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Molecular epidemiology of influenza A virus infection in Cyprus in four consecutive seasons (2009 pandemic–2013)

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SUMMARY

The aim of this study was to investigate the epidemiology of influenza A virus infection in Cyprus from the 2009 pandemic until 2013. Pandemic influenza A(H1N1)2009 virus infections outnumbered infections with other respiratory viruses until the end of 2009. The pandemic virus was also the prevalent influenza strain during influenza season 2010–2011; however, it was completely replaced by H3N2 subtype in the next season. During the most recent influenza season, 2012–2013, the pandemic strain was once again the only influenza A virus circulating in Cyprus. Full-length neuraminidase gene sequencing revealed mutations that had previously been identified as permissive. No significant difference in the expression of the IFN-inducible genes *OAS* and *IFIT1* were observed. The phylogenetic analysis of the neuraminidase gene sequences revealed a picture of continuous importation of influenza strains in the island of Cyprus with local circulation playing only a minor role in determining the prevalent strain of the next influenza season.

Key words: Epidemiology, infectious disease epidemiology, influenza.

INTRODUCTION

In April 2009, a previously undescribed influenza A(H1N1) virus was isolated from humans in Mexico and the USA. This novel virus, which is thought to have been generated and circulated in pig populations before cross-species transmission to humans, spread to more than 200 countries causing illness and death, leading the World Health Organization to declare the outbreak of a pandemic in June 2009 (http://www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/index.html).

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Two unusual features of pandemic A(H1N1)2009 virus were its efficient human-to-human transmission and its ability to cross the species barrier [1]. To reach functional receptors on target cells present in the respiratory tract, influenza virus uses its neuraminidase (NA) to cleave sialic (neuraminic) acid associated with mucins, thus allowing viral penetration through the mucosa [2]. In addition to that, NA's most important function is the release of progeny viruses by cleaving terminal sialic acid residues on the surface of infected cells. Therefore, NA was chosen as a suitable drug target. NA inhibitors (NAIs) are synthetic analogues of sialic acid with proven activity against pandemic A(H1N1)2009 virus but with documented emergence of resistance as is the case with seasonal H1N1 viruses [3].

In Cyprus, all pandemic A(H1N1)2009 virus infections were reported to the Ministry of Health.

As elsewhere, most infected individuals experienced predominantly mild uncomplicated, self-limiting upper respiratory tract illness, typically presenting with fever, malaise, myalgia, headache and dry cough. Most hospitalizations occurred in individuals aged <2 years and ⩾65 years. Individuals with certain underlying conditions such as diabetes, morbid obesity, pregnancy and cardiovascular, neurological and pulmonary diseases, including asthma, appeared to be at greater risk of severe disease progression. Frequently reported complications included pneumonia, bacterial co-infection and exacerbation of underlying medical conditions such as congestive heart failure [4].

The aim of the current study was to gain an insight into the pandemic period in Cyprus and monitor the circulation of the new influenza strain in the following three influenza seasons. In addition, the presence of other viruses known to cause respiratory tract infections with influenza-like symptoms, co-circulating with the pandemic influenza virus, was investigated. Sequencing of the NA gene of a number of isolates was performed in order to assess the presence of mutations in the NA gene that could lead to NAI resistance or facilitate the introduction of resistance. Finally, the expression of interferon (IFN)-induced genes with documented antiviral activity was investigated and compared between cells from nasopharyngeal swabs positive for pandemic A(H1N1)2009 virus and cells positive for other respiratory viruses.

MATERIALS AND METHODS

Samples

Between July and December 2009, 652 nasopharyngeal swab samples (Becton, Dickinson and Company, USA) of patients with fever (temperature ⩾38 °C) and/or other acute respiratory symptoms (rhinorrhoea, cough, wheezing, respiratory distress, etc.) were sent to the Department of Molecular Virology of the Cyprus Institute of Neurology and Genetics from state hospitals or private clinics. Samples from individuals who had close contact with confirmed cases or had travelled abroad were also investigated. In addition, several influenza A-positive samples from the three subsequent influenza seasons (2010–2013) were included in this study.

