



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



Evaluation of a polymerase chain reaction–electrospray ionization time-of-flight mass spectrometry for the detection and subtyping of influenza viruses in respiratory specimens



Catherine Mengelle^{a,*}, Jean-Michel Mansuy^a, Isabelle Da Silva^a, Jean-Luc Guerin^b, Jacques Izopet^{a,c}

^a Department of Virology, CH Toulouse, 330 Avenue de Grande Bretagne, TSA 40031, 31059 Toulouse Cedex 09, France

^b Vet-school of Toulouse, 23 Chemin des capelles, BP87614 Toulouse, France

^c Department of Physiopathology, Toulouse Purpan, Unité Inserm U563, Toulouse, France

ARTICLE INFO

Article history:

Received 14 November 2012

Received in revised form 27 February 2013

Accepted 6 March 2013

Keywords:

Influenza
Mass spectrometry
Respiratory viruses
Multiplex PCR
Direct diagnosis

ABSTRACT

Background: PCR coupled to electrospray ionization mass spectrometry technology (PCR/ESI-TOF-MS) (PLEX-ID system, Abbott Ibis Biosciences) was developed to characterize microbial pathogens.

Objectives: To evaluate the performance of the PLEX-ID flu detection™ kit for detecting Influenza viruses by comparison with the multiplex RespiFinder® Kit (PathoFinder).

Study design: Acute-phase respiratory samples ($n = 293$) were analysed for this purpose. A subpopulation of influenza type A positive samples, identified with the RespiFinder® kit ($n = 64$), were subtyped with the RealTime ready Inf A/H1N1 Detection Set® (Roche Molecular Diagnostics) and results were compared to the PLEX-ID Flu Detection™ kit.

Results: 274 samples gave concordant results (93.5%, $p < 0.0001$): 65 influenza A-positive, 18 influenza B-positive and 191 negative samples. Of these, 7 samples were PLEX-ID positive/RespiFinder® negative (5 influenza A and 2 influenza B) and 12 were PLEX-ID positive/RespiFinder® negative (10 influenza A and 2 influenza B). PLEX-ID showed one sample as an influenza A and B co-infection while the RespiFinder® assay showed it to be influenza A-positive. The sensitivity, specificity, positive and negative predictive values of the PLEX-ID™ system were 87.4%, 96.5%, 92.2% and 94.1% respectively. Thirteen of 19 discordant samples available for retesting were investigated further with the Anyplex™ II RV16 Detection kit (Seegene): seven were RespiFinder® concordant, while six were PLEX-ID™ concordant. Subtyping of 61/64 influenza A samples was concordant (95.3%): 55 were H1N1pdm09 and six were non-H1N1pdm09. Three samples gave negative PLEX-ID™ results (one H1N1pdm09 and two non-H1N1pdm09).

Conclusions: PCR/ESI-TOF-MS technology showed good diagnostic performances to detect and subtype influenza viruses.

© 2013 Elsevier B.V. All rights reserved.

1. Background

Influenza viruses are responsible for winter epidemics among children and adults, with huge potential costs for society.¹ Influenza viruses are detected directly from respiratory samples.² That was originally done using cell cultures or direct antigen detection³ but culture-based methods are time-consuming and direct antigen detection is not sensitive enough to provide a reliable

diagnosis. Molecular diagnostics, which is globally the current standard technology, overcomes these limitations.⁴ The majority of molecular approaches to influenza viruses detection are based upon real-time reverse-transcription PCR targeting the segment encoding the conserved matrix protein (*M*) gene segment.⁵ Downstream assays can be used to type influenza A or B or further subtype influenza A.

Some influenza A subtypes (H1N1pdm09, non-H1N1pdm09, H3N2, avian H5N1) can be detected using current molecular techniques that target hemagglutinin (*HA*) and neuraminidase (*NA*).⁶ However, it is still difficult to detect and identify emerging viruses with reassorted internal genes.

