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Our first impact factor

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It's here: the first impact factor for *Eurosurveillance*. On 28 June 2012, Thomson Reuters published the 2011 impact factors: an exciting 6.15 places *Eurosurveillance* at rank 6 among the 70 journals in the category Infectious Diseases [1] and thus puts us prominently on the global scientific community map.

'We don't have one' used to be the answer when we were asked about the impact factor for the journal. This changed in 2009, when *Eurosurveillance* was accepted and listed for the impact factor, and we were able to say 'We'll get it'. Now we have it, we are more than pleased with the encouraging 6.15, which is similar to that of leading journals in the field. We regard it as an endorsement of our work and editorial policy. It also justifies the confidence shown by our contributors in the many strengths of *Eurosurveillance* and our ability to reach the right audience in the years when we did not yet have an impact factor. It is also a testament to the dedication of the Editors-in-chief and various editorial teams during the journal's 15-year history [2], whose hard work has led to making the journal what it is today.

The figure of 6.15 gives us a firm basis on which to build in the future. It will help us to attract the best and most relevant papers for our readers in the field of infectious disease surveillance and epidemiology. *Eurosurveillance* will, of course, continue to serve as a platform for the public health community across Europe, in order to ensure that all relevant findings are shared internationally. We will keep on striving to provide timely information that supports infectious disease prevention and control. In so doing, we aim to influence the public health agenda and stimulate scientific debate.

In 1955, Eugene Garfield first presented the concept of an impact factor in a paper in *Science* [3] and the concept evolved over time [4]. The 2011 impact factor is computed using the total number of citable articles published in a given journal in 2009 and 2010 as the denominator and the number of times these articles are cited in indexed journals in 2011 as the numerator. The resulting figure is widely considered as an indicator of the scientific impact of a journal, and publishing in journals with a high impact factor is a requirement for an academic career in many institutions.

It is not news that the impact factor is a much debated and controversial indicator [5-7], but commonly accepted alternative measures remain to be developed. One way of assessing the impact of articles and journals is the ranking of the Faculty of 1000, which selects articles for post-publication peer review [8]. *Eurosurveillance* is proud to have had five of its papers selected and ranked as 'must read' [9] and 'recommended' [10-13]. Other suggested ways to measure journal impact are the h-index [14] and the Scopus-based SCImago Journal Rank (SJR) [7]. The latter gives an indication of how the average article of a journal influences science: citations from highly cited journals weigh more than those from low-cited ones. Thus the average article published in a journal with a higher SJR is considered more central to the scientific discussion than those of journals with a lower SJR. The 2011 SJR ranks *Eurosurveillance* at 61 of 1,597 journals in the field of medicine [15].

While equipped with a good impact factor and ranking in the SJR and well positioned among other journals in our field, *Eurosurveillance* aims to progress still further and we expect to attract a number of new contributors. An online submission system, due to be launched later this year, will help us to process submissions more efficiently. We have already started using a plagiarism-detection software. Moreover, we have entered the world of social media by setting up a Twitter account earlier this year. A steady increase of 'followers' of the journal, by on average two to three per day, shows we are on the right track. We also plan to set up *Eurosurveillance* pages on several social network sites soon. Watch out for them.

In times when open access 'mega journals' – publishing several thousands of articles a year – are evolving, more journals are moving towards offering open access and new business models are emerging, such as offering authors publishing flat rates. Having a non-commercial publisher allows us to provide a platform also for authors with limited financial resources or those from less-resourced institutions. In this way, we

contribute to broadening the evidence base within our scope.

Even if the impact factor boosts the reputation of the journal, we will keep on doing things the *Eurosurveillance* way. We have developed many strong personal ties with our contributors and have built a solid network of collaborators and advisors: we are grateful for their involvement over the years and thank them once again for their support. By our commitment to serving authors, reviewers and the greater scientific community, we actively participate in providing high-quality information for the benefit of public health.
Acknowledgments

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First report of sandfly fever virus infection imported from Malta into Switzerland, October 2011

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We report the first documented cases of sandfly fever virus infection in travellers returning from Malta to Switzerland in autumn 2011. These cases illustrate the importance of considering sandfly-borne viral infection in the differential diagnosis of febrile patients from the Mediterranean island Malta. Raising awareness among physicians is relevant especially now at the beginning of the summer tourist season.

