Development and Implementation of Influenza A Virus Subtyping and Detection of Genotypic Resistance to Neuraminidase Inhibitors

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Influenza virus hemagglutinin and neuraminidase, surface glycoproteins with an essential role in viral pathogenesis, are important antigen determinants and essential markers for epidemiological surveillance. Neuraminidase is also a suitable target for designing antiviral drugs. The introduction into clinical practice of neuraminidase inhibitors and the development of random point mutations have increased the emergence of drug-resistant viruses. A universal RT nested PCR-based system has been developed for subtyping H1, H3, N1 and N2, in influenza A viruses of human or animal origin. The subsequent sequencing and analysis of the hemagglutinin and neuraminidase templates reveal antigenic and receptor binding changes in the HA1 subunit and mutations of clinical relevance concerning resistance to neuraminidase inhibitors. The specificity and sensitivity of the method were evaluated using 113 influenza A isolates, 105 influenza A positive respiratory samples obtained from patients and 29 prototype strains of both human and animal origin. The resulting analytical sensitivity of the subtyping techniques is one to at least 100 molecules of cloned DNA product in a final reaction volume of 50 μl. In the course of implementing the method, two H1N1 isolates with the H274Y mutation in the neuraminidase segment have been detected and their molecular features analyzed. The emergence of influenza virus resistance makes the neuraminidase genetic characterization and surveillance activities to detect antiviral resistance necessary. *J. Med. Virol. 82:843–853, 2010.* © 2010 Wiley-Liss, Inc.

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INTRODUCTION

Influenza virus is an RNA virus of the *Orthomyxoviridae* family comprised of three medically important genera: *Influenzavirus* A, *Influenzavirus* B, and *Influenzavirus* C. Influenza A virus is the only species in the genus *Influenzavirus* A. Influenza A viruses are further classified, based on their viral surface proteins hemagglutinin (HA) and neuraminidase (NA). Sixteen HA subtypes (or serotypes) and nine NA subtypes of influenza A virus have been identified. Different influenza A viruses have been isolated from many hosts including humans, pigs, horses, marine mammals and a wide range of domestic and wild birds. Influenza surveillance studies, performed with humans and animals, have provided a wealth of data on the ecology and evolution of influenza viruses related to public health and economic impact [Fouchier et al., 2003; Govorkova et al., 2005; de Jong et al., 2006]. Influenza surveillance requires national and global monitoring of viral activity to determine the antigenic type and subtype of the circulating influenza A viruses. It is also important to detect the emergence of novel viruses, which may cause unexpected outbreaks or produce a

pandemic. It is generally accepted that numerous outbreaks in domestic and wild animals, humans, and human influenza pandemics have resulted from interspecies transmission of avian influenza viruses [Neumann and Kawaoka, 2006]. During the last decade, avian influenza A(H5N1) has caused several outbreaks in poultry and infected humans resulting in hundreds of deaths [WHO, 2009]. Nevertheless, the potential of avian influenza A(H5N1) to cause a global pandemic is uncertain and cannot be predicted [Holmes et al., 2005].

Although vaccination coverage varies markedly across continents [de Lataillade et al., 2009], the control of seasonal influenza infection in humans is based on annual vaccination of several risk groups and antiviral treatment of acute infection as well as by prophylaxis in unvaccinated people at high risk, such as the elderly or immunosuppressed patients [Couch, 2000]. Nevertheless, the overall prescription rates between 2002 and 2007 for oseltamivir remained at low levels (six prescriptions/1,000 inhabitants per year in the EU Member States) compared to those reported in Japan, where the prescription rate in 2005 was 70.9/1,000 inhabitants per year [Kramarz et al., 2009]. To select the vaccine strains, laboratory methods for characterizing influenza viruses have been primarily focused on changes occurring in the hemagglutinin (HA) genome segment that encodes the major surface antigen of the virus. The characterization of the genome segment coding the neuraminidase (NA) protein is currently much less frequent, even though immunity against this surface protein is also protective [Nicholson et al., 2003]. In addition, the introduction into clinical practice of NA inhibitors (NAIs) and, particularly, the natural variation of the NA sequence have increased the frequency of viral resistance. Prior knowledge of antiviral resistance features in potentially pandemic influenza viruses is necessary for global pandemic preparedness, stockpiling, and treatment. In spite of the recent circulation and the low incidence in humans of the potentially pandemic virus A(H5N1), oseltamivir resistance has been detected in infected patients [de Jong et al., 2005; Le et al., 2005]. In the case of seasonal epidemic influenza viruses, resistance to zanamivir has only been found in one, immunocompromised child infected with influenza B virus [Gubareva et al., 1998], although resistance to oseltamivir in patients infected with influenza A virus was found in 4% of adults [Gubareva et al., 2001], and 4–8% of children [Regoes and Bonhoeffer, 2006] and increases to 18% in Japanese children admitted to hospital [Kiso et al., 2004]. Recently, during the 2007–2008 influenza season, an unusually high incidence of oseltamivir-resistant A(H1N1) viruses was reported in many countries from the Northern and Southern hemispheres [WHO, 2008]. Due to the high rate of circulating resistant strains observed in regions where the use of oseltamivir is low (i.e., Europe), the origin of this increased oseltamivir resistance does not seem to be related to the use of this antiviral drug.

In summary, the genetic drift of influenza A viruses is often a determinant of virulence, contributing to the capacity to evade the immune response [Smith et al., 2004] and antiviral drug resistance. These factors increase the urgency of determining the genetic composition of circulating and emerging variants and the implementation of versatile and universal methods in virological laboratories to detect and characterize different influenza A subtypes, derived from different host species. In the present study, a universal RT nested PCR-based system was developed to subtypes N1 and N2 in influenza A viruses of animal or human origin. In addition, a method for universal subtyping of H1 and H3 is also described. This simple sequence based method is a useful and less time consuming alternative than phenotypic techniques for the assessment of NAIs susceptibility and should contribute to molecular surveillance of the genus *Influenzavirus* A.

MATERIALS AND METHODS

Clinical Samples and Virus Isolates

A total of 105 influenza A positive respiratory samples containing upper (pharyngeal and nasopharyngeal exudates, nasopharyngeal aspirates, nasal lavages) and lower (brochoalveolar lavages) respiratory tract samples, collected from 2004 to 2008, were analyzed using the HA and NA RT-nested PCR assay. These specimens were received for virological study at the Influenza and Respiratory Viruses Laboratory of the Spanish National Center for Microbiology. All samples were initially checked for Influenza A virus and another 13 different respiratory viruses using the PCR assays described previously [Coiras et al., 2003, 2004], human metapneumovirus [López-Huertas et al., 2005] and specimens collected from mid-2006 were also tested for human bocavirus [Pozo et al., 2007]. Further 113 isolates included in this study were received from the primary laboratories of the Spanish influenza surveillance network for characterization during the 2004–2008 seasons. The selected isolates were found to be positive for influenza A virus by RT-nested PCR [Coiras et al., 2003] and/or by isolation in Mardin–Darby canine kidney (MDCK) cell culture.