Viral nucleic acid extraction and real-time RT–PCR

Detection of the pandemic A(H1N1)2009 virus was performed following the guidelines published by the WHO Collaborating Centre for influenza at CDC Atlanta, USA (http://www.who.int/csr/resources/publications/swineflu/CDCrealtimeRTPCRprotocol_20090428.pdf). Briefly, viral nucleic acids were extracted from 200 µl of the nasopharyngeal swab medium using the QIAamp Viral RNA Mini kit (Qiagen, USA). Real-time RT–PCR (Invitrogen SuperScript™ III Platinum® One-Step Quantitative kit; Invitrogen, USA) was performed on a 7500 Real-time PCR system (Applied Biosystems, USA). Detection of the H3N2 subtype was performed as described previously [5].

Samples negative for the pandemic A(H1N1)2009 virus, were analysed further, using published protocols, for the presence of the following viruses: influenza B [6], respiratory syncytial virus (RSV) [7], coronaviruses OC43, NL63 and 229E, human metapneumovirus, human bocavirus [8], adenovirus [9], human enteroviruses [10], rhinoviruses [11] and parainfluenza viruses 1–4 [12, 13].

Full-length NA gene sequencing

For the sequencing of the NA gene, five pairs of primers and a one-step RT–PCR protocol recommended by the WHO were used to generate five overlapping fragments of the full-length NA gene (1410 bp) (http://www.who.int/csr/resources/publications/swineflu/GenomePrimers_20090512.pdf). Twenty-nine pandemic A(H1N1)2009 virus positive samples from different time points of the pandemic as well as representative samples with a sufficient viral load from the rest of the study period (one sample from 2010, five samples from the beginning of 2011 and three samples from the beginning of 2013) were chosen and subjected to Sanger sequencing (CEQ 8000, Beckman Coulter, USA) using the universal primers m13for and m13rev as sequencing primers. Sequences were submitted to Genbank and assigned accession numbers KF687910–KF687947.

Phylogenetic analysis

Phylogenetic analyses were conducted in MEGA 5.2.1 [14]. Full-length NA gene alignment of 38 influenza isolates and the vaccine strain A/California/07/2009 (H1N1) was performed using CLUSTALW. Bayesian Information Criterion (BIC) scores were calculated in MEGA 5.2.1 for different models to determine the best-fitting nucleotide substitution model. In addition,

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jModelTest [15, 16] was used for evaluating the best-fitting nucleotide substitution model under BIC yielding the same result [Hasegawa-Kishino-Yano (HKY)]. This model was then used to construct a maximum-likelihood (ML) phylogenetic tree with 1000 bootstrap replicates. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. The topology of the ML tree was compared with a neighbour-joining phylogenetic tree obtained using the maximum composite likelihood method showing no significant differences.

Gene expression

Total RNA from cells of the nasopharynx was used to investigate gene expression of 2′-5′-oligoadenylate synthetase 2 (OAS2) and interferon-induced protein with tetratricopeptide repeats 1 (IFIT1 or P56). Aliquots from nucleic acids extracted for virus detection, were treated with DNase I (Invitrogen) to remove genomic DNA. The purity of total RNA was assessed by NanoDrop ND-1000 (Thermo Scientific, USA). One hundred ng of total RNA were used for one-step RT–PCR using three TaqMan Gene Expression assays (Applied Biosystems), according to the manufacturer's specifications. The assay identification numbers for the genes to be investigated were: (i) *OAS2* Hs00942643_m1 and (ii) *IFIT1* Hs00356631_g1. Co-amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs03929097_g1) was used to normalize the amount of total RNA present using the threshold cycle relative quantification according to the supplier's guidelines. The fold change in studied gene expression, normalized to endogenous control, was calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical analyses were performed with GraphPad Prism 5.00 for Windows (GraphPad Software, USA). Differences in *OAS2* and *IFIT1* expression in cells from ten nasopharyngeal swabs infected with pandemic A(H1N1) 2009 virus and cells from nine samples infected with other respiratory viruses were assessed using the non-parametric Mann–Whitney test. Cells from nasopharyngeal swabs negative for all investigated respiratory viruses were used as negative control.

RESULTS

Real-time PCR for respiratory viruses

Nasopharyngeal swabs from 652 individuals collected during the pandemic period were tested for influenza A and 403 samples were found positive (62%). All influenza A virus-positive samples were identified as pandemic A(H1N1)2009. The age distribution of positive patients, shown in Fig. 1, revealed a maximum incidence rate in younger people aged between 16 and 25 years. The incidence rate remained elevated until the age of 35 years and then decreased steeply to almost no incidence of infection in people aged > 50 years.