On 17 October 2011, a Swiss citizen was hospitalised with fever, nausea, vomiting and intensifying headache two days after his return from the Mediterranean island Malta, where he had spent two weeks on Gozo Island. Nine days before his admission, the patient had suffered from back pain, tiredness and subfebrile temperatures, but had recovered after medication with the non-steroid inflammatory drug ibuprofen. The patient's wife had suffered from similar, less intense symptoms. Both had multiple insect bites and were diagnosed with laboratory-confirmed sandfly fever. The couple's two accompanying children and two further travel companions had been frequently bitten by small flying insects, but suffered no symptoms.

Case 1

Case 1, a man in his late 40s, was admitted to hospital in Switzerland two days after his return from Malta and presented in a reduced general condition. He had fever (38.4 °C), a generalised rash and complained of severe headache, without meningism. Multiple skin lesions due to insect bites were visible on the extremities (Figure).

Laboratory tests for this patient, including C-reactive protein, electrolytes and transaminases revealed no abnormalities, and a complete blood cell count showed relative lymphopenia (17.9%, normal range: 20–52%).

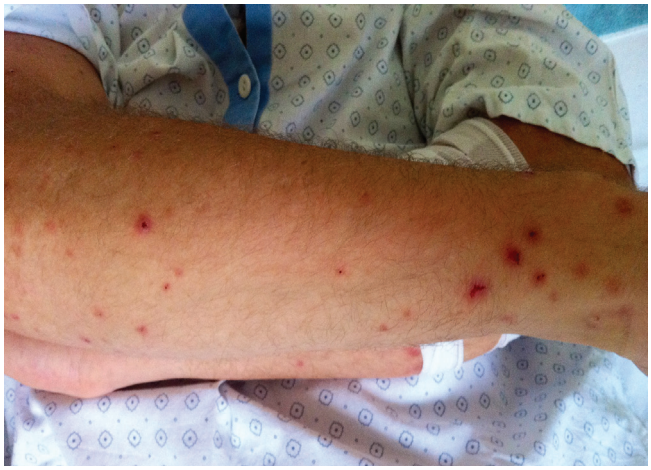
Because of the recent stay in a Mediterranean country and the multiple skin lesions, a sandfly fever virus (SFV) infection (pappataci fever) was assumed. Serology for SFV was positive on a serum sample taken on the day after admission, i.e. 10 days after onset of first symptoms on Malta. An immunoblot (IB) for bunyaviruses (Mikrogen, Munich, Germany) showed reactivity with the TOSV bands, with stronger intensity than the cut-off reference, indicating the presence of IgM and IgG antibodies against TOSV. An indirect immunofluorescence test (IIFT) (Sandfly fever virus Mosaic 1, Sandfly fever virus serotypes Sicilian, Naples, Toscana, Cyprus; Euroimmun, Lübeck, Germany) revealed high serum antibody titres against Toscana and Naples SFV (Table), indicating an infection with a phlebovirus belonging to the SFNV serological complex.

In a convalescent serum, taken 52 days after onset of illness, an eight-fold increase of anti-TOSV IgM was demonstrated, accompanied by a five-fold increase for anti-TOSV IgG (Table).

The patient gradually improved under analgesic and anti-emetic therapy. On day 15 after onset of illness he could be transferred to a rehabilitation centre and fully

Figure

Multiple skin lesions due to the insect bites on arm of sandfly fever patient, Switzerland, October 2011



TABLE

Serology results for two patients with sandfly fever, Switzerland ex Malta, October 2011

	Case 1		Case 2	
Days post-onset of illness	10	52	11	52
TOSV IgM IB	++	++	+/-	++
TOSV IgG IB	++	++	-	++
TOSV IgM IIFT	400	3,200	100	1,000
TOSV IgG IIFT	2,000	10,000	-	1,000
SFNV IgM IIFT	400	1,000	100	320
SFNV IgG IIFT	1,000	1,000	-	100
SFSV IgM IIFT	-	-	-	-
SFSV IgG IIFT	-	-	-	-
SFCV IgM IIFT	-	-	-	-
SFCV IgG IIFT	-	-	-	-

IB: bunyavirus immunoblot, IIFT: indirect immunofluorescence test; SFCV: sandfly fever Cyprus virus; SFNV: sandfly fever Naples virus; SFSV: sandfly fever Sicilian virus; TOSV: Toscana virus.

++ strong reactivity,

+/- weak reactivity,

- no reactivity/no immunofluorescence.