Specificity Controls

Twenty-four additional prototype strains were used to test the assay specificity (Table II), using 17 different HA/NA combinations of influenza A subtypes from animal origin; they were cultured for 48 h in allantoic fluid of 9-day-old embryonated chicken eggs at 34°C. The allantoic fluids were stored at −80°C under biosafety rules. In addition, five different influenza A and two different influenza B human reference strains harvested in MDCK cell culture were included.

Sensitivity Controls

To assess the analytical sensitivity of each PCR assay, controls were prepared in serial dilutions that contained

a known number of cloned amplified product. Cloned controls were obtained for each HA and NA subtype of A/New Caledonia/20/99 (H1N1) and A/Panama/2007/99 (H3N2). The TOPOTA Cloning System (Invitrogen Corporation, San Diego, CA) was used to clone PCR products. Selection of transformants was made on LB/ampicillin plates. The number of copies per tube was adjusted after measurement of the OD260 from plasmid DNA purified with QIAprep Spin Miniprep Kit System (Qiagen, Hilden, Germany).

WHO Quality Controls

All the simulated RNA samples contained in the 1st, 2nd, and 3rd quality control panels for 2007 and 2008 in the WHO External Quality Assessment Programme (EQAP) and prepared for detection of Influenza virus type A were also used for further sensitivity and specificity evaluation. These panels consisted of coded samples containing vacuum-dried influenza RNA from H5N1 strains, representing genetic clusters of clade 1 and three different genetic clusters of clade 2, H1N1 strains, H3N2 strains and negative samples. The RNA concentrations ranged from 10^3 to 10^4 copies per µl.

Primer Set Design and Optimization

A complete selection of NA sequences available in the U.S. National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) public database, including every NA and HA influenza A subtype from different host species was selected for designing the primer sets. Multiple alignments were constructed using the Macaw 2.0.5 program (NCBI, Bethesda, MA) for this purpose.

In order to amplify NA, a universal PCR primer set (NA+ and NA−) was designed in a highly conserved region of the NA segment. To increase the annealing temperature, a 6-nucleotide tail sequence was added at the 5′-end of the NA+ primer. The alignments of distinct N1 and N2 subtypes from different hosts, geographical settings, and temporal sources allowed the design of the specific primer set N1T+, N1T− and N2T+, N2T−.

For the HA, modifications at the 3′-end of published primers [Hoffmann et al., 2001] were included in the original primers. A couple of universal primers that are able to hybridize with every influenza A viral RNA segment (FluARNA+ and FluARNA−) were obtained. Specific consensus primers for all the genetic variations of H1 (H1-SEQ+ and H1-SEQ−) and H3 (H3-SEQ+ and H3-SEQ−) subtypes were designed after alignment with ClustalX (EMBL, Heidelberg, Germany).

RNA Extraction and Amplification

The RNA present in clinical samples, MDCK infected cell cultures, or allantoic fluid were extracted with the automated nucleic acid extraction instrument BioRobot M48 Workstation and MagAttract Virus Mini M48 kit (Qiagen) according to the manufacturer's instructions. Prior to the extraction procedure, the samples were inactivated with the MagAttract Virus Mini M48 kit lysis buffer containing guanidine hydrochloride within a class II biological safety cabinet. The input sample volume was 200 µl to produce an output elution volume of 50 µl. All the amplification assays were carried out in a PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA).

NA RT-PCR

The Qiagen one-step RT-PCR kit was used in a 45 µl RT-PCR mixture under the following conditions: 0.4 µM of each primer (NA+ and NA−), 0.4 mM of each deoxynucleotides triphosphate (dNTP) and 2 µl of enzyme mixture including Omniscript™ reverse transcriptase, Sensiscript™ reverse transcriptase and HotStarTaq® DNA polymerase in a 1× buffer. Five microliters of sample were added to 0.2 ml thin-walled tubes containing the reaction mixture. The RT step and first round amplification were performed at 45°C for 45 min and 95°C for 15 min followed by 45 cycles: 94°C for 1 min, 48°C for 2 min and 68°C for 1 min, and a final incubation of 68°C for 5 min.

NA Multiplex Nested PCR

The 48 µl nested PCR mixture contains 0.4 µM of each primer (N1T+, N1T−, N2T+, and N2T−), 12 mM Tris–HCl (pH 8.5), 3 mM $(NH_4)_2SO_4$ and 2 mM $MgCl_2$, 200 µM of each dNTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and 2.4 units of AmpliTaq® DNA polymerase (Applied Biosystems, Foster City, CA). An aliquot of 2 µl of RT-PCR product was added to the reaction mixture. The nested PCR step conditions consisted of 95°C for 3 min followed by 35 cycles: 94°C for 30 sec, 63°C for 1 min and 72°C for 1 min, and a final extension of 72°C for 5 min.

HA RT and Nested PCR

The Qiagen one-step RT-PCR kit was employed with a 45 µl reaction mixture containing 0.4 µM of each primer (FluARNA+ and FluARNA−), 0.4 mM of each dNTP, 1× buffer and 2 µl of enzyme mixture. Five microliters of extracted nucleic acid was added to the PCR reaction mixture. The RT conditions were 50°C for 45 min and 95°C for 15 min followed by 45 cycles: 94°C for 30 sec, 50°C for 30 sec and 72°C for 2 min, and a final incubation of 72°C for 5 min.

Due to the similar molecular weights of the HA1 amplification product of H1 and H3, the second round of the nested PCR was performed in two different thin-walled tubes, corresponding to H1 and H3 subtyping. The 48 µl nested PCR mixture contained 2.5 units of AmpliTaq® DNA polymerase, 60 mM Tris–HCl (pH 8.5), 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, and 200 µM of each dNTP (Amersham Pharmacia Biotech). The H1 master mix contained 0.1 µM of each primer (H1 + SSEQ and H1 − ASEQ) and the H3 mixture 0.6 µM (H3 + SSEQ and H3 − ASEQ). Two microliters of RT-PCR product was added to each reaction mixture. The nested

PCR step conditions for the H1 and H3 subtyping tubes consisted of 95°C for 3 min followed by 35 cycles: 94°C for 30 sec, 52°C for 2 min and 72°C for 30 sec, and a final extension of 72°C for 5 min.

PCR Product Analysis

PCR products were analyzed by gel electrophoresis on 2% SeaKem® LE agarose (Lonza, Basel, Switzerland) containing 5 mg/ml of ethidium bromide in 0.5% Tris–borate–EDTA buffer.

Sequencing

All sequencing reactions were carried out by mixing 4 μl of BigDye terminator v3.1 (Applied Biosystem), 2 μl of either primer and 4 μl of purified amplified products. The sequencing reaction was performed in a PTC-200 Peltier Thermal Cycler as follows: 94°C for 3 min to denature the target and 24 cycles of 96°C for 10 sec, 50°C for 10 sec and 60°C for 4 min. Products were run on an ABI PRISM 3700 (Applied Biosystem) sequencer using the dideoxynucleotide chain termination method.