All negative samples were analysed further for the presence of 14 other respiratory viruses (Table 1). Human rhinovirus was found in 84 (13%) samples, coronavirus OC43 in eight (1·2%), followed by coronavirus 229E and human enterovirus in six (0·9%) and metapneumovirus in three (0·5%). Human adenovirus and parainfluenza virus 3 were each detected in two (0·3%) samples, whereas parainfluenza viruses 1, 2 and 4 were detected in only one (0·15%) sample. No influenza B, RSV, coronavirus NL63 or bocavirus were detected.

Pandemic A(H1N1)2009 virus was the prevalent strain until the end of the 2009–2010 influenza season with all influenza A being of the same type until April 2010. This was also the case for the next influenza season (2010–2011), while the trend changed completely in the 2011–2012 season with all influenza A being of the H3N2 type. During the most recent influenza season (2012–2013) the pandemic strain was once again the only influenza A virus circulating in Cyprus (Table 2).

NA gene sequencing

Full-length NA gene sequences were obtained for 38 isolates covering the period 2009–2013. Alignments were built using MEGA 5. Two amino-acid substitutions, V106I and N248D were found in almost all isolates. Besides these two mutations, other sporadic mutations also occurred in certain isolates, the most frequent of which were I8L, I46 T, V241I, and R257 K. Several other mutations were observed as shown in Figure 2.

Phylogenetic analysis

The phylogenetic tree shown in Fig. 3 was inferred using the ML method based on the HKY model, which was determined as the best-fitting nucleotide substitution model according to the BIC score. Vaccine strain A/California/07/2009 was used as outgroup. The analysis involved 38 nucleotide sequences

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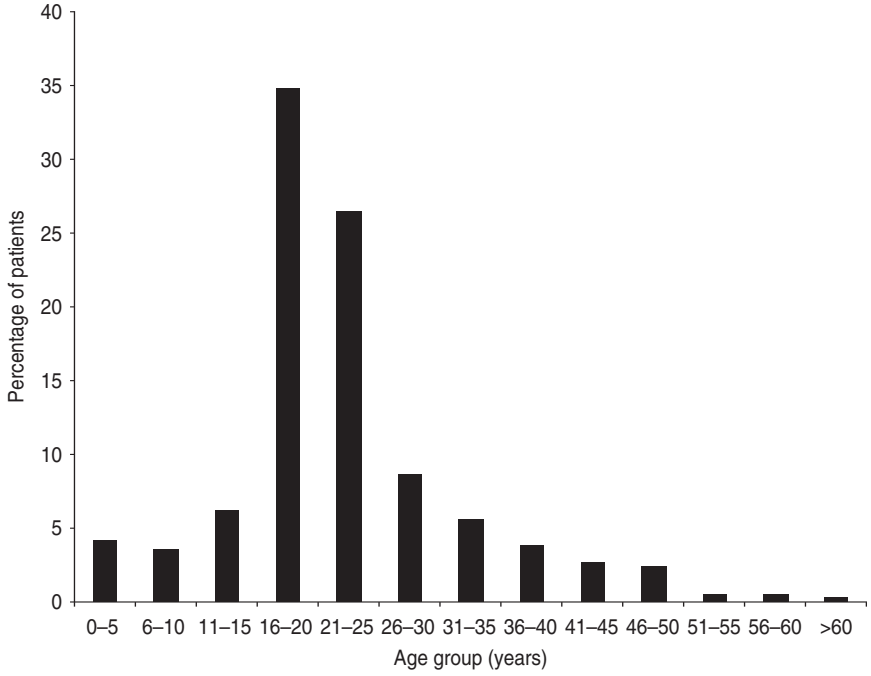

Fig. 1. Infection and age. Age distribution of pandemic H1N1-positive patients between July 2009 and December 2009.

Table 1. *Detection rates and distribution by pathogen of viral respiratory infections in 652 samples between July and December 2009*

Cases	Number (%)*
Total positive	517 (79)
Influenza A	403 (62)
Rhinovirus	84 (13)
OC43	8 (1·2)
229E	6 (0·9)
Enteroviruses	6 (0·9)
Parainfluenza viruses 1–4	5 (0·75)
Metapneumovirus	3 (0·45)
Adenovirus	2 (0·3)

* % values are of total number of patients.

