was evaluated statically by bootstrap analysis with 1000 bootstrap samples. The genomic sequences of vaccine strains recommended by the World Health Organization (WHO) were used as reference sequences and were retrieved from the National Center for Biotechnology Information (NCBI) Influenza Virus Resource (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) and the Global Initiative on Sharing All Influenza Data (GISAID, http://www.gisaid.org).

Nucleotide sequence accession numbers

The hemagglutinin nucleotide sequences of the 23 influenza A viruses isolated in this study have been deposited in the GISAID database and assigned accession numbers **EPI944520**–**EPI944542**.

Results

Surveillance

From November 2016 to January 2017, a total of 60 samples from three sentinel sites in Taipei City were collected and sent to the Taiwan CDC. Among the subjects, 27 (45%) were male and 33 (55%) were female, and they ranged in age from 1 to 96 years. The most common clinical manifestation was fever >38.5 $^\circ$C. Overall, a total of 52 samples were positive for respiratory pathogens; no dual infections were found in any patient. Of these 52 samples, 24 (40%) were influenza A-positive (23 cases of A/H3 and one case of A/H1). The other non-influenza viruses were RV/HEV (n=13, 21.6%), parainfluenza virus (n=3, 5%), human coronavirus OC43 (n=1, 1.7%), Mycoplasma pneumoniae (n=2, 3.3%), human metapneumovirus (n=1, 1.7%), and human adenovirus (n=5, 8.3%) (Table 1).

Table 1

Age distribution of the patients and clinical identification of respiratory pathogens during the 2016–2017 influenza season, Taiwan.

	Patients (n = 60)		Symptoms	Clinical identification							
	Male	Female	Fever (>38.5 $^\circ$C)	Influenza A virus	Adenovirus	Parainfluenza virus	Human rhinovirus/ enterovirus	Human metapneumovirus	Human coronavirus OC43	RSV	*Mycoplasma pneumoniae*
Age group	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
0–5	2 (3.3)	7 (11.6)	9 (15.0)	2 (3.3)	1 (1.7)	1 (1.7)	3 (5.0)	1 (1.7)			
6–18	19 (31.7)	14 (23.3)	33 (55.0)	14 (23.3)	3 (5.0)	2 (3.3)	8 (13.3)			2 (3.3)	
19–24	2 (3.3)	4 (6.6)	4 (6.6)	1 (1.7)					1 (1.7)		2 (3.3)
25–49	3 (5.0)	6 (10.0)	9 (15.0)	6 (10.0)	1 (1.7)		1 (1.7)			1 (1.7)	
50–64	0 (0.0)	2 (3.3)	2 (3.3)				1 (1.7)				
65+	1 (1.7)	0 (0.0)	1 (1.7)	1 (1.7)							
Total	27 (45.0)	33 (55.0)	58 (96.6)	24 (40.0)	5 (8.3)	3 (5.0)	13 (21.6)	1 (1.7)	1 (1.7)	3 (5.0)	2 (3.3)

RSV, respiratory syncytial virus.

Table 2
Performance of the FilmArray assay on clinical specimens compared to other laboratory diagnostic assays.

Pathogens	Genotype	Number of positive[a]		
		Multiplex PCR (FilmArray)	ESPLINE Influenza A&B-N assay	Cell culture/IFA
		n (%)	n (%)	n (%)
Influenza A virus	Total	24 (40.0)	22 (36.7)	24 (40.0)
	H3N2	23 (38.3)	21 (35.0)	23 (38.3)
	pdmH1N109	1 (1.7)	1 (1.7)	1 (1.7)
Other respiratory viruses	Total	28 (46.6)		26 (43.3)
Human rhinovirus/enterovirus		13 (21.6)		13 (21.6)
Adenovirus		5 (8.3)		5 (8.3)
RSV		3 (5.0)		3 (5.0)
Parainfluenza virus		3 (5.0)		3 (5.0)
Human metapneumovirus		1 (1.7)		1 (1.7)
Human coronavirus OC43		1 (1.7)		1 (1.7)
Mycoplasma pneumoniae		2 (3.3)		

IFA, immunofluorescence assay; RSV, respiratory syncytial virus.
[a] Total test: 60 specimens.