Sequence Analysis

Sequences were arranged using the EditSeq and SeqMan programs of the DNASTAR package (Lasergene, Inc., Madison, WI) and multiple alignments were made using ClustalX (EMBL). Molecular analyses, and translation into amino acids were conducted using the MEGA version 3.1 program [Kumar et al., 2004]. The consensus sequences were constructed using the Bio-Edit Sequence Alignment Editor© [Hall, 1999]. The consensus sequences were obtained analyzing a total of 83 H1 and 34 N1 amplification products of epidemic strains circulating in Spain during the 2007/2008 influenza season and that were without the H274Y mutation. The threshold frequency for inclusion of a residue in the consensus sequence was 95%. Comparisons of the sequences were made to vaccine strains for the 2005/2006, 2006/2007, 2007/2008, and 2008/2009 influenza seasons in the Northern hemisphere. The universal numbering for N2 NA was used for the N1 and N2 subtypes. The amino acid numbering for H1 was according to the alignment of Winter et al. [1981].

RESULTS

Primer Design and Optimization

The universal primers for the NA RT-PCR were designed to assure the amplification of the nine NA subtypes. To achieve this goal multiple alignments were made with sequences available in the Genbank Database for each NA subtype from all the existing HA-NA combinations. A similar approach was used to design specific inner primers for N1, N2, H1, and H3, thus allowing NA and HA subtyping from both human and animal influenza A viruses. The sequences and properties of every primer used in the PCR assays are shown in Table I. Primer annealing temperatures were established theoretically and then standardized by experimentation to achieve maximum product yield and specificity. Thermocycling parameters (denaturing, annealing, and extension times) were optimized systematically. To accomplish a robust HA RT-PCR and NA multiplex RT-PCR, a biochemical optimization (concentration of Mg^{2+}, dNTPs, enzymes and buffer) was performed for each primer set. The NA subtyping primers were designed to ensure that the final reaction products could be differentiated easily on the basis of their different size by agarose gel electrophoresis.

Analytical Specificity

The universality of the method was tested with different influenza virus subtypes isolated from samples of human and animal sources. The specificity of the

TABLE I. Primer Set Used for Amplification

Specificity	Primer	Sequence (5′–3′)	Nucleotide position in the target sequence (5′–3′)	
			A/New Caledonia/20/99	A/Panama/2007/99
Influenza A RNA	FluARNA+	<u>TATTCGTCTCAGGG</u>AGCAAAAGC	1–9 of the eight RNA segments	1–9 of the eight RNA segments
	FluARNA−	<u>ATATCGTCTCGTATT</u>AGTAGAAA-CAAGG	The last 13 nucleotides of the eight RNA segments	The last 13 nucleotides of the eight RNA segments
H1 subtype	H1 + SSEQ	CAATATGTATAGGCTACCATGC	56–77	—
	H1 − ASEQ	CCCTCAATRAAACCRGCAAT	1,045–1,064	—
H3 subtype	H3 + SSEQ	GACACCATGCAGTGCCAA	—	124–141
	H3 − ASEQ	CCCTCCCAACCATTTTCTAT	—	1,092–1,111
N1–N9 subtypes	NA+	GGGGTTAGCAAAAGCAGG	1–10	1–12
	NA−	CCTCTGATYAIYTCIACIYARAARCA	1,279–1,304	1,280–1,305
N1 subtype	N1T+	GCAAGYGCWTGYCATGATGGC	559–579	—
	N1T−	CCTGACCAATCAGTTATTGC	1,220–1,201	—
N2 subtype	N2T+	CAAGTARTGCYRTGTGARCC	—	164–183
	N2T−	AGYCCTGAGCACACATAAC	—	981–963

The underlined nucleotides at the 5′-end correspond to recognition sequences for the restriction endonuclease *Bsm*BI. The dotted underlined nucleotides at the 5′-end are not complementary to the *Influenzavirus* A sequences. The expected size of the PCR products is based on HA and NA genomes of A/New Caledonia/20/99 (accession no. AY289929, AJ518092, and EF620020) and A/Panama/2007/99 (accession no. DQ487340, DQ487337, and DQ487338).

multiplex nested PCR for HA and NA was analyzed with a panel of different influenza A viruses containing 15 HA and 9 NA subtypes in order to verify the presence of H1, H3, N1, and N2 subtypes in the absence of other HA and NA subtypes or nonspecific amplification (Table II). A product of the expected size was obtained for each viral template by the NA nested PCR when the two couples of the primer sets were present (Fig. 1). When specimens containing circulating influenza B viruses and B prototype strains were analyzed, no amplified product was detected in any case. The cross reactivity of the assay was assessed using a panel of nucleic acids from respiratory pathogens derived from cell culture or clinical specimens. This panel included parainfluenza viruses 1, 2, 3, and 4, adenovirus, rhinovirus, coronaviruses 229E and OC42, respiratory syncytial viruses A and B, and human metapneumovirus. All the results obtained with the nested PCR for H1 versus H3, of different H1N1 and H3N2 strains, and N1 versus N2 of the diverse H1N1, H3N2, and H5N1 strains included in the WHO EQAP 2007 and 2008 agreed with the expected outcomes.

Analytical Sensitivity

Evaluation of sensitivity was carried out by amplifying cloned HA and NA products from H1N1 (A/New Caledonia/20/99) and H3N2 (A/Panama/2007/99) influenza strains. When serial dilutions were tested separately in 50 µl of final reaction volume, 1–10 molecules per tube of N1 and 10–100 molecules per tube of N2 were detected, indicating the respective limit of sensitivity for each NA subtype (Fig. 2). After mixing cloned N1 and N2 products in the same tube, the sensitivity limits were estimated as 10–100 molecules per tube for N1 and 1–10
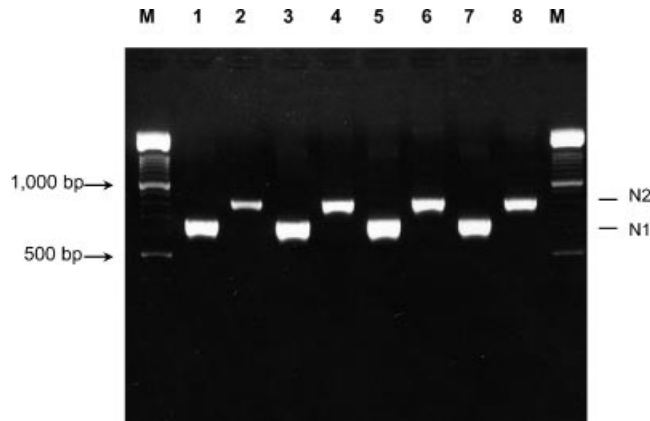

Fig. 1. Ethidium bromide stained agarose gel of NA multiplex-nested PCR amplified product from a tissue grown culture of the influenza strains (**lanes 1–8**): A/swine/Wisconsin/1/67 (H1N1), A/Tokyo/3/67 (H1N2), A/New Caledonia/20/99 (H1N1), A/California/7/04 (H3N2), A/Solomon Islands/3/06 (H1N1), A/Wisconsin/67/05 (H3N2), A/duck/Vietnam/TG24-01/05 (H5N1) and A/turkey/Massachusetts/3740/65 (H6N2). M: molecular size marker.

molecules per tube for N2 (Fig. 2). Analogous assays were performed for H1 and H3, and the limit of detection for both was 1–10 molecules per tube (Fig. 3).