Table 2. *Influenza A typing in four consecutive seasons in Cyprus (2009 pandemic–2013)*

Season	Pandemic A(H1N1)2009	A(H3N2)	Total
2009/2010	406	0	406
2010/2011	48	0	48
2011/2012	0	25	25
2012/2013	19	0	19

of 1410 bp length in the final dataset. Sample names are composed of the year, month and day when the sample was received plus an internal laboratory code. It appears that pandemic A(H1N1)2009 was introduced into Cyprus on multiple occasions. Most of the samples from the beginning of the outbreak, i.e. July 2009, form one clade (clade no. 1). Two months later, in September 2009, it appears that a slightly different strain had been introduced into Cyprus which also dominated the months of November and December (clade 3). Samples isolated in 2011 belong to one of two clades, either clade 2, which again appears to be a new introduction and clade 5, which could have evolved from a strain already introduced in July 2009. H1N1 was absent in the 2011/2012 winter season, where all influenza isolates belonged to the H3N2 type, but reappeared in the following 2012/2013 season. All three isolates cluster together and are most closely related to the previous 2011 isolates (clade 5).

Gene expression

Levels of expression of IFN-induced genes *IFIT1* and *OAS2* were assessed in cells from nasopharyngeal swabs with regard to the virus they have been infected with and are shown in Fig. 4. Results were statistically analysed using the Mann–Whitney *U* test. A significant increase of the median expression of both genes was observed (*OAS2*: 47-fold for influenza A and

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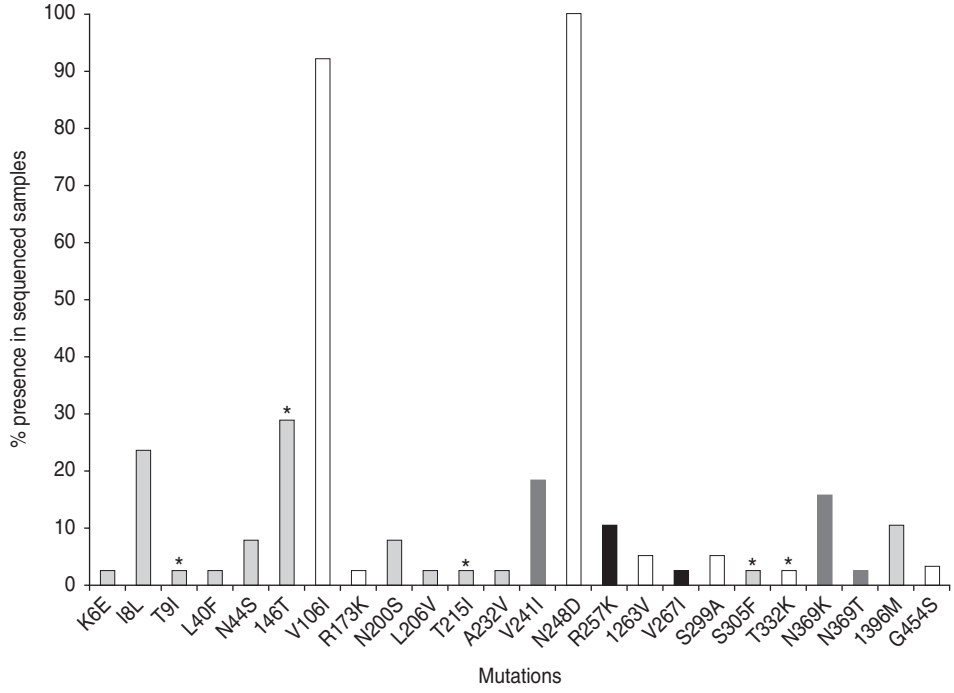

Fig. 2. Prevalence of NA mutations. Frequencies of the NA mutations identified in 35 pandemic A(H1N1)2009 positive samples from July 2009 until January 2013 in Cyprus. Different shading indicates different functions for mutations: ■, possible permissive mutations; □, mutations in antigenic regions of NA; ■, mutations that are both possibly permissive and located in antigenic regions of NA; ■, mutations of unidentified function. An asterisk indicates a non-conservative amino-acid substitution.