The results of the FilmArray assay, the rapid diagnostic test ESPLINE Influenza A&B-N, and viral culture/IFA are shown in Table 2. Of the 24 influenza-positive cases, the rapid test identified 22 and FilmArray identified all 24, with a positive predictive value of 100%. The rapid test showed lower sensitivity than the FilmArray assay. All of the samples that were positive by FilmArray also yielded positive isolates by cell culture in combination with the IFA method; the results of the FilmArray assay and cell culture were identical.

Phylogenetic analysis of influenza A H3N2 virus

Although the relative prevalence rates of influenza viruses vary from season to season, influenza A H3N2 virus was the major epidemic strain isolated during the study period. Phylogenetic analysis of the influenza A H3N2 virus HA1 genes indicated that all recently circulating influenza A H3N2 viruses in Taiwan fell into clade 3C.2a, and the majority of them represented a new drifted A H3N2 genetic type, subclade 3C.2a1 (Figure 1).

Three new phylogenetic subclades of A H3N2 emerged in 2014, one in subdivision 3C.2 (3C.2a) and two in 3C.3 (3C.3a and 3C.3b). Viruses in subclade 3C.2a have predominated in most regions of the world in recent years, and viruses with a further change N171K in the HA1 gene are referred to as subclade 3C.2a1 (Harvala et al., 2017; World Health Organization, 2016; European Centre for Disease Prevention and Control (ECDC), 2016). Antigenic characterization of these 3C.2a viruses continues to be technically challenging due to their low or undetectable hemagglutination activity, as described in many studies (Lednicky et al., 2016; Lin et al., 2017; Skowronski et al., 2016). In Taiwan, these viruses were first detected in early 2016. In addition, two influenza A H3N2 isolates were identified with two additional substitutions I58V and S219Y forming clade 3C.2a and belonging to a novel subclade 3C.2a2 cluster I; this new subclade emerged and was proposed in London recently (Harvala et al., 2017).

Discussion

This study is the first to evaluate the suitability of the FilmArray multiplex PCR method to survey respiratory pathogens among patients with ILI during the influenza season in Taiwan. Influenza surveillance in Taiwan is based on sentinel physician and laboratory isolation of influenza viruses (Lin et al., 2008). However, other respiratory viruses cause upper respiratory tract infections at rates similar to those of influenza A virus and account for a substantial percentage of all ILI cases (Fowlkes et al., 2014; Otomaru et al., 2015). Data from this study showed that

around 60% of ILI cases seen at sentinel sites were not caused by influenza virus infections. Even during the influenza season there are still many other respiratory pathogens, not only viruses but also bacteria, that co-circulate with influenza virus. Of note, HRV/HEV most part of the non-influenza infections in Taiwan, and data from further laboratory investigations showed that 11 (84.6%) of these cases were caused by enteroviruses CA2, CA6, and CA10. The rate of positivity for HRV/HEV infection in the present study is higher than that reported in previous studies (Liao et al., 2015; Lin et al., 2004).

The ILI surveillance conducted in Taiwan during the 2016–2017 influenza season is presented in this study, including the involvement of other respiratory viruses, and the results suggest the need for more sensitive and specific laboratory diagnostic systems along with clinician prescription to avoid the misuse or overuse of medical resources and the spread of communicable diseases.

With regard to seasonal influenza virus activity, previous studies have shown that it is possible to capture a significant proportion of influenza virus diversity in Taiwan, as well as the early emergence of new phylogenetic lineages and antigenic variants in neighboring countries, which are likely to be the progenitor epidemic strains in the following season (Chiu et al., 2014; Lin et al., 2011). In this study, the concurrent existence of European strains in the 3C.2a2 subclade was identified (Harvala et al., 2017), and this genetic observation showed that epidemic strains of the same serotype isolated in Taiwan were comparable to strains isolated elsewhere; however, the origin and effects of strains introduced from outside Taiwan could not be addressed in this study. It remains unclear why Taiwan is an important region for viral diversity. However, the geographical position of Taiwan provides an ideal environment for viral migration between tropical and temperate regions. Furthermore, among the countries of East Asia, Taiwan is particularly highly interconnected with the world via air travel. Thus the continuous monitoring of Taiwanese isolates may provide a complete profile of viral antigenic and genetic evolution patterns that may be important to assist in the early prediction of potential new regional or pandemic strains.