Numbers refer to reciprocal titres, according to the dilution scheme of the manufacturer. End-point titres were adjusted to the strength of immunofluorescence, e.g. end-point titre 1:3,200, if serum-dilution 1:1,000 showed medium strength of immunofluorescence.

recovered 21 days after onset of symptoms. The patient suffered no relapse in the following eight months.

Case 2 and accompanying persons

Case 2, a woman in her early 50s, showed positive IgM antibodies against TOSV and SFNV in a serum taken 11 days after onset of illness, with a 10-fold increase in anti-TOSV IgM and a seroconversion for anti-TOSV IgG antibodies in a follow-up serum, taken on day 52 after onset of illness (Table).

Serum samples were also taken from the couple's children and the two accompanying travellers on day 52 after onset of illness of patients 1 and 2. All samples were tested by IIFT and IB and turned out negative for IgM and IgG antibodies against TOSV, SFNV, SFSV, and SFCV.

Leishmania species are known to be transmitted by *Phlebotomus perniciosus* in Malta [5]. Patients were informed to remain alert for late appearing symptoms of leishmaniasis, which may not manifest until months after infection [5]. However, in the following eight months, none of the travellers developed clinical signs of leishmaniasis.

Background

Phleboviruses of the *Bunyaviridae* family, transmitted to humans by arthropods, are found in Europe, Africa, central Asia, and the Americas. In Mediterranean Europe, the phleboviruses Toscana virus (TOSV), sandfly fever Naples virus (SFNV), sandfly fever Sicilian virus (SFSV), and sandfly fever Cyprus virus (SFCV) are transmitted by phlebotomine sandflies. Among these, TOSV circulates in countries around the Mediterranean Sea (Algeria, Cyprus, France, Greece, Italy, Portugal, Spain and Turkey) [1,2].

TOSV may cause an acute, nonfatal, influenza-like symptomatology or even aseptic meningitis and meningoencephalitis [2-4]. SFNV, SFSV and other related viruses can cause the so-called 'three-day fever' or 'pappataci fever'. Patients present with influenza-like symptoms including fever, retro-orbital pain, myalgia and malaise and usually recover fully within a week. However, infections with these viruses, even when mild, have been shown to be highly incapacitating during the time the patients are affected [2].

Discussion

Viruses in the genus *Phlebovirus* can cause a variety of clinical syndromes ranging from a brief, self-limiting febrile illness to encephalitis, meningoencephalitis and fatal haemorrhagic fever. Of our cases, the male patient suffered from symptoms of a SFV infection that incapacitated him for two weeks, while his wife had less severe symptoms. The other four travellers accompanying the couple were not infected, despite multiple insect bites.

The *Phlebovirus* genus consists of more than 60 distinct virus serotypes. While antigenically unrelated to members of other genera in the family *Bunyaviridae*, various degrees of cross-reactivity in serological tests can occur within the genus, as reported for the SFNV and SFSV antigenic complexes [6,7]. Our patients showed

cross-reactions in the IIFT using SFNV and TOSV antigens, both members of the SFNV antigenic complex. Although IIFT using SFSV and SFCV as antigens yielded negative results, reactions with other serotypes in the genus *Phlebovirus* cannot be ruled out.

Viral neutralisation tests (VNT) using early convalescent sera remain the serological reference method to identify these viruses or to assess the specificity of the antibody response [8]. Although generally regarded as the gold standard assay for specificity, the VNT is relatively labour-intensive and only established in few laboratories. SFV-specific commercial assays such as IIFT, enzyme-linked immunoassay and IB are much more commonly used. For the diagnosis of the cases described here, we used IIFT for screening purposes and the IB as confirmatory assay [8].

Direct viral diagnosis by isolation and RT-PCR from blood or cerebrospinal fluid is only possible in the early stages of infection, i.e. the first two days after onset of symptoms and before seroconversion [2]. Case 1 presented on day 9 after symptom onset, at a time when SFV-specific serology was already positive. Diagnosis was therefore attempted by serological investigation, using two commercial kits. That the increase in anti-TOSV IgM and IgG in the convalescent serum of Case 1 was higher than the increase in antibodies against SFNV, was suggestive of an infection with TOSV rather than with SFNV. Similarly in Case 2, anti-TOSV IgM increased more than anti-SFNV IgM and was accompanied by higher seroconversion for anti-TOSV IgG than for SFNV-IgG.