Surveillance and Diagnosis

To assess the performance with circulating human seasonal viruses of this method for surveillance or diagnostic purposes, a total of 105 positive upper and lower respiratory clinical samples for influenza A virus determined by PCR [Coiras et al., 2003] and/or antigen detection, and 113 isolates from the Spanish influenza surveillance network during the 2004–2008 influenza

TABLE II. Prototype Strains of Influenza Viruses Used as Specificity Controls in the HA Nested PCR, Universal NA RT-PCR, and NA Multiplex Nested PCR

Type	Influenza viruses	HA nested PCR	NA RT-PCR	NA multiplex nested PCR
H1N1	A/swine/Wisconsin/1/67	H1	+	N1
	A/New Caledonia/20/99	H1	+	N1
	A/Solomon Islands/3/06	H1	+	N1
H2N2	A/Tokyo/3/67	−	+	N2
H3N2	A/Panama/2007/99	H3	+	N2
	A/California/7/04	H3	+	N2
	A/Wisconsin/67/05	H3	+	N2
H4N6	A/duck/Czechoslovakia/56	−	+	−
H5N1	A/duck/Vietnam/TG24-01/05	−	+	N1
H5N3	A/tern/South Africa/61	−	+	−
H6N2	A/turkey/Massachusetts/3740/65	−	+	N2
H7N7	A/equine/Prague/1/56	−	+	−
H8N4	A/turkey/Ontario/6118/68	−	+	−
H10N7	A/chick/Germany/N/49	−	+	−
H10N8	A/quail/Italy/1117/65	−	+	−
H11N6	A/duck/England/1/56	−	+	−
H12N5	A/duck/Alberta/60/76	−	+	−
H13N6	A/gull/Maryland/704/77	−	+	−
H14N5	A/mallard/Astrakhan/263/82	−	+	−
H15N9	A/shearwater/Western A/Australia/2576/79	−	+	−
H16N3	A/yellow-legged gull/Spain/1/07	−	+	−
B	B/Jiangsu/10/03	−	−	−
	B/Malaysia/2506/04			

Fig. 2. Ethidium bromide stained agarose gel showing serial 10-fold dilutions from 10,000 copies of cloned amplified fragment corresponding to N1 (**lanes 1–5**), N2 (**lanes 6–10**) and a mixture of 10,000 to 1 copy of N1 and from 1 to 10,000 copies of N2 (**lanes 11–15**) of cloned product. M: molecular size marker. NC: negative control.

seasons were selected for HA and NA subtyping. The specific products could be easily separated and identified on a 2% agarose gel for both culture tissue adapted viruses and clinical samples containing wild-type strains. All the H1 influenza viruses only tested positive for the N1 NA subtype and all the H3 influenza viruses only tested positive for N2 NA subtype. Therefore, no H1N2 recombinant viruses were found and none of the clinical samples and cultures tested positive for N1 plus N2 subtype.

Nucleotide Sequencing Analysis

To assay the capacity of the method to identify mutations involved in the resistance to NAIs, 36 N1 and 39 N2 influenza A subtype viruses were selected for sequencing on the basis of their different geographical origin, as well as the immunologic compromise status and/or treatment with NAIs of the patients. All the amplification products of the N1 subtype sequenced belonged to the 2007/2008 influenza season. The
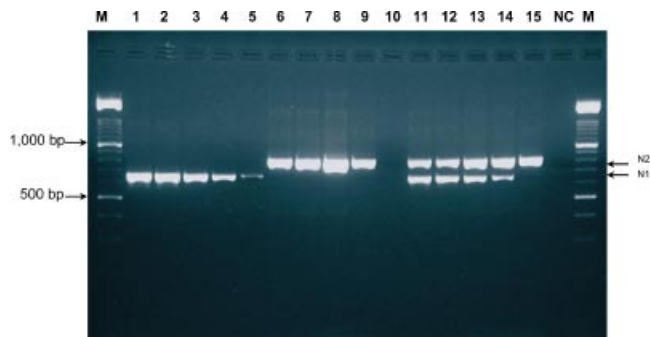

Fig. 3. Ethidium bromide stained agarose gel showing serial 10-fold dilutions from 10,000 copies of cloned amplified fragment corresponding to H1 (**lanes 1–5**) and H3 (**lanes 6–10**). M: molecular size marker.

sequencing of the NA gene revealed an H274Y substitution, associated with resistance to oseltamivir although sensitive to zanamivir, in two viruses isolated during the 2007/2008 influenza season. The first sample containing the H274Y mutation (GenBanK accession no. FJ573473) was obtained at the 52nd epidemiological week from an influenza vaccinated 13-year-old female patient with type 1 (insulin-dependent) diabetes mellitus. The second sample containing the virus with the H274Y substitution (GenBanK accession no. FJ573474) was collected at the 6th epidemiological week from a 4-month-old male patient who attended at the emergency department with clinical symptoms of bronchiolitis. Neither the patients nor their relatives reported prior treatment with NAIs or close contact with treated patients. The Gly at position 353 (G354 in N1 numbering) detected in the FJ573473 sequence is the same as observed in the vaccine strain A/Solomon Islands/3/06 used for the 2007/2008 influenza season in the Northern hemisphere (Fig. 4). Although the second oseltamivir resistant strain showed the mutation G353D in the FJ573474 sequence, this change is also present in the N1 sequence of the recommended vaccine virus for the 2008/2009 influenza season A/Brisbane/59/07. The HA from both viruses were sequenced (GenBanK accession nos. FJ573475 and FJ573476). The most remarkable amino acid change in the HA was found in the first virus sequence (FJ573475) which present the E94K mutation. This mutation was absent in the vaccine viruses and the 2007/2008 influenza season consensus sequence (Fig. 5). The H1 consensus sequence for the 2007/2008 influenza season showed polymorphism at position 240 with the mutation G240R in 7 of the 83 (8%) HA sequences examined.

DISCUSSION

The role of the laboratory in influenza surveillance in humans and animals is comprised of viral isolation, antigenic and genetic characterization and supplementary techniques based on molecular virology. Although virus isolation and phenotypic assays continue to be essential in reference laboratories for further epidemiological characterization and the discovery of new resistance markers, genetic characterization is a useful tool and the molecular approach is an alternative for influenza surveillance as well as diagnostic purposes. The last few years have also seen attempts to extend the surveillance to antiviral susceptibility [Meijer et al., 2006].