This study has some limitations. First, the surveillance was implemented at three sentinel sites and surrounding areas in Taipei City, which may limit the ability to generalize the findings to the entire country. Second, in the case of negative results for any of the tested pathogens, no further tests were performed to identify other etiological agents including bacteria. Third, it is possible that these observations were limited by the particular strains of respiratory viruses circulating in the study region.
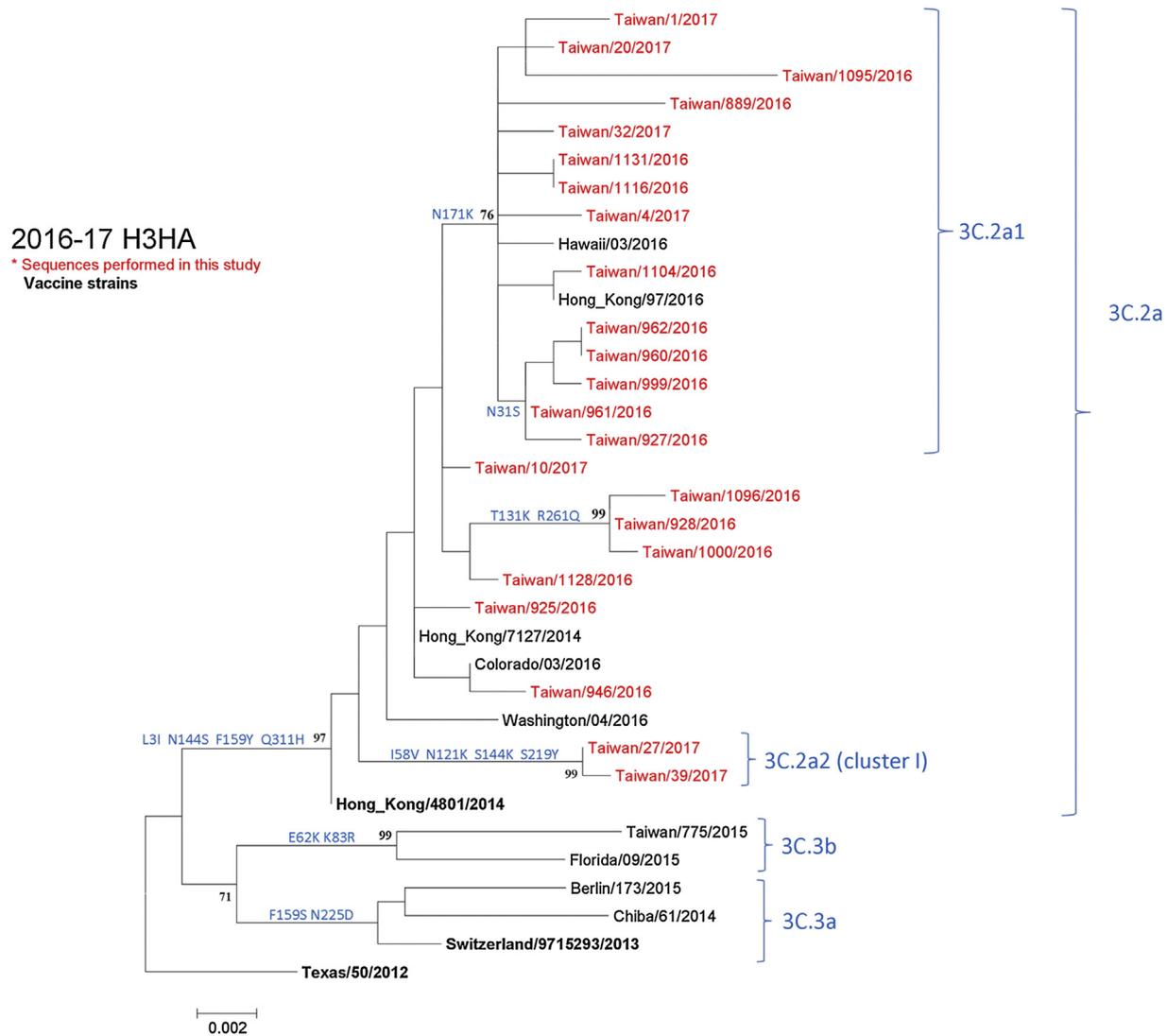
Figure 1. Molecular phylogenetic analysis by maximum likelihood method. The evolutionary history was inferred using the maximum likelihood method based on the Tamura–Nei model. Numbers to the left of the nodes are bootstrap percentages (1000 replications). Bootstrap values of less than 70 are not shown. Initial tree(s) for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA 6.