15-fold for other respiratory viruses; *IFIT1*: median increase 175-fold for influenza and 57 for other respiratory viruses); however, the analysis revealed no statistically significant differences in the median increase in expression levels of these two genes between individuals infected with influenza and individuals infected with another respiratory virus (P values $>0{\cdot}05$).

DISCUSSION

Pandemic A(H1N1)2009 virus, a new strain of influenza virus identified in April 2009, spread quickly in humans worldwide to cause the first influenza pandemic of the 21st century. The pandemic started late in Cyprus as the first confirmed case of infection was reported on 2 June 2009 [17]. After the first case, however, the infection rate increased markedly, coinciding with the beginning of the tourist season. The virus might have been imported to the island either by tourists visiting Cyprus or by residents of Cyprus returning home from their vacations.

Although there are no laboratory-confirmed results regarding the presence of cross-reactive antibodies in our study population, the lower attack rate of pandemic A(H1N1)2009 virus in older people, supports the view of cross-immunity within this age group due to previous influenza infections, as shown previously [18]. The low percentages of detection of other viruses that caused respiratory infections, during the study period (July–December 2009), could be explained by the unusual prevalence of the pandemic A(H1N1)2009 virus. In fact, several papers have reported changes in respiratory virus circulation patterns due to the activation of innate immunity by the most prevalent virus [19, 20]. These findings are further confirmed by the fact that during September and the beginning of October 2009, when the influenza epidemic declined, infections from rhinovirus increased only to be again outnumbered by pandemic A(H1N1)2009 virus infections until the end of the study period (data not shown). Viral interference may have been the reason why another very prevalent respiratory virus, RSV, was not detected until the end of 2009. Furthermore, RSV infections do not usually present with influenza-like symptoms.

The phylogenetic analysis of the NA gene sequences revealed a picture of continuous importation of influenza strains on the island and that

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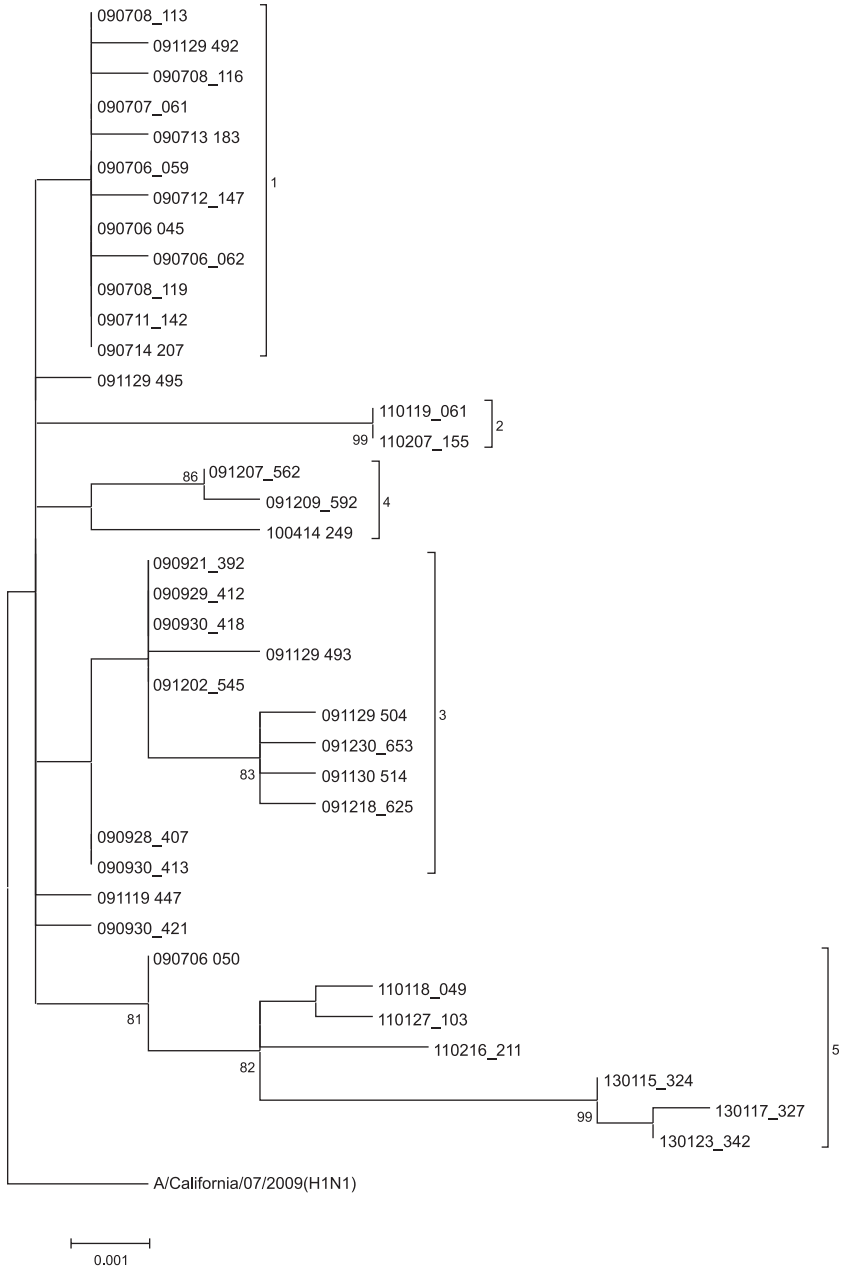