Most cases of TOSV infection have been reported in residents of or travellers to central Italy and Spain, and sporadically in other Mediterranean regions such as Portugal, Cyprus, southern France and Greece [9]. Asymptomatic infections have also been described [9]. Unlike the other SFV serotypes, TOSV shows a peculiar neurotropism. It can cause meningitis or meningoencephalitis from which patients generally recover within seven to 10 days [9]. TOSV infections occur particularly during the summer and correlate with the life cycle of the insect vectors *Phlebotomus perniciosus* and *P. perfiliewi* [9].

TOSV has been isolated from *P. perfiliewi* and *P. perniciosus*. The latter vector is distributed throughout the Mediterranean region as two races. SFNV has been isolated in Italy from *P. perniciosus*, in Serbia from *P. perfiliewi* and in Egypt from *P. papatasi* [10]. The typical *P. perniciosus* race occurs in Italy as well as in Tunisia, Morocco and in Malta [10]. However, to the best of our knowledge, neither TOSV nor SFNV infections have been reported from Malta so far. Thus, in connection with the beginning of the summer season, it is now of particular relevance to consider sandfly-borne phleboviruses in the differential diagnosis of patients returning from Malta and presenting with febrile illness.

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Réunion, a sentinel territory for influenza surveillance in Europe

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In Réunion, a French overseas territory located in the southern hemisphere, increase in influenza activity is generally observed several months earlier than in Europe. Influenza activity is monitored in Réunion through a multi-source surveillance system including sentinel practitioners network, hospital emergency department, laboratory and mortality. Since 2009, three successive influenza epidemics occurred on the island. The largest was observed in 2009 while epidemics in 2010 and 2011 were much weaker. In terms of circulating strains, B viruses were predominant at the beginning of the 2009 epidemic but they were completely evicted once A(H1N1)pdm09 circulation started. In 2010, A(H1N1)pdm09 virus was predominant again, but a constant co-circulation of B viruses was observed. In 2011, A(H3N2) virus circulated. The same viruses were identified a few months later in mainland France in the respective seasons. Since 2009, virus circulation, epidemiological trends and health impact of influenza have been similar to those observed in Europe. Influenza surveillance in Réunion may therefore give reliable early information which should be considered apart from the surveillance in mainland France. Then, it might be even a more suitable predictor for Europe than other temperate southern hemisphere countries.

Introduction

Réunion, a French overseas territory with 840,000 inhabitants (2011 estimate [1]), is located in the southern hemisphere in the south-western Indian Ocean. It is 700 km east of Madagascar and 200 km south-west of Mauritius, above the Tropic of Capricorn. The island benefits from a healthcare system similar to mainland France and epidemiological surveillance has been developed by the regional office of the French Institute for Public Health Surveillance (Cire OI) based on the surveillance system of mainland France [2]. Despite the distance of 9,300 km between Réunion and France, the island is directly connected to Europe with four daily flights to France.

The interest of monitoring influenza in temperate southern hemisphere countries has been recently underlined because it may give an indication of what will happen in Europe during the following winter [3]. Because of its location and a surveillance system similar to the one in France, Réunion can also provide suitable information in terms of prevision. Indeed, as seasons are inverted compared with those in the northern hemisphere, increase in influenza activity is observed on the island during the 'austral' winter (June–July), i.e. several months before Europe. Information collected