New technologies like the polymerase chain reaction (PCR) electrospray ionization time-of-flight mass spectrometry (PCR/ESI-TOF-MS) should be able to identify these pathogens. The method

Abbreviations: PCR–ESI–TOF–MS, PCR-electro-spray ionization time-of-flight mass spectrometry; RT-PCR, reverse-transcription PCR; MLPA, multiplex ligation-dependent probe amplification; RSV, respiratory syncytial virus.

* Corresponding author. Tel.: +33 5 676 90 919; fax: +33 5 676 90425.

E-mail address: mengelle.c@chu-toulouse.fr (C. Mengelle).

was originally applied in microbiology,⁷ for identifying species of bacteria in respiratory samples,⁸ but it has also been used to identify viruses including coronavirus⁹ and adenovirus.¹⁰ PCR/ESI-TOF-MS technology has more recently been used to identify and characterize the recombinant influenza A H1N1pdm09^{11–14} and to identify the origin of the gene segments of that reassortant virus of porcine, human and avian influenza viruses.^{15,16} The technique has also been independently evaluated for its ability to type influenza in retrospective nasopharyngeal specimens.¹⁷ The present study is the first showing results of samples collected prospectively and compared to a multiplex reference method different from any previously published method.

2. Objectives

This study evaluates the suitability of the PLEX-ID flu detectionTM assay based upon a reverse-transcription (RT) coupled to PCR/ESI-TOF-MS, for detecting influenza viruses in prospective clinical respiratory samples and determining their subtypes.

Samples tested with the multiplex RespiFinder kit[®]^{18,19} were used as a reference for comparison and discrepant results were tested with a third RT-PCR multiplex molecular technique for confirmation of results.

We discriminated between H1N1pdm09 and non-H1N1pdm09 influenza A subtypes in a subpopulation of samples that were influenza A positive with the RespiFinder kit[®] using the RealTime ready Inf A/H1N1 Detection Set[®]. The results were compared to those obtained with the PLEX-ID Flu detection[®] assay.

3. Study design

3.1. Materials

The Department of Virology, CHU Toulouse, France, tested 293 respiratory samples prospectively collected during the 2010–2011 winter season when the Flu epidemic was on its peak. Of these, 148 were from the French Flu survey network and 145 were collected from children attending the Pediatric Unit of the Toulouse University Hospital.

Nasopharyngeal swab samples were collected on Virocult[®] swabs (Kitvia, Labarthe Inard, France) and stored at $-80 \pm 5^\circ\text{C}$.

3.2. Methods

3.2.1. Multiplex ligation-dependent probe amplification (MLPA)

Respiratory samples were tested prospectively with the MLPA. Total nucleic acid was extracted with the MagNA Pure LC Total Nucleic Kit[®] (Roche) on the MagNA Pure LCTM instrument according to the manufacturer's instructions. Input sample volume was 200 μl and output elution volume was 100 μl (2-fold concentration).

Respiratory viruses were detected with the RespiFinder[®] DC kit, which can detect 15 respiratory viruses: influenza virus type A, influenza virus type B, avian influenza virus A/H5N1, respiratory syncytial viruses (RSV) A and B, parainfluenza viruses 1, 2, 3 and 4, coronaviruses OC43, 229E and NL63, rhinovirus, adenovirus and human metapneumovirus.

The method is based on amplifying viral genomes with a multiplex RT-PCR. Its sensitivity is due to hybridization, ligation and PCR of the probe. The various amplicons are detected by size-fractionation on a capillary electrophoresis system with the sequencing analyzer ABI 3130 XLS (Applied Biosystems). The internal amplification control is an RNA transcript from the encephalomyocarditis (EMC) virus: this checks for the presence of sample addition and the absence of PCR inhibitors.

3.2.2. Influenza A virus subtyping using real time PCR

The MLPA test does not differentiate between influenza A H1N1pdm09 and influenza A non-H1N1pdm09 viruses. The influenza type A viruses detected were subtyped by testing influenza A positive MagNA pure total nucleic acid extracts with the RealTime ready Inf A/H1N1 Detection Set[®] (Roche Diagnostics) employing the RNA virus master[®] one-step RT-PCR kit (Roche Diagnostics) according to manufacturer's instructions on the Light Cycler 480TM system (Roche Diagnostics, Meylan, France). The set contained specific primer/probe mixes for detecting influenza A matrix protein 2 (M2) and the H1N1pdm09-specific hemagglutinin HA1 (H1) in a single reaction.