Larger studies involving specimens from different geographical areas would be helpful to assess the reliability and validity of the study findings.

The facilities available for the rapid diagnosis of these microorganisms in our laboratory include an automated NucliSens easyMAG instrument (bioMérieux, Marcy l'Etoile, France) for nucleic acid extraction, a LightCycler thermal cycler (Roche, Molecular Biochemical) for real-time PCR detection, and a Perkin Elmer 2700 thermocycler (Applied Biosystems, Forest City, CA, USA) for conventional RT-PCR reactions. The total runtime is approximately 6.5 h and the cost of testing eight specimens for all 20 respiratory pathogens is about USD 623.80, including reagents and supplies. Assuming the use of a single FilmArray instrument for diagnosis, it takes about 8.5 h to test eight specimens. The list price for eight FilmArray Respiratory Panel kits is USD 1032.00 (USD 129.00/test), and no other reagents or supplies are needed. Thus, the cost of the FilmArray assay is higher than that of the previously used conventional methods for respiratory pathogen testing. However, the FilmArray Respiratory Panel offers significant advantages such as accuracy, ease of use, faster turnaround time, and less hands-on time, and this test may be more favorable than other available methods.

Although advances in diagnostic testing and improvements in clinical care have been made over the years, limitations remain in the area of rapid testing for multiple pathogens and there is a need to shorten the turnaround time to results. The FilmArray system has the ability to perform high-order multiplex testing yet is simple to use and requires minimal hands-on time. Previous studies have shown the rapid evolution of Taiwanese influenza isolates in terms of antigenic and genetic diversity, which may result in epidemic strains that have the capacity to become the dominant strains worldwide (Chiu et al., 2014; Lin et al., 2011; Lin et al., 2007). Of note, the data from this study show that the FilmArray system was able to detect new genetic variants of the influenza A H3N2 virus even in the small number of samples tested. Thus, the system could be used as the first-line diagnostic test for the sensitive and accurate detection of known respiratory pathogens.
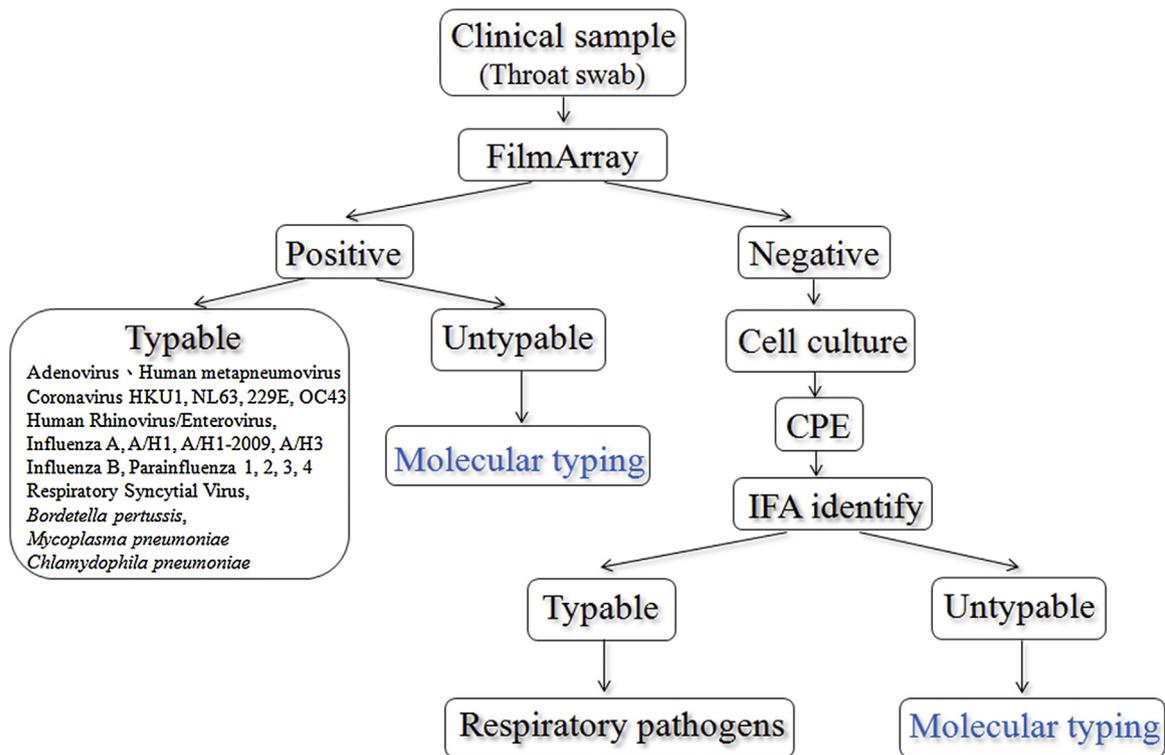
Figure 2. Schematic illustration of the workflow for the detection of common upper respiratory infection pathogens. Sample processing steps include virus isolation from the clinical sample, followed by immunofluorescence assay (IFA) identification when a cytopathic effect (CPE) is observed. The IFA untypeable viruses or pathogens are then detected by RT-PCR and sequencing.