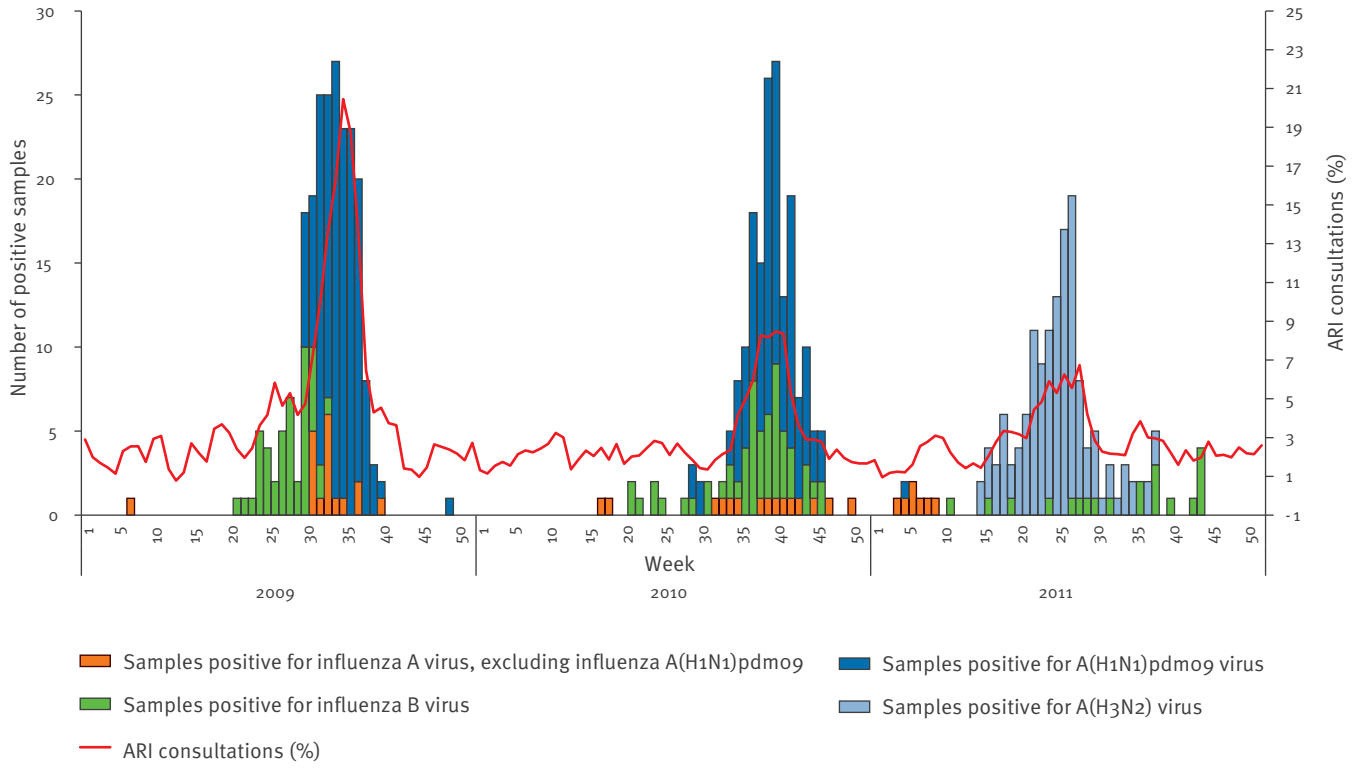
TABLE 1

Indicators collected continuously through influenza surveillance system, Réunion, France

Source	Indicators	Frequency	Starting year
Mortality records	• Total number of deaths • Number of death certificates with mention of influenza • Death in intensive care units and detection of influenza virus (PCR for influenza)	Daily	2006 2008 2009
Emergency departments and hospital wards	• Total number of emergency room visits • Emergency room visits for acute respiratory infections (number and percentage) • Severe cases of influenza (number, severity and outcome)	Daily	2009
Sentinel practitioners	• Total number of consultations • Consultations for influenza syndrome (number and consultation rates)	Weekly	1996
Reference hospital laboratory	• Total number of samples for influenza analysis • Number of positive tests for influenza virus (PCR for influenza virus) • Type of influenza virus (PCR for influenza virus)	Weekly	1998

Figure

Weekly consultations for acute respiratory infections and number of samples positive for influenza viruses, Réunion, France, 2009–2011



ARI: acute respiratory infection.
Source: sentinel practitioner network.

can thus be the same as that observed in Europe a few months later.

The aim of this paper is to present the main results from the surveillance system for influenza in Réunion from 2009 to 2011 in order to assess whether this territory should be considered as an interesting sentinel for influenza surveillance.

Methods

In Réunion, influenza activity has been monitored since 1996 through a sentinel practitioner network [4]. During the influenza A(H1N1)pdm09 pandemic, a multisource surveillance system has been developed [2] to complete this sentinel network. Indicators continuously collected through this surveillance system are summarised in Table 1. Statistical analysis is carried out using Stata and Excel.

Epidemiological and virological surveillance of acute respiratory infections by the sentinel practitioner network

Forty general practitioners and two paediatricians participate in the influenza sentinel network (4.9% of the total general practitioners). They are located all over the island and report on a weekly basis their total number of consultations and the number of acute respiratory infections (ARI) according to the following case definition: sudden onset of fever >38° C, cough, associated or not with other symptoms (breathing difficulty, headache, etc.). Weekly ARI consultation rates are calculated and monitored in order to follow the epidemiological situation of influenza. Furthermore, every physician of the sentinel network is expected to collect a nasal swab from the first two patients of the week presenting with ARI symptoms with an onset of less than three days. This sampling allows surveillance of the viruses circulating in Réunion. Swabs are tested by RT-PCR influenza in the reference hospital laboratory (CHU, Réunion).