3.2.3. PCR-electrospray ionization time-of-flight mass spectrometry (PCR/ESI-TOF MS)

Respiratory samples were analyzed with the PLEX-ID system (Abbott Molecular, IL, USA). RNA was extracted from nasopharyngeal swabs using the PLEX-ID Viral RNA Isolation Kit (Abbott Molecular, IL, USA) on the PLEX-ID FH and SP instruments in a fully automated manner. Input sample volume was 300 μl and output elution volume was 200 μl (1.5-fold concentration).

The PLEX-ID Fluid handler was used to distribute the nucleic acids and enzymes for PCR into the PLEX-ID Flu Detection plates. Viral nucleic acids were amplified using the PLEX-ID Flu DetectionTM kit (Abbott Molecular, IL, USA). Segments of the influenza genome were amplified by PCR on the PLEX-ID TC (Mastercycler ProS-Eppendorf) using nine primer-pairs located at core conserved sites.

The regions targets are shown in Table 1. The Pan-influenza-PB1 primer-pair detects all influenza viruses (A, B and C types). Primer-pairs targeting nucleoprotein (NP), matrix protein (M1), polymerase (PA), non-structural 1 (NS1) and polymerase basic 2 protein (PB2) gene segments were designed to amplify sequences of influenza A viruses only. Primer-pairs located on HA and NA glycoproteins were designed to amplify only influenza H1N1pdm09. A specific PB2 primer was selected to amplify influenza B in addition to the pan-influenza primer. The last well contained the PB2 and the influenza A-NA (N1-TFR) which covers the most common mutation (N1-TFR) associated with oseltamivir (Tamiflu) resistance (H275Y).^{20,21}

Each amplicon is automatically desalted and purified on a weak anion exchanger.²² Methanol-based aerosols containing denatured ionized amplicons are sprayed into the mass spectrometer. The time-of-flight to the detector is determined giving the mass of the amplicon.¹² By database analysis, the base composition (BC) of each PCR amplicon derived from each viral gene segment is determined. Influenza viruses are then typed and subtyped by bioinformatic analysis of the BC signatures produced by the nine target genes. The BC signatures are compared to known BC signatures in the reference database (Influenza Research Database, NCBI Influenza Virus Resource, Systems Influenza.org.) and viruses are identified by matching them to the closest signatures in the database.

3.2.4. Analysis of discordant results

The AnyplexTM II RV16 Detection kit (Seegene) was used as a third analytical technique for samples that gave discordant results. This multiplex RT-PCR test detects 16 viruses, including influenza A and B.

3.2.5. Statistical methods

Data were analyzed using StatView 5.0 StataTM software (StatCorp, Texas). The Kappa-Cohen test was used to compare assays. *P* values less than 0.05 were considered significant. The sensitivities (percentages of reactive samples) of these assays were compared using the McNemar's chi-squared test.

Table 1
Locations and sequences of the nine primer-pairs in each of the 8 wells employed for amplification and identification of influenza virus gene segments by electrospray ionization and mass spectrometry.

Primer identification	Location	Type of influenza virus	Sequence	Length
2798	PB1	A, B, C	Forward (F): TGTCTGGAATGATGATGGGCATGTT Reverse (R):TCATCAGAGGATTGGAGTCCATCCC	128
1266	NP	A	F: TACATCCAGATGTGCACTGAACTCAAATCA R: TCGTCAAATGCAGAGAGCACCATTCTCTCTA	111
1279	M1	A	F: TCTTGCCAGTTGATGGCCTCATATAC R: TGGGAGTCAGCAATCTGCTCACA	115
1287	PA	A	F: TGGGATTCTTTCGTCTCAGTCCGA R: TGGAGAAGTTCGGTGGGAGACTTTGGT	122
2775	NS1	A	F: TCCAGGACATACTGATGAGGATGTCAAAAATGCA R: TGCTTCCCAAGCGAATCTCTGTA	129
1259	PB2	A	F: TACCACTGTGGACCATATGGCCATAAT R: TCGGATATTTTCAATGCCATCATCCACTTCAT	105
5101	HA	AH1N1pdm09	F: TGGACAGGGATGGTAGATGGATGGTACGG R: TCTCGTCAATGGCATTCTGTGTCT	103
1261	PB2	B	F: TCCCATGTACTGGCATAACATGCTTGA	91
4998	N1-TFR	A	R: TATGAACTCAGCTGATGTTGCTCCTGC F: TCAGTCCGAATTGGATGCCCTCAAITA R: TATCAGGATAACAGGAGCACTCTCAT	58