A shortcoming of the FilmArray system is that it does not detect other clinically important respiratory viruses such as the Middle East respiratory syndrome coronavirus (MERS-CoV), human avian influenza viruses, and enterovirus D68, and it does not differentiate between human rhinovirus and enterovirus. Thus, a second detection system would be required if a full diagnostic profile is needed. The recommended analytical scheme for laboratory diagnosis is shown in Figure 2.

Due to its high sensitivity and simplicity of performance, the FilmArray can be applied at the front line of diagnostics. The use of the FilmArray assay facilitates not only urgent testing but also laboratory surveillance for common viral and bacterial respiratory pathogens.

In conclusion, this study demonstrated the use of a highly sensitive assay using clinical samples that is feasible for application worldwide. This may lead to an increased rate of diagnosis of viral infections and to improved patient outcomes, and in particular to a reduction in the overuse of antibiotics and antivirals.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

References

BioFire Diagnostics. BioFire FilmArray respiratory panel package insert. Salt Lake City, UT: BioFire Diagnostics; 2013.

Chang HL, Hsu JF, Tsai YM, Lin SY, Kuo HF, Yang CJ. Acute respiratory distress syndrome and acute myocarditis developed in a previously healthy adult with influenza B. BMC pulmonary medicine 2016;16:1.

Chiu SC, Lin JH, Wang HC, Wu HS, Chang HW, Lin YC, et al. Molecular epidemiologic and clinical characteristics of influenza B-associated complications among hospitalized patients during an outbreak in Taiwan. International Journal of Infectious Diseases: IJID: official publication of the International Society for Infectious Diseases 2014;23:94–100.

Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic acids research 2004;32:1792–7.

European Centre for Disease Prevention and Control (ECDC). Influenza virus characterisation, summary Europe. Stockholm: ECDC; 2016 September 2016.

Fowlkes A, Giorgi A, Erdman D, Temte J, Goodin K, Di Lonardo S, et al. Viruses associated with acute respiratory infections and influenza-like illness among outpatients from the Influenza Incidence Surveillance Project, 2010-2011. The Journal of Infectious Diseases 2014;209:1715–25.

Freymuth F, Vabret A, Brouard J, Toutain F, Verdon R, Petitjean J, et al. Detection of viral, Chlamydia pneumoniae and Mycoplasma pneumoniae infections in exacerbations of asthma in children. Journal of clinical virology: the official publication of the Pan American Society for Clinical Virology 1999;13:131–9.

Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 1999;13:131–9.