Hospital emergency departments and surveillance of severe cases of influenza

There are four emergency departments in Réunion. In all of them, a computerised medical file is filled in during each medical consultation, regardless of the diagnosis. Medical files are automatically extracted and transmitted daily to the French Institute for Public Health Surveillance (InVS, Paris, France). The regional office (Cire OI) can then monitor daily number of visits for all causes including ARI. In addition to this surveillance, all severe influenza cases observed in Réunion are reported by clinicians to the Cire OI through a standardised form including epidemiologic, demographic

TABLE 2

Comparison of circulating influenza viruses, Réunion and mainland France, influenza seasons[a] during 2009–2012

Location	2009		2010/11		2011/12	
	Epidemic period	Influenza virus isolated	Epidemic period	Influenza virus isolated	Epidemic period	Influenza virus isolated
Réunion	Week 30 to week 42 (2009)	• A(H1N1)pdm09 • B	Week 35 to week 47 (2010)	• Predominantly A(H1N1)pdm09 • B	Week 16 to week 30 (2011)	• Predominantly A(H3N2) • B
Mainland France	Week 43 to week 52 (2009)	• A(H1N1)pdm09 • B	Week 51 (2010) to week 7 (2011)	• Predominantly A(H1N1)pdm09 • B	Week 6 to week 14 (2012)	• Predominantly A(H3N2) • B • Occasionally A(H1N1)pdm09

[a] In the southern hemisphere the influenza season is between May and October. In the northern hemisphere it is during the winter months.

and clinical data. A severe case of influenza is defined as a patient with a laboratory-confirmed influenza infection (positive RT-PCR for influenza virus) admitted for more than 24 hours to an intensive care unit (ICU) or as a patient who died.

Mortality surveillance

The National Institute for Statistics (Institut National de la Statistique et des Etudes Économiques, Insee) conducts the administrative recording of deaths from all causes in France. For several years, Insee has been monitoring and centralising daily mortality in France including Réunion. In case of an influenza epidemic on the island, we analyse this total number and excess of deaths from all causes. This system is completed by analysis of all death certificates received by the regional public health authority that mention 'influenza'. These certificates are recorded as influenza-associated deaths. Electronic death certification which is being implemented in France is being used by the Intensive Care Department of Saint-Denis Hospital, and is analysed in real time by the Cire.

Results

Data from the physician sentinel network and from virological surveillance for 2009–2011 are presented in the Figure.

Since 2009, three successive influenza epidemics occurred in Réunion. The largest was observed in 2009, with consultation rates for ARI reaching 21%. In 2010 and especially in 2011, epidemics were much weaker with a maximal percentage of ARI of 8.5% and 6.7%, respectively. The three of them started during the southern hemisphere winter (between June and August), and lasted between 8 and 10 weeks. Numbers of visits from emergency rooms show the same pattern of consultation rates of sentinel network [4-5].

Regarding circulating strains, B viruses were predominant at the beginning of the 2009 epidemic, but they were rapidly evicted once A(H1N1)pdm09 circulation started (Table 2).

In the 2010 influenza season in Réunion, the pandemic virus was predominant again, but a constant co-circulation of B viruses was observed. In 2011, the A(H3N2) virus has been circulating almost exclusively; A/Victoria/210/2009 strain was notably identified. No A(H1N1)pdm09 circulation was detected and only few instances of B virus circulation were identified. During the three influenza seasons, the same viruses were identified in mainland France, except in 2011/12 when A(H1N1)pdm09 was occasionally identified.

During the influenza A(H1N1)pdm09 pandemic in 2009, the Réunion surveillance system showed a nine-week epidemic period, with a peak of consultation rate for ARI during week 35 (24–30 August 2009). The number of patients with A(H1N1)pdm09 infection who consulted a physician was estimated at 66,915 (cumulative attack rate: 8.26%) [3]. After 2009, epidemic periods of influenza were observed in Réunion but they were weaker.

Characteristics of severe cases during 2009-2011 are presented in Table 3.