4. Results

The 293 respiratory samples were collected from 156 females and 137 males (mean age: 13.1; median: 2.7; range: 0 months to 99 years). The mean age from those 148 from the French Flu survey network was 24.5 years and median was 16.9 years. The mean age from those 145 collected from children attending the Pediatric Unit of the Toulouse University Hospital was 1.6 years and the median was 0.4 years.

4.1. Detection of influenza viruses

The samples were tested by both the reference technique, the RespiFinder® DC kit, and by PCR–electrospray ionization–time-of-flight–mass spectrometry (PCR/ESI-TOF-MS) with the PLEX-ID Analyzer and the PLEX-ID™ Flu Detection™ kit (Table 2). A total of 274 samples gave concordant results (93.5% – $p < 0.0001$): 65 were influenza A positive and 18 were influenza B positive; 191 samples were negative by both techniques. One sample which was RespiFinder® Flu A positive showed a influenza A and B coinfection on the PLEX-ID™. The sensitivity was 87.4% (95%CI: 85.2–89.6)% and the specificity was 96.5% (95%CI: 93.9–99.1)%. The positive and negative predictive values were 92.2% and 94.1% respectively.

The PLEX-ID™ and the RespiFinder® reference assay gave discrepant results for 19 samples. Of these, 7 were PLEX-ID™ positive and RespiFinder® negative. There were 5 influenza A: three H1N1 pdm09, one non-H1N1pdm09 and one H3N2 influenza A virus. Three of these samples were positive for RSV with the RespiFinder® DC kit assay and were collected from children attending the emergency unit. Twelve samples were PLEX ID™ negative and RespiFinder® positive: ten samples were influenza A positive and two were influenza B positive.

4.2. Characterization of influenza A subtypes

We determined the subtypes of 64 samples that tested positive for influenza A viruses with the RespiFinder® using the RealTime ready Inf A/H1N1 Detection Set® (Roche Molecular Diagnosis): 56 samples were H1N1pdm09 and eight were influenza A non-H1N1pdm09 viruses.

We compared these results to those obtained with the PLEX-ID™ Flu detection assay (Table 3) and found that 61/64 samples gave concordant results (95.3%): 55 were H1N1pdm09 and 6 were non-H1N1pdm09 viruses (i.e. H3N2 Flu A with the RespiFinder® assay). Three samples gave negative PLEX-ID™ results. One of

these samples was H1N1pdm09 and two were non-H1N1pdm09 influenza A viruses.

4.3. Analysis of discrepant result

Thirteen discordant samples were tested with the Anyplex™II RV16 Detection kit: seven were RespiFinder® concordant, while six were PLEX-ID™ concordant (Table 4). Six discordant samples were not tested due to a lack of material.

5. Discussion

This study reported an evaluation of the PLEX-ID technology for detecting and identifying influenza genomes from clinical samples with the PLEX-ID™ Flu detection kit on the PLEX-ID™ Analyzer.

The emergence of reassortant influenza A viruses due to the accumulation of mutations in the *H* and the *N* genes²³ may lead to the appearance of new viruses that may spread throughout the whole human population as was the case for the H1N1pdm09 virus. This is a triple reassortant porcine H1N1 that has acquired the PB1 gene from the human influenza A H3N2virus.¹⁵ This virus has been sequenced, but its porcine origin was only discovered by PCR coupled to electrospray ionization time-of-flight mass spectrometry.²⁴

The suitability and sensitivity of PCR/ESI-TOF-MS technology for use in clinical diagnosis have yet to be determined. We therefore evaluated the suitability of this new technique for detecting and subtyping the influenza genomes in prospective clinical samples.