Fig. 3. Phylogenetic analysis. Maximum-likelihood phylogenetic tree based on the complete NA gene sequences of 38 H1N1 samples isolated in Cyprus between 2009 and 2013. Sample names are composed of the year, month and day that the sample was received plus an internal laboratory code. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Only bootstrap values >80% are shown.

local long-term circulation plays a minor role in determining the prevalent strain of the next influenza season. This phenomenon is highlighted by the sudden replacement of H1N1 by H3N2 in the 2011/2012 season and the total replacement of H3N2 by H1N1 in the 2012/2013 season and may be explained by the small population size on one hand and a large number of travellers and tourists visiting the island on the other. The circulation of only one influenza A subtype during each influenza season in Cyprus is in sharp contrast with the data from other European countries where H1N1 and H3N2 co-circulate.

The catalytic site of the NA enzyme has been shown to be conserved in all influenza A and B viruses, therefore NA has been considered as a suitable target for designing anti-influenza agents for both prophylactic

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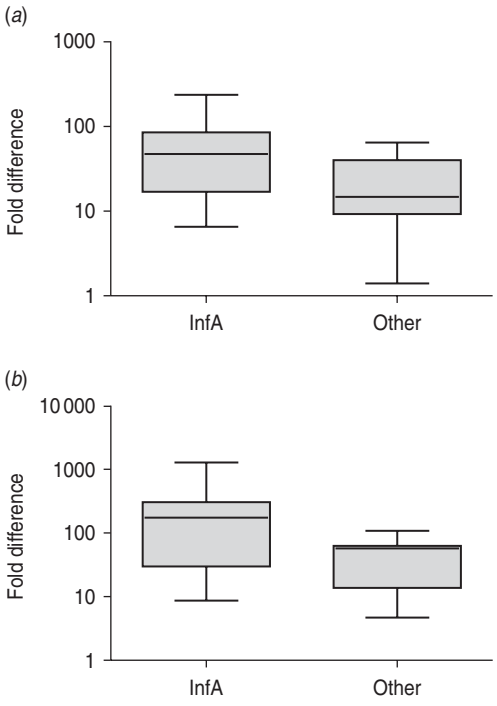

Fig. 4. Expression regulation of IFN-inducible genes. Relative gene expression of (*a*) *OAS2* and (*b*) *IFIT1* in patients with influenza-like illness in the presence of pandemic A(H1N1)2009 or another respiratory virus. Whiskers represent median, minimum and maximum values, boxes represent the lower and upper quartiles.