Chronic respiratory disease was the most common comorbidity every year. The non-negligible proportion of obese patients has to be noted, as well as the presence of one pregnant woman in 2009 and two in 2010, without any other risk factor for severity. In 2010, a particularly high severity among patients hospitalised in ICU could be observed: all 14 patients needed respiratory assistance. Half of them needed extracorporeal membrane oxygenation or high frequency oscillation. Half of the patients hospitalised in ICU died.

Discussion

Since 2009, the surveillance system of influenza in Réunion allows to have a good and real-time view of the epidemiological situation through monitoring a large range of indicators [5-8]. The epidemiological situation of the pandemic influenza in 2009 has been described in other temperate countries of the southern hemisphere [9] and showed that the epidemiological pattern in Réunion compared well with that of other

TABLE 3

Characteristics of severe influenza cases[a], Réunion, France, 2009–2011

Characteristic	2009 (n=24)	2010 (n=14)	2011 (n=8)
Sex (female)	13	8	3
Mean age in years (range)	38 (0–75)	43 (19–76)	52 (0–76)
Risk factors / comorbidities			
Chronic respiratory disease	10	6	3
Obesity[b]	3	5	3
Diabetes	3	3	2
Pregnancy	1	2	0
Age ≥65 years	4	2	0
Cardiac disease	4	1	4
Immunodeficiency	1	1	0
None	4	0	2
Indicators or signs of severity			
Acute respiratory distress syndrome	13	14	3
Respiratory assistance[c]	15	13	3
Extracorporeal membrane oxygenation[d]	3	5	1
High frequency oscillation[e]	0	2	0
Death	5	7	1

[a] Patients with a biologically-confirmed influenza infection admitted to an intensive care unit.

[b] Body mass index >30.

[c] The patient undergoes assisted ventilation by mechanical pump and endotracheal intubation.

[d] Application of a life support system that circulates the blood through an oxygenating system, which may consist of a pump, a membrane oxygenator, and a heat exchanger.

[e] High frequency oscillation ventilation is the delivery of small tidal volumes to the infant at fast frequencies. Both Inspiration and expiration are active, therefore reducing the likelihood of gas trapping.

temperate southern hemisphere countries such as New Zealand [10], South Africa [11], Australia [12-14], with A(H1N1)pdm09 virus circulating predominantly. This pattern was indicative for what was going to happen in the forthcoming influenza season in the northern hemisphere. In 2010, a weaker epidemic was observed with also a non-negligible number of severe cases [15]. Similar patterns were then observed in Europe [16], confirming that surveillance of influenza in Réunion can also provide useful data to anticipate what can be expected a few months later in northern hemisphere countries in terms of dynamics, severity and circulating viruses. During the last three years, our epidemiological data identified obesity and diabetes as risks factors of severe form of influenza. This was confirmed a few months later in Europe [17,18].

During the 2011 season, we did not observe any A(H1N1)pdm09 circulation, which is very specific to our island since all the other southern hemisphere countries detected it [19]. In Réunion, the A(H3N2) virus circulated almost exclusively whereas it was not the predominant influenza A subtype in any temperate southern hemisphere countries considered as sentinels for the northern hemisphere [3]. A few months later, in France, Ireland, Spain and the United Kingdom, the influenza season 2011/12 started during the last weeks of 2011 and has been dominated by influenza A(H3) viruses with minimal circulation of influenza A(H1N1)pdm09 and B viruses [20]. Since 2009, virus circulation, epidemiological trends and health impact in Réunion were therefore similar to those observed in Europe. It confirms that Réunion might be even a more suitable predictor for Europe than other southern hemisphere countries.

In conclusion, influenza surveillance in Réunion may give reliable timely information which should be considered apart from the surveillance in mainland France. In addition to data from other southern hemisphere temperate countries, influenza surveillance in Réunion should be taken into consideration in order to make predictions of what can be expected in the corresponding winter season in northern hemisphere countries.

Furthermore, this information can be very useful for epidemiological surveillance in the Indian Ocean. An international network was initiated in 2006 by the Indian Ocean Commission: the epidemiological surveillance and alert control (SEGA - Surveillance Epidémiologique Gestion des Alerte) [21]. One of its objectives is to exchange epidemiological information on influenza

surveillance. Real-time data on the epidemiological situation of influenza and circulating viruses are therefore available through this network for Comoros, Réunion, Madagascar, Mauritius and Seychelles.