The reference technique was the widely used RespiFinder® DC commercial kit that can detect 15 respiratory viruses, including Influenza A, B and H5N1 avian virus. As this kit does not identify H1N1 pdm09, we used a group of samples that were influenza A positive by the RespiFinder® to distinguish between H1N1pdm09 and non-H1N1pdm09 viruses using the RealTime ready Inf A/H1N1 Detection Set® assay.

The RespiFinder® and the PLEX-ID™ Flu detection assays performed comparably and gave similar results. Samples that gave discrepant results were tested with a third (confirmatory) technique, resulting in seven reference/confirmatory assay positives and six PLEX-ID/confirmatory assay positives. This could be attributed to intermittent or stochastic positivity at the lower limit of detection of the methodologies in low titre samples. This was confirmed by the high crossing point values (Ct) obtained with the confirmatory technique (38.74–43.13 cycles).

The PLEX-ID™ Flu detection assay detected both influenza A and influenza B genomes and also distinguished between H1N1pdm09

Table 2

Detection and identification of influenza genomes in 293 prospective clinical respiratory samples with the RespiFinder® and PLEX-ID™ Flu assays.

		RespiFinder® assay Influenza A positive	Influenza B positive	Negative	Total
PLEX-ID™ Flu detection assay	Influenza A Positive	65 ^a	0	5 ^b	70
	Influenza B Positive	1 ^a	18	2	20
	Negative	10	2	191	203
	Total	75	20	198	293

Number in bold are the concordant results between the PLEX-ID™ Flu detection assay and the RespiFinder® reference assay.

^a One sample with influenza A and influenza B coinfection was diagnosed by the PLEX-ID™ Flu detection kit.^b Three H1N1 pdm09, one non-H1N1 pdm09 and one H3N2 influenza A virus.**Table 3**

RealTime ready Inf A/H1N1 Detection Set® and PLEX-ID™ FLU detection assay: Distinction between H1N1pdm09 and non-H1N1pdm09 viruses in a sub population of 64 Flu A RespiFinder® positive samples.

PLEX-ID™ Flu detection assay	RealTime ready Inf A/H1N1 Detection Set® Influenza A sub-typing	H1N1pdm09	A non-H1N1pdm09	Total
	A H1N1pdm09	55	0	55
A H3N2	0	6	6	
Negative	1	2	3	
Total	56	8	64	

Number in bold are the concordant results between the PLEX-ID™ Flu detection assay and the RespiFinder® reference assay.

Table 4

Anyplex™ II RV16 Detection kit: results obtained from 19 discrepant samples.

		RespiFinder® positive/PLEX-ID™ Flu detection assay negative: N = 7	RespiFinder® negative/PLEX-ID™ Flu detection assay positive: N = 12
Anyplex™ II RV16 Detection kit	Positive	2	5
	Negative	2	4
	Not tested	3	3

and non-H1N1pdm09 viruses as shown by comparison with the RealTime ready Inf A/H1N1 Detection Set®. The PLEX-ID™ system also detected both influenza A and B types in a single sample, which could not be done with the reference technique. The possibility of coinfection has also been demonstrated by testing 145 samples with another technique (Influenza A/B r-gene™ – ARGENE-bioMérieux–Verniolle–France). Thirty-eight samples were positive for influenza A or B and one sample tested positive for influenza A & B (data not shown).

The RespiFinder® technique did not detect the influenza A genome in three samples that were positive for RSV. As the primers can compete with each other, some viruses present in minor quantities in clinical samples may not be amplified. The PLEX-ID™ system suffered no such restriction as it employs multiple primer-pairs targeting core regions of the viruses in multiplex end-point RT-PCRs.