Harvala H, Frampton D, Grant P, Raffle J, Ferns RB, Kozlakidis Z, et al. Emergence of a novel subclade of influenza A(H3N2) virus in London, December 2016 to January 2017. Euro surveillance: bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin 2017;22.

Kim YJ, Hong JY, Lee HJ, Shin SH, Kim YK, Inada T, et al. Genome type analysis of adenovirus types 3 and 7 isolated during successive outbreaks of lower respiratory tract infections in children. Journal of clinical microbiology 2003;41:4594–9.

Lednicky JA, Iovine NM, Brew J, Loeb JC, Sugimoto JD, Rand KH, et al. Hemagglutinin Gene Clade 3C.2a Influenza A(H3N2) Viruses, Alachua County, Florida, USA, 2014-15. Emerging infectious diseases 2016;22:121–3.

Liao X, Hu Z, Liu W, Lu Y, Chen D, Chen M, et al. New Epidemiological and Clinical Signatures of 18 Pathogens from Respiratory Tract Infections Based on a 5-Year Study. PloS one 2015;10:e0138684.

Lin TY, Huang YC, Ning HC, Tsao KC. Surveillance of respiratory viral infections among pediatric outpatients in northern Taiwan. Journal of clinical virology: the official publication of the Pan American Society for Clinical Virology 2004;30:81–5.

Lin JH, Chiu SC, Shaw MW, Lin YC, Lee CH, Chen HY, et al. Characterization of the epidemic influenza B viruses isolated during 2004-2005 season in Taiwan. Virus research 2007;124:204–11.

Lin JH, Chiu SC, Lee CH, Su YJ, Tsai HC, Peng YT, et al. Genetic and antigenic analysis of epidemic influenza viruses isolated during 2006-2007 season in Taiwan. Journal of medical virology 2008;80:316–22.

Lin JH, Chiu SC, Cheng JC, Chang HW, Hsiao KL, Lin YC, et al. Phylodynamics and molecular evolution of influenza A virus nucleoprotein genes in Taiwan between 1979 and 2009. PloS one 2011;6:e23454.

Lin Y, Wharton SA, Whittaker L, Dai M, Ermetal B, Lo J, et al. The characteristics and antigenic properties of recently emerged subclade 3C.3a and 3C.2a human influenza A(H3N2) viruses passaged in MDCK cells. Influenza and other respiratory viruses 2017;11:263–74.

Otomaru H, Kamigaki T, Tamaki R, Opinion J, Santo A, Daya E, et al. Influenza and other respiratory viruses detected by influenza-like illness surveillance in Leyte Island, the Philippines, 2010-2013. PloS one 2015;10:e0123755.

Poritz MA, Blaschke AJ, Byington CL, Meyers L, Nilsson K, Jones DE, et al. FilmArray, an automated nested multiplex PCR system for multi-pathogen detection: development and application to respiratory tract infection. PloS one 2011;6: e26047.

Poutanen SM, Low DE, Henry B, Finkelstein S, Rose D, Green K, et al. Identification of severe acute respiratory syndrome in Canada. The New England journal of medicine 2003;348:1995–2005.

Rose TM, Henikoff JG, Henikoff S. CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) PCR primer design. Nucleic acids research 2003;31:3763–6.

Schmidt HA, Strimmer K, Vingron M, von Haeseler A. TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics 2002;18:502–4.

Skowronski DM, Sabaiduc S, Chambers C, Eshaghi A, Gubbay JB, Krajden M, et al. Mutations acquired during cell culture isolation may affect antigenic characterisation of influenza A(H3N2) clade 3C.2a viruses. Euro surveillance: bulletin Europeen sur les maladies transmissibles=European communicable disease bulletin 2016;21:30112.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular biology and evolution 2013;30:2725–9.

Tsai HP, Kuo PH, Liu CC, Wang JR. Respiratory viral infections among pediatric inpatients and outpatients in Taiwan from 1997 to 1999. Journal of Clinical Microbiology 2001;39:111–8.

van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, et al. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nature medicine 2001;7:719–24.

World Health Organization, 2016. Recommended composition of influenza virus vaccines for use in the 2017 southern hemisphere influenza season. Weekly Epidemiological Record 91, 469–484.

Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. The New England journal of medicine 2012;367:1814–20.