This report describes a first round universal primer set that allows reliable amplification of all NA subtypes followed by specific N1 and N2 detection in a second round multiplex nested PCR. The N1 and N2 specific primers set was carefully designed to amplify all N1 and N2 genetic strains regardless of the HA subtype and virus source (human or animal). The strategic location of the primers in the NA gene gives this method the capacity to detect the specific substitutions concerned with the acquisition of clinical resistance to NAIs. At
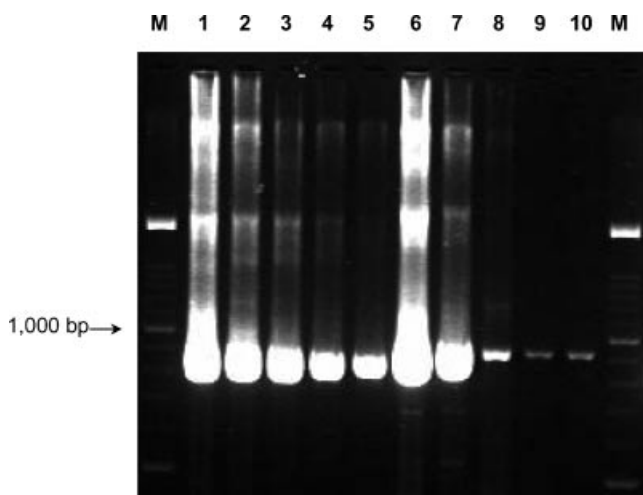
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                          188
                          |
#A/NewCaledonia/20/99    GWLTIGISGP  DNGAVAVLKY  NGIITETIKS  WKKRILRTQE  SECVCVNGSC
#A/SolomonIslands/3/06   ..........  ..........  ..........  ..........  .....M....
#A/Brisbane/59/07        ..........  ..........  .....G....  ...Q......  .....M....
#FJ573473                ..........  ..........  .....G....  ...Q......  .....M....
#FJ573474                ..........  ..........  .....G....  ...Q......  .....M....
#N1 Consensus 2007/08    GWLTIGISGP  DNGAVAVLKY  NGIITGTIKS  WKKQILRTQE  SECVCMNGSC


#A/NewCaledonia/20/99    FTIMTDGPSN  GAASYKIFKI  EKGKVTKSIE  LNAPNFHYEE  CSCYPDTGTV
#A/SolomonIslands/3/06   ..........  ..........  .......T..  ..........  ..........
#A/Brisbane/59/07        ..........  K.........  ..........  ..........  .........I.
#FJ573473                ..........  K.........  ..........  ......Y...  .........I.
#FJ573474                ..........  K.........  ..........  ......Y...  .........I.
#N1 Consensus 2007/08    FTIMTDGPSN  KAASYKIFKI  EKGKVTKSIE  LNAPNFHYEE  CSCYPDTGIV


#A/NewCaledonia/20/99    MCVCRDNWHG  SNRPWVSFNQ  NLDYQIGYIC  SGVFGDNPRP  KDGEGSCNPV
#A/SolomonIslands/3/06   ..........  ..........  ..........  ..........  ..........
#A/Brisbane/59/07        ..........  ..........  ..........  ..........  E.........
#FJ573473                ..........  ..........  ..........  ..........  E.........
#FJ573474                ..........  ..........  ..........  ..........  E.........
#N1 Consensus 2007/08    MCVCRDNWHG  SNRPWVSFNQ  NLDYQIGYIC  SGVFGDNPRP  EDGEGSCNPV


#A/NewCaledonia/20/99    TVDGADGVKG  FSYKYGNGVW  IGRTKSNRLR  KGFEMIWDPN  GWTDTDSDFS
#A/SolomonIslands/3/06   ..........  ..........  ..........  ..........  ...N......
#A/Brisbane/59/07        .....N....  .....D....  ..........  ..........  ...N......
#FJ573473                .....N....  ..........  ..........  ..........  ...N......
#FJ573474                .....N....  .....D....  ..........  ..........  ...N......
#N1 Consensus 2007/08    TVDGANGVKG  FSYKYDNGVW  IGRTKSNRLR  KGFEMIWDPN  GWTNTDSDFS


                          394
                          |
#A/NewCaledonia/20/99    VKQDVVA
#A/SolomonIslands/3/06   .......
#A/Brisbane/59/07        .......
#FJ573473                .......
#FJ573474                .......
#N1 Consensus 2007/08    VKQDVVA
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Fig. 4. Alignment of N1 amino acid sequences from the isolates and the vaccine strains A/New Caledonia/20/99, A/Solomon Islands/3/06 and A/Brisbane/59/07 used in the 2006/2007, 2007/2008, and 2008/2009 influenza seasons, respectively. The amino acids are numbered according to the N2 numbering. The GenBank numbers FJ573473 and FJ573474 correspond to the first and second isolate in that order. Dots indicate identity. Bold type amino acid highlights the H274Y mutation conferring oseltamivir resistance.

present these substitutions have been described at the residues E119G/A/D/V in the H3N2 subtype, H274Y in the H1N1 and H5N1 subtypes, R292K in the H3N2 subtype and N294S in the H3N2 and H5N1 subtypes [Kiso et al., 2004; Le et al., 2005; Yen et al., 2006]. In addition, this assay allows the detection of mutations at codon D151 in A(H3N2) viruses. Substitutions at this position have been reported in the H1N1 and H3N2 subtypes but most often in H3N2 viruses [Monto et al., 2006]. Nevertheless, the relevance of substitution at D151 for in vivo resistance has not been demonstrated [Sheu et al., 2008].

A similar approach was used to develop the H1 and H3 amplification oligonucleotides. Independently of NA subtype or human−animal origin, the H1 and H3 set of primers amplify the entire HA1 domain of the HA gene together with the fusion peptide-encoding segment. The HA1 domain is more informative than the HA2 domain

[Fanning and Taubenberger, 1999] and this feature makes it possible to recognize amino acid changes affecting the main antigenic sites (antigenic drift), compare them with vaccine strains and compile evolution studies [Bush et al., 1999].