The PLEX-ID platform that we evaluated is a complete system that includes extraction, PCR and analysis with the PLEX-ID™ analyzer. The PCR analysis is twice as fast as with the Ibis T5000²⁵ (Ibis Biosciences, Inc., Carlsbad, CA, USA) which was the first platform used for PCR/ESI-TOF-MS technology. The PLEX-ID™ input stacker can take 15 plates of PCR and the platform can process approximately 300 samples per 24-hour. The new kit used in this study can also identify H1N1pdm09 viruses, which the previous one^{12,26,27} could not.

Influenza A viruses are identified and subtyped by bioinformatic analysis of detected amplicons. This signature is then referred to a regularly updated database that contains about 10,000 reference BC signatures.

This means that all genotypes of the influenza A genome may be recognised and diagnosed, as well as new zanamivir antiviral resistance mutation²⁸ and newly emerging swine variants (H3N2 v).²⁷ An “open-mode” reference database system that is more easily accessed would be a great help, as it would enable the detection of

changes in a virus during a winter season.¹² These new signatures could then be added to keep the database up-to-date.

In conclusion, our results obtained from clinical samples collected prospectively show that the assay can detect and identify influenza A and B genomes, as well as coinfections with the two viruses. It can also successfully subtype the influenza A genomes. The assay results were well correlated with those obtained with the reference method, showing that the primer-pairs designed based on core conserved regions of the virus genome are suitable for identifying and characterizing influenza viruses.

Funding

The PLEX-ID Flu Detection™ kits were provided by Abbott, Ibis Biosciences.

Competing interests

None.

Ethical approval

This was a non-interventional study with no addition to the usual procedures. Biological material and clinical data were obtained only for standard viral diagnosis following physicians' orders (no specific sampling, no modification of the sampling protocol, no supplementary question to the national standardized questionnaire). Data analyses were carried out using an anonymized database. According to the French Law of Public Health (CSP Art L 1121-1.1), such protocol is exempt from written informed consent.

Acknowledgments

We thank D. Favy, M. Picard-Maureau, J. Smith and P. Leroux for assistance. The English text was checked by Dr Owen Parkes.