and therapeutic purposes. NAIs target the active centre of the influenza NA molecule that consists of eight functional (R-118, D-151, R-152, R-225, E-277, R-293, R-368, Y-402; N1 numbering) and 11 framework (E-119, R-156, W-179, S-180, D-199, I-223, E-228, H-275, E-278, N-295, E-425; N1 numbering) residues, and as a result they inhibit the release of newly formed virions from infected cells, cause viral aggregation, reduce infectivity and limit the ability of viruses to penetrate mucus found in the airways [21]. The main NA functional mutation, H275Y, conferring NAI resistance, causes a substantial decrease in the total protein and its activity both in seasonal and in pandemic H1N1 [22]. The ability of pandemic H1N1 H275Y viruses to transmit is especially alarming because seasonal H1N1 and H3N2 viruses prior to 2007 were attenuated by the aforementioned mutation, suggesting that secondary mutations acquired by the virus increased transmissibility [23]. Frequently, in molecular evolution, an initial occurrence of a secondary mutation enables the protein to tolerate the subsequent functional mutation, therefore the secondary mutation is referred to as 'permissive' [24]. To investigate the role of potentially permissive NA mutations that may be responsible for offsetting the negative effects of the H275Y substitution, a computational analysis of NA protein stability was conducted in a recent Australian study. The results showed that two of these mutations, V241I and N369 K, both of which emerged in pandemic A (H1N1)2009 viruses in 2010 and are now present in over 80% of currently circulating strains, could restore ~50% of the protein stability that was lost as a result of the H275Y mutation [25]. These results partly confirmed the results of a previous study, where computationally identified secondary NA mutations were tested for their effect on NA protein levels and activity impaired by the H275Y mutation. Several of the secondary mutations partially buffered this defect, with the strongest effects being mediated by R257 K, T289M, N369 K and V234M [26]. Possible molecular markers of reduced oseltamivir susceptibility in avian and swine N1 viruses are amino-acid changes located outside the active side such as V267I, N307D and V321I that potentially distort hydrophobic pockets and indirectly affect the NA catalytic and framework residues. More specifically, residues V267 and N307 are near a hydrophobic patch that may be important for stabilizing framework residues H275 and E278 [27]. Although the H275Y mutation was not detected in our sequenced samples, the existence of several permissive mutations is alarming due to the fact that NA could tolerate a possible subsequent introduction of resistance mutations. Thus, monitoring for resistance in influenza viruses should take into consideration not only NA resistance mutations themselves but also secondary mutations, especially in patients at risk of developing resistance, such as patients having prolonged post-exposure prophylaxis or treatment with subtherapeutic dosages [28].

There are also NA mutations that affect vaccine development through altering antibody interactions as well as antigenic regions. Several of the mutations identified in our study are found within or near the antigenic regions of the NA that are comprised of amino-acid residues 83–99, 103–144, 156–190, 252–303, 330, 332, 340–345, 368, 370, 386–395, 400, 431–435 and 448–468. The ubiquitous N248D substitution changes a central part at an antibody-binding site [29].

Type 1 interferons (IFN-α/β) are an important component of the innate immune system and are a major component in host defence against viral infections. In virus-infected cells, a signalling pathway is activated

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by viral dsRNA that is produced during infections by both RNA and DNA viruses. The resulting up-regulation of IFN-α/β gene expression activates signal transduction pathways [30], leading to the transcriptional induction of more than 300 IFN-stimulated genes that cause suppression of viral replication, clearance of virus-infected cells and facilitation of adaptive immune response [31]. Most of the individuals infected with pandemic influenza virus presented with high fever, indicating an elevated response of the innate immune system. To our knowledge, our study is the first to have compared the expression regulation of two of the most important IFN-stimulated genes, *OAS2* and *IFIT1* (P56), between cells infected with pandemic A(H1N1)2009 virus and cells infected with other viruses that infect the respiratory tract. OAS is the first component of the OAS/RNase L antiviral pathway and even though it is IFN-inducible, it requires binding to dsRNA for activation of its enzymatic activity [32]. IFIT1 was one of the first IFN-inducible proteins whose cDNA was cloned [33]. The induction of IFIT1 mRNA by IFNs is transient and the mRNA has a relatively short half-life, indicating that the protein may have a potential regulatory role [34]. One of the best characterized interactions of IFIT1 has been with the eukaryotic translation factor eIF3 [35].

On August 2010, the WHO announced that the 2009 pandemic had moved into the post-pandemic period, but based on experience with past pandemics, pandemic A(H1N1)2009 virus was expected to take on the behaviour of a seasonal influenza virus and continue to circulate for some years to come (http://www.who.int/mediacentre/news/statements/2010/h1n1_vpc_20100810/en/index.html). There are concerns that this virus may mutate or reassort with other existing influenza viruses to give rise to more readily transmittable or more pathogenic viruses. Therefore, active molecular surveillance is important to monitor the pathogenicity of circulating strains and to update pandemic preparedness and response plans accordingly.

DECLARATION OF INTEREST

None.

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