The use of highly degenerated, even inosinated, primers requires extensive optimization but provides universality and robustness in the amplification over time, avoiding the need for periodic checking for mismatches, primer modifications and re-standardization of the PCR when a new strain of virus starts to circulate. Using the first universal NA RT-PCR amplification, every one of the nine subtypes of NA obtained from animal influenza viruses harvested in embryonated chicken eggs or from human influenza strains cultured in MDCK cells was detected, probably due to the high viral load obtained in culture. After the second round, the nested PCR described here was able to detect

```
                            32                        53a                      77a
                            |                         ↓                        ↓
#A/NewCaledonia/20/99       KNVTVTHSVN LLEDSHNGKL CLLKGIAPLQ LGNCSVAGWI LGNPECELLI
#A/SolomondIsland/03/06     .......... .......... .......... .......... ..........
#A/Brisbane/59/07           .......... ...N...... .......... .......... ..........
#FJ573475                   .......... ...N...... .......... .......... ..........
#FJ573476                   .......... ...N...... .......... .......... ..........
#H1 Consensus 2007/08       KNVTVTHSVN LLEDSHNGKL CLLKGIAPLQ LGNCSVAGWI LGNPECELLI

                                       94a                      125a,b,c
                                       ↓                        ↓↓↓
#A/NewCaledonia/20/99       SKESWSYIVE TPNPENGTCY PGYFADYEEL REQLSSVSSF ERFEIFPKES
#A/SolomondIsland/03/06     .R........ K......... ..H....... .......... ..........
#A/Brisbane/59/07           .......... K......... ..H....... .......... ..........
#FJ573475                   .......... K...K..... ..H....... .......... ..........
#FJ573476                   .......... K......... ..H....... .......... ..........
#H1 Consensus 2007/08       SKESWSYIVE KPNPENGTCY PGHFADYEEL REQLSSVSSF ERFEIFPKES

                                       137a
                                       ↓
#A/NewCaledonia/20/99       SWPNHTVTGV SASCSHNGKS SFYRNLLWLT GKNGLYPNLS KSYVNNKEKE
#A/SolomondIsland/03/06     ......T... ........E. ...K...... .......... ...A......
#A/Brisbane/59/07           .......... ........E. .......... .......... ...A......
#FJ573475                   .......... ........E. .......... .......... ...A......
#FJ573476                   .......... ........E. .......... .......... ...A......
#H1 Consensus 2007/08       SWPNHTVTGV SASCSHNGES SFYRNLLWLT GKNGLYPNLS KSYANNKEKE


#A/NewCaledonia/20/99       VLVLWGVHHP PNIGNQRALY HTENAYVSVV SSHYSRRFTP EIAKRPKVRD
#A/SolomondIsland/03/06     .......... ....D..... .......... ......K... ..........
#A/Brisbane/59/07           .......... ....I.K... .......... ......K... ..........
#FJ573475                   .......... ....D.K... .......... ......K... ..........
#FJ573476                   .......... ....D.K... .......... ......K... ..........
#H1 Consensus 2007/08       VLVLWGVHHP PNIGDQKALY HTENAYVSVV SSHYSRKFTP EIAKRPKVRD


#A/NewCaledonia/20/99       QEGRINYYWT LLEPGDTIIF EANGNLIAPW YAFALSRGFG SGIITSNAPM
#A/SolomondIsland/03/06     R......... .......... .........R .......... ....N.....
#A/Brisbane/59/07           .......... .......... .........R .......... ....N.....
#FJ573475                   .......... .......... .........R .......... ....N.....
#FJ573476                   .......... .......... .........R .......... ....N.....
#H1 Consensus 2007/08       QEGRINYYWT LLEP-DTIIF EANGNLIAPR YAFALSRGFG SGIINSNAPM


                                                                     322
                                                                     |
#A/NewCaledonia/20/99       DECDAKCQTP QGAINSSLPF QNVHPVTIGE CPKYVRSAKL RMVTGLR
#A/SolomondIsland/03/06     .......... .......... .......... .......... .......
#A/Brisbane/59/07           .K........ .......... .......... .......... .......
#FJ573475                   .K........ .......... .......... .......... .......
#FJ573476                   .K........ .......... .......... .......... .......
#H1 Consensus 2007/08       DKCDAKCQTP QGAINSSLPF QNVHPVTIGE CPKYVRSAKL RMVTGLR
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Fig. 5. Alignment of H1 amino acid sequences from the isolates and the vaccine strains A/New Caledonia/20/99, A/Solomon Islands/3/06, and A/Brisbane/59/07 used in the 2006/2007, 2007/2008, and 2008/2009 influenza seasons, respectively. The amino acids are numbered to correspond to the numbering of the H3 subtype, according to the alignment of Winter et al. [1981], with additional amino acid residues present in the H1 subtype sequence marked by an arrow. These additional residues are numbered as the previous residue followed by a letter designation. The GenBank numbers FJ573475 and FJ573476 correspond to the first and second isolate in that order. Dots indicate identity. Hyphen indicates no consensus position.

and distinguish N1 and N2 subtypes directly from clinical respiratory samples. The RNA isolated from a cloacal swab sample, obtained from a great-crested grebe (*Podiceps cristatus*) infected with highly pathogenic H5N1 and sent to the Influenza and Respiratory Viruses Laboratory for further molecular analysis, gave positive results also after the first round amplification, probably because of the high replication efficiency and elevated viral load reported in animals and humans infected with influenza H5N1 viruses [Govorkova et al., 2005; de Jong et al., 2006]. The H5N1 viruses can be divided into genetic clusters named clade 1 and clade 2

and the latter can be further subdivided into three subclades (1, 2, and 3) [Webster and Govorkova, 2006]. The results of the EQAP 2007 and 2008 showed the flexibility of this method for the NA detection of the different H5N1 clades and subclades circulating from 1996 until now as well as the H1 and H3 strains included in each panel.

Even though the use of DNA plasmids for sensitivity evaluation does not take into account extraction and retro-transcription efficiency, the analytical sensitivity of the techniques examined for HA and NA subtyping with cloned products was high enough to detect 1 to at least 100 copies of genome equivalents. The sensitivity of this nested RT-PCR technique is comparable to that of several reported real-time PCR protocols for HA and/or NA subtyping in H1N1 and H3N2 seasonal influenza viruses [Schweiger et al., 2000], swine influenza viruses [Richt et al., 2004], H5N1 avian influenza [Aguero et al., 2007], and H5N1 of avian and human origin [Payungporn et al., 2006]. The capacity of real-time PCR is very limited in as far as detecting multiple targets (e.g., influenza subtyping) or several single mutations in a multiplexed assay because the most recent platforms generally only allow four-color probes [Mackay et al., 2002]. The design of oligoprobes is almost nonfeasible in conserved sites of genes with high rates of variability such as the HA and NA in influenza viruses. Furthermore, the amplicon size (<200 base pairs for most of the assays) is also a restrictive factor. Nevertheless, real-time PCR with TaqMan probes to detect oseltamivir resistance has been reported but only for the recognition of the H274Y mutation in H5N1 influenza viruses [Chutinimitkul et al., 2007]. Up until now, NA subtyping by the conventional PCR [Wright et al., 1995; Poddar, 2002; Takao et al., 2002] and real-time PCR [Schweiger et al., 2000] assays described at present for influenza A viruses do not permit the detection of all the clinical mutations conferring resistance to NAIs. In 2005, a pyrosequencing assay was developed to monitor adamantane resistance [Bright et al., 2005] and, in 2008, pyrosequencing-based technology was also used to detect NAIs resistance [Lackenby et al., 2008]. Although pyrosequencing is not yet available in most laboratories, this assay is a promising method that permits detection of resistance markers in viruses in clinical specimens and allows the differentiation of quasi species (resistant or sensitive) in the same samples.