References

- Lipatov AS, Govorkova EA, Webby RJ, Ozaki H, Peiris M, Guan Y, et al. Influenza: emergence and control. *J Virol* 2004;**78**(17):8951–9.
- De Gascun CF, Carr MJ, Hall WW. Influenza viruses. In: Cohen J, Powderly W, editors. *Infectious diseases*. 3rd ed Amsterdam: Elsevier; 2010.
- Petric M, Comanor L, Petti CA. Role of the laboratory in diagnosis of influenza during seasonal epidemics and potential pandemics. *J Infect Dis* 2006;**194**(Suppl 2):S98–110.
- Ginocchio CC, Zhang F, Manji R, Arora S, Bornfreund M, Falk L, et al. Evaluation of multiple test methods for the detection of the novel 2009 influenza A (H1N1) during the New York City outbreak. *J Clin Virol* 2009;**45**(3):191–5.
- Fouchier RA, Bestebroer TM, Herfst S, Van Der Kemp L, Rimmelzwaan GF, Osterhaus AD. Detection of influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene. *J Clin Microbiol* 2000;**38**(11):4096–101.
- Lee MK, Kim HR. Comparison of molecular assays for the rapid detection and simultaneous subtype differentiation of the pandemic influenza A (H1N1) 2009 virus. *J Microbiol Biotechnol* 2012;**22**(8):1165–9.
- Ecker JA, Massire C, Hall TA, Ranken R, Pennella TT, Agasino Ivy C, et al. Identification of *Acinetobacter* species and genotyping of *Acinetobacter baumannii* by multilocus PCR and mass spectrometry. *J Clin Microbiol* 2006;**44**(8):2921–32.
- Ecker DJ, Sampath R, Blyn LB, Eshoo MW, Ivy C, Ecker JA, et al. Rapid identification and strain-typing of respiratory pathogens for epidemic surveillance. *Proc Natl Acad Sci U S A* 2005;**102**(22):8012–7.
- Sampath R, Hofstadler SA, Blyn LB, Eshoo MW, Hall TA, Massire C, et al. Rapid identification of emerging pathogens: coronavirus. *Emerg Infect Dis* 2005;**11**(3):373–9.
- Blyn LB, Hall TA, Libby B, Ranken R, Sampath R, Rudnick K, et al. Rapid detection and molecular serotyping of adenovirus by use of PCR followed by electrospray ionization mass spectrometry. *J Clin Microbiol* 2008;**46**(2):644–51.
- Sampath R, Hall TA, Massire C, Li F, Blyn LB, Eshoo MW, et al. Rapid identification of emerging infectious agents using PCR and electrospray ionization mass spectrometry. *Ann N Y Acad Sci* 2007;(1102 (April)):109–20.
- Sampath R, Russell KL, Massire C, Eshoo MW, Harpin V, Blyn LB, et al. Global surveillance of emerging Influenza virus genotypes by mass spectrometry. *PLoS One* 2007;**2**(5):e489.
- Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature* 2009;**459**(7250):1122–5.
- Metzgar D, Baynes D, Myers CA, Kammerer P, Unabia M, Faix DJ, et al. Initial identification and characterization of an emerging zoonotic influenza virus prior to pandemic spread. *J Clin Microbiol* 2010;**48**(11):4228–34.
- Shinde V, Bridges CB, Uyeki TM, Shu B, Balish A, Xu X, et al. Triple-reassortant swine influenza A (H1) in humans in the United States, 2005–2009. *N Engl J Med* 2009;**360**(25):2616–25.
- Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* 2009;**325**(5937):197–201.
- Cordey S, Thomas Y, Suter P, Kaiser L. Pilot evaluation of RT-PCR/electrospray ionization mass spectrometry (PLEX-ID/Flu assay) on influenza-positive specimens. *Open Virol J* 2012;**6**:64–7.
- Reijmans M, Dingemans G, Klaassen CH, Meis JF, Keijnders J, Mulders B, et al. RespiFinder: a new multiparameter test to differentially identify fifteen respiratory viruses. *J Clin Microbiol* 2008;**46**(4):1232–40.
- Schouten JP, McElgunn CJ, Waaijjer R, Zwiijnenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res* 2002;**30**(12):e57.
- Gubareva LV. Molecular mechanisms of influenza virus resistance to neuraminidase inhibitors. *Virus Res* 2004;**103**(1,2):199–203.
- Herlocher ML, Truscon R, Elias S, Yen HL, Roberts NA, Ohmit SE, et al. Influenza viruses resistant to the antiviral drug oseltamivir: transmission studies in ferrets. *J Infect Dis* 2004;**190**(9):1627–30.
- Jiang Y, Hofstadler SA. A highly efficient and automated method of purifying and desalting PCR products for analysis by electrospray ionization mass spectrometry. *Anal Biochem* 2003;**316**(1):50–7.
- Nelson MI, Holmes EC. The evolution of epidemic influenza. *Nat Rev Genet* 2007;**8**(3):196–205.
- Faix DJ, Sherman SS, Waterman SH. Rapid-test sensitivity for novel swine-origin influenza A (H1N1) virus in humans. *N Engl J Med* 2009;**361**(7):728–9.
- Ecker DJ, Sampath R, Massire C, Blyn LB, Hall TA, Eshoo MW, et al. Ibis T5000: a universal biosensor approach for microbiology. *Nat Rev Microbiol* 2008;**6**(7):553–8.
- Deyde VM, Sampath R, Garten RJ, Blair PJ, Myers CA, Massire C, et al. Genomic signature-based identification of influenza A viruses using RT-PCR/electro-spray ionization mass spectrometry (ESI-MS) technology. *PLoS One* 2010;**5**(10):e13293.
- Deyde VM, Sampath R, Gubareva LV. RT-PCR/electrospray ionization mass spectrometry approach in detection and characterization of influenza viruses. *Expert Rev Mol Diagn* 2011;**11**(1):41–52.
- Hurt AC, Holien JK, Parker M, Kelso A, Barr IG. Zanamivir-resistant influenza viruses with a novel neuraminidase mutation. *J Virol* 2009;**83**(20):10366–73.