When N1 and N2 plasmids were mixed, the resulting sensitivity was in the same range of detection (1−100 copies per tube) as that obtained when the cloned products were tested separately. The assay using mixed plasmids was repeated five times. The result for three of five assays is shown in Figure 2 (lanes 11−15), but the results with the other two runs were the same as obtained when using the separate primers. These differences might be because the technique is close to the lowest detection threshold. Therefore, the multiplex PCR format made it possible to detect of both NA subtypes in the same sample without significant loss of the sensitivity. Although infection with different subtypes is not well documented in the literature [Stockton et al., 1998] and still remains basically unidentified, this feature is useful for the recognition of a hypothetical co-infection with N1 and N2 viruses (H1N1, H5N1, or H3N2 subtypes). The clinical sensitivity of the assay was assessed with the detection and subtyping of influenza viruses directly from clinical samples. Since the replication efficiency of NAIs resistant viruses might be compromised [Yen et al., 2005], molecular detection directly from clinical samples allows genetic characterization independently of the viral replication rate in cell culture and avoids the identification of mutations that are not present in wild-type viruses but are originated by adaptation to cell culture.

For surveillance purposes, different H1N1 and H3N2 subtypes of epidemic viruses circulating in humans from the 2004 to 2008 influenza seasons were tested. Although in past seasons H1N2 human influenza virus circulated world wide [Cheung et al., 2006], this reassortant virus was not found in the clinical samples or cultures analyzed in Spain. Furthermore, throughout the 2007/2008 influenza season an unusual phenomenon of world wide circulation of oseltamivir-resistant A(H1N1) viruses containing the H274Y mutation was reported. The frequency of resistance ranged from 0% to 69% depending on the geographical location [WHO, 2008]. The same as was reported in other countries, neither of the two cases described here had received oseltamivir, been exposed to patients who had received oseltamivir treatment or who had traveled abroad. Besides, the oseltamivir-resistant rates were different in neighboring countries. These epidemiological data indicate the emergence of an oseltamivir-resistant strain in absence of selective drug pressure. The high rates of oseltamivir-resistant H1N1 viruses observed during 2007/2008 influenza season in some countries (e.g., 67% in Norway) contradict on the basis of the H274Y mutation affects the viral fitness. In fact, some studies reported reduced viral replication and transmissibility and other shown similar fitness than the wild-type viruses [Hurt et al., 2006]. The NA sequencing of the 75 selected influenza A viruses allowed the detection of the H274Y mutation in two A(H1N1) strains recovered from two patients during the 2007/2008 season. This mutation confers oseltamivir resistance but does not affect susceptibility to zanamivir [Gubareva, 2004]. Regarding the Gly → Asp mutation detected at position 353 (354 in N1 numbering), preliminary analysis of the NA sequence data suggest that most of the A(H1N1) viruses with the H274Y mutation studied during the 2007/2008 influenza season had lost the G353D substitution. The characterization of the A(H1N1) 1918 influenza NA gene showed that this position is also aspartic acid in A/Brevig Mission/1/18 and glycine in nearly all other NA proteins, regardless of subtype. Although it has not been proven to date experimentally, the change from G353D/E could result in a conformational change of the protein affecting NA activity and virulence [Reid et al., 2000]. Nevertheless,

this mutation was mainly found at the beginning of the influenza season and reverted in the later isolates analyzed in Europe [Hay, 2008]. The HA analysis of the two resistant viruses did not show modifications affecting epitopes or receptor-binding sites. Only one hypothetical neutral polymorphism (E94K) was found in the first oseltamivir-resistant virus but it was absent in the second isolate. The HA1 sequencing of the sensitive viruses circulating during the 2007/2008 influenza season showed one significant polymorphism in Ca antigenic site [Caton et al., 1982] at position 240 (Gly → Arg) in 8% of the isolates. The homogeneity observed in the HA gene suggests that the resistant A(H1N1) viruses do not form a particular group with regard to the susceptible viruses and there are no amino acid variations that distinguish the HA of resistant from those of sensitive viruses.

In conclusion, this work reports the development and implementation of an easy and useful tool for the subtyping and recognition of antigenic and binding sites in HA1 and the detection of resistance markers in NA. The improvements compared with phenotypic methods include high sensitivity, rapidity, ease of use and, additionally does not require infectious virus in the laboratory. The emergence of influenza virus resistance to NAIs, regardless of the acquisition mechanism, necessitates the implementation of genetic characterization for the NA segment and the expansion of surveillance and diagnostic activities for the detection of resistance to NAIs.

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REFERENCES

Aguero M, Sanchez A, San Miguel E, Gomez-Tejedor C, Jimenez-Clavero MA. 2007. A real-time TaqMan RT-PCR method for neuraminidase type 1 (N1) gene detection of H5N1 Eurasian strains of avian influenza virus. Avian Dis 51:378–381.

Bright RA, Medina MJ, Xu X, Perez-Oronoz G, Wallis TR, Davis XM, Povinelli L, Cox NJ, Klimov AI. 2005. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: A cause for concern. Lancet 366:1175–1181.

Bush RM, Bender CA, Subbarao K, Cox NJ, Fitch WM. 1999. Predicting the evolution of human influenza A. Science 286:1921–1925.

Caton A, Brownlee GG, Yewdell JW, Gerhard W. 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell 31:417–427.

Cheung CL, Rayner JM, Smith GJ, Wang P, Naipospos TS, Zhang J, Yuen KY, Webster RG, Peiris JS, Guan Y, Chen H. 2006. Distribution of amantadine-resistant H5N1 avian influenza variants in Asia. J Infect Dis 193:1626–1629.

Chutinimitkul S, Suwannakarn K, Chieochansin T, Mai le Q, Damrongwatanapokin S, Chaisingh A, Amonsin A, Landt O, Songserm T, Theamboonlers A, Poovorawan Y. 2007. H5N1 oseltamivir-resistance detection by real-time PCR using two high sensitivity labeled TaqMan probes. J Virol Methods 139: 44–49.

Coiras MT, Perez-Brena P, Garcia ML, Casas I. 2003. Simultaneous detection of influenza A, B, and C viruses, respiratory syncytial virus, and adenoviruses in clinical samples by multiplex reverse transcription nested-PCR assay. J Med Virol 69:132–144.

Coiras MT, Aguilar JC, Garcia ML, Casas I, Perez-Brena P. 2004. Simultaneous detection of fourteen respiratory viruses in clinical specimens by two multiplex reverse transcription nested-PCR assays. J Med Virol 72:484–495.

Couch RB. 2000. Prevention and treatment of influenza. N Engl J Med 343:1778–1787.

de Jong MD, Tran TT, Truong HK, Vo MH, Smith GJ, Nguyen VC, Bach VC, Phan TQ, Do QH, Guan Y, Peiris JS, Tran TH, Farrar J. 2005. Oseltamivir resistance during treatment of influenza A (H5N1) infection. N Engl J Med 353:2667–2672.

de Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, Hoang DM, Chau NV, Khanh TH, Dong VC, Qui PT, Cam BV, Ha do Q, Guan Y, Peiris JS, Chinh NT, Hien TT, Farrar J. 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat Med 12:1203–1207.

de Lataillade C, Auvergne S, Delannoy I. 2009. 2005 and 2006 seasonal influenza vaccination coverage rates in 10 countries in Africa, Asia Pacific, Europe, Latin America and the Middle East. J Public Health Policy 30:83–101.

Fanning TG, Taubenberger JK. 1999. Phylogenetically important regions of the influenza A H1 hemagglutinin protein. Virus Res 65:33–42.

Fouchier RA, Osterhaus AD, Brown IH. 2003. Animal influenza virus surveillance. Vaccine 21:1754–1757.

Govorkova EA, Rehg JE, Krauss S, Yen HL, Guan Y, Peiris M, Nguyen TD, Hanh TH, Puthavathana P, Long HT, Buranathai C, Lim W, Webster RG, Hoffmann E. 2005. Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. J Virol 79:2191–2198.

Gubareva LV. 2004. Molecular mechanisms of influenza virus resistance to neuraminidase inhibitors. Virus Res 103:199–203.

Gubareva LV, Matrosovich MN, Brenner MK, Bethell RC, Webster RG. 1998. Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. J Infect Dis 178:1257–1262.

Gubareva LV, Kaiser L, Matrosovich MN, Soo-Hoo Y, Hayden FG. 2001. Selection of influenza virus mutants in experimentally infected volunteers treated with oseltamivir. J Infect Dis 183: 523–531.

Hall TA. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98.

Hay AJ. 2008. Characteristics of human influenza AH1N1, AH3N2, and B viruses isolated September 2007 to February 2008. London: WHO Collaborating Centre for Reference and Research on Influenza.

Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 146:2275–2289.

Holmes EC, Taubenberger JK, Grenfell BT. 2005. Heading off an influenza pandemic. Science 309:989.

Hurt AC, Ho HT, Barr I. 2006. Resistance to anti-influenza drugs: Adamantanes and neuraminidase inhibitors. Expert Rev Anti Infect Ther 4:795–805.

Kiso M, Mitamura K, Sakai-Tagawa Y, Shiraishi K, Kawakami C, Kimura K, Hayden FG, Sugaya N, Kawaoka Y. 2004. Resistant influenza A viruses in children treated with oseltamivir: Descriptive study. Lancet 364:759–765.

Kramarz P, Monnet D, Nicoll A, Yilmaz C, Ciancio B. 2009. Use of oseltamivir in 12 European countries between 2002 and 2007—Lack of association with the appearance of oseltamivir-resistant influenza A(H1N1) viruses. Euro Surveill 14: Pii:19112.

Kumar S, Tamura K, Nei M. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform 5:150–163.

Lackenby A, Democratis J, Siqueira MM, Zambon MC. 2008. Rapid quantitation of neuraminidase inhibitor drug resistance in influenza virus quasispecies. Antivir Ther 13:809–820.

Le QM, Kiso M, Someya K, Sakai YT, Nguyen TH, Nguyen KH, Pham ND, Ngyen HH, Yamada S, Muramoto Y, Horimoto T, Takada A, Goto H, Suzuki T, Suzuki Y, Kawaoka Y. 2005. Avian flu: Isolation of drug-resistant H5N1 virus. Nature 437:1108.

López-Huertas MR, Casas I, Acosta-Herrera B, García ML, Coiras MT, Pérez-Breña P. 2005. Two RT-PCR based assays to detect human metapneumovirus in nasopharyngeal aspirates. J Virol Methods 129:1–7.

Mackay IM, Arden KE, Nitsche A. 2002. Real-time PCR in virology. Nucleic Acids Res 30:1292–1305.

Meijer A, Brown C, Hungnes O, Schweiger B, Valette M, van der Werf S, Zambon M. 2006. Programme of the Community Network of Reference Laboratories for Human Influenza to improve Influenza Surveillance in Europe. Vaccine 24:6717–6723.

Monto AS, McKimm-Breschkin JL, Macken C, Hampson AW, Hay A, Klimov A, Tashiro M, Webster RG, Aymard M, Hayden FG, Zambon M. 2006. Detection of influenza viruses resistant to neuraminidase inhibitors in global surveillance during the first 3 years of their use. Antimicrob Agents Chemother 50:2395–2402.

Neumann G, Kawaoka Y. 2006. Host range restriction and pathogenicity in the context of influenza pandemic. Emerg Infect Dis 12:881–886.

Nicholson KG, Wood JM, Zambon M. 2003. Influenza. Lancet 362:1733–1745.

Payungporn S, Chutinimitkul S, Chaisingh A, Damrongwantanapokin S, Buranathai C, Amonsin A, Theamboonlers A, Poovorawan Y. 2006. Single step multiplex real-time RT-PCR for H5N1 influenza A virus detection. J Virol Methods 131:143–147.

Poddar SK. 2002. Influenza virus types and subtypes detection by single step single tube multiplex reverse transcription-polymerase chain reaction (RT-PCR) and agarose gel electrophoresis. J Virol Methods 99:63–70.

Pozo F, García-García ML, Calvo C, Cuesta I, Pérez-Breña P, Casas I. 2007. High incidence of human bocavirus infection in children in Spain. J Clin Virol 40:224–228.

Regoes RR, Bonhoeffer S. 2006. Emergence of drug-resistant influenza virus: population dynamical considerations. Science 312: 389-391.

Reid AH, Fanning TG, Janczewski TA, Taubenberger JK. 2000. Characterization of the 1918 "Spanish" influenza virus neuraminidase gene. Proc Natl Acad Sci USA 97:6785–6790.

Richt JA, Lager KM, Clouser DF, Spackman E, Suarez DL, Yoon KJ. 2004. Real-time reverse transcription-polymerase chain reaction assays for the detection and differentiation of North American swine influenza viruses. J Vet Diagn Invest 16:367–373.

Schweiger B, Zadow I, Heckler R, Timm H, Pauli G. 2000. Application of a fluorogenic PCR assay for typing and subtyping of influenza viruses in respiratory samples. J Clin Microbiol 38:1552–1558.

Sheu TG, Deyde VM, Okomo-Adhiambo M, Garten RJ, Xu X, Bright RA, Butler EN, Wallis TR, Klimov AI, Gubareva LV. 2008. Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. Antimicrob Agents Chemother 52:3284–3292.

Smith DJ, Lapedes AS, de Jong JC, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. 2004. Mapping the antigenic and genetic evolution of influenza virus. Science 305:371–376.

Stockton J, Ellis JS, Saville M, Clewley JP, Zambon MC. 1998. Multiplex PCR for typing and subtyping influenza and respiratory syncytial viruses. J Clin Microbiol 36:2990–2995.

Takao S, Shimazu Y, Fukuda S, Kuwayama M, Miyazaki K. 2002. Neuraminidase subtyping of human influenza a viruses by RT-PCR and its application to clinical isolates. Jpn J Infect Dis 55:204–205.

Webster RG, Govorkova EA. 2006. H5N1 influenza—Continuing evolution and spread. N Engl J Med 355:2174–2177.

WHO. 2008. Influenza A(H1N1) virus resistance to oseltamivir (Sep 29, 2008).

WHO. 2009. Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO (Jan 14, 2009).

Winter G, Fields S, Brownlee GG. 1981. Nucleotide sequence of the haemagglutinin gene of a human influenza virus H1 subtype. Nature 292:72–75.

Wright KE, Wilson GA, Novosad D, Dimock C, Tan D, Weber JM. 1995. Typing and subtyping of influenza viruses in clinical samples by PCR. J Clin Microbiol 33:1180–1184.

Yen HL, Herlocher LM, Hoffmann E, Matrosovich MN, Monto AS, Webster RG, Govorkova EA. 2005. Neuraminidase inhibitor-resistant influenza viruses may differ substantially in fitness and transmissibility. Antimicrob Agents Chemother 49:4075–4084.

Yen HL, Hoffmann E, Taylor G, Scholtissek C, Monto AS, Webster RG, Govorkova EA. 2006. Importance of neuraminidase active-site residues to the neuraminidase inhibitor resistance of influenza viruses. J Virol 80:8787–8795.