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Frequency of oseltamivir resistance in Sydney, during the Newcastle outbreak of community transmitted oseltamivir-resistant influenza A(H1N1)pdm09 virus, Australia, June to August 2011

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Although oseltamivir-resistant pandemic influenza A(H1N1)pdm09 is uncommon in immunocompetent individuals, a recent report from Newcastle, Australia, showed the first sustained community spread, from June to August 2011, of oseltamivir-resistant influenza A(H1N1)pdm09 virus carrying the H275Y neuraminidase (NA) mutation. To determine the frequency and the extent of this viral variant spread in the nearest major city to Newcastle, we performed a sequence-based genotypic assessment on samples from 143 oseltamivir untreated and 23 oseltamivir post-treatment individuals with influenza collected contemporaneously in Sydney, 120 km southwest of Newcastle. The detection of two of 143 (1.4%) community-derived samples containing H275Y suggests a low prevalence of oseltamivir-resistant influenza A(H1N1)pdm09 virus in the general community and no convincing evidence of spread of the NA H275Y-bearing influenza A(H1N1) pdm09 virus. In oseltamivir treated patients, oseltamivir-resistant influenza A(H1N1)pdm09 virus continue to emerge with three of 23 (13%) post-treatment samples containing the H275Y mutation. The observation of signature mutations and distinct phylogenetic relationship in full-length sequences of haemagglutinin and neuraminidase genes derived from 2011 strains against 2009 strains indicates continued genetic evolution and antigenic drift of the influenza A(H1N1) pdm09 viruses circulating in Australia.

Introduction

Although the world has moved into the post-influenza pandemic period after 2009, local outbreaks and transmission of the pandemic influenza A(H1N1)pdm09 virus remained intense in the southern hemisphere 2011 winter [1].

During the 2009 influenza pandemic, almost all tested influenza A(H1N1)pdm09 viruses remained susceptible to oseltamivir and zanamivir [2], but oseltamivir-resistant variants bearing the H275Y neuraminidase (NA) mutation emerged from individuals receiving prophylaxis, and from immunocompromised patients receiving treatment [3-5]. The frequency of oseltamivir resistance mutations was relatively high in immunocompromised adults and young children when under drug selection pressure, suggesting perhaps a relatively low genetic barrier for NA H275Y to emerge in influenza A(H1N1)pdm09 viruses [5].

Oseltamivir-resistant influenza A(H1N1)pdm09 virus with the NA H275Y mutation may present equivalent viral fitness and transmissibility compared to wild-type viruses in animal models, indicating its potential transmission in the general community (similar to NA H275Y-bearing seasonal influenza A(H1N1) viruses circulating prior to 2009) [6], although others failed to confirm these results, and data derived from animal models may not be directly applicable to humans [7].

Currently, the detection of oseltamivir-resistant influenza A(H1N1)pdm09 virus in untreated individuals in the community remains uncommon (generally less than 1%) and transmission has been documented only in closed settings or where there is close contact with an infected individual [8-10]. However, a recent report of the first sustained community transmission of oseltamivir-resistant influenza A(H1N1)pdm09 viruses (detected in 16% of isolates), in Newcastle, Australia, between June and August 2011 [11], has highlighted the potential of widespread movement of oseltamivir-resistant influenza A(H1N1)pdm09 virus.

The same study also observed the genetically related oseltamivir-resistant influenza A(H1N1)pdm09 virus in Sydney, the largest city and transport hub in Australia, and other areas, suggesting the spread of oseltamivir-resistant influenza A(H1N1)pdm09 virus had occurred [11]. To determine the frequency and the extent of spread, a sequence-based genotypic assessment of influenza A(H1N1)pdm09 viruses circulating at the same time as the Newcastle outbreak was performed in Sydney.

Methods

Patient samples

Respiratory tract samples from 143 oseltamivir treatment-naive individuals infected with influenza A(H1N1) pdm09 virus, detected using an in-house nucleic acid test (NAT) [12] were collected between June and August 2011, which covered the time period during the Newcastle outbreak. For comparison, samples from an additional 23 individuals infected with influenza A(H1N1) pdm09 virus (confirmed on laboratory testing) who had completed a five-day course of oseltamivir during same period were also included. This study was approved by the Sydney West Area Health Service Human Research Ethics Committee (HREC2009/7/4.17(3031)).

Genetic analysis

Viral ribonucleic acid (RNA) was extracted from respiratory tract samples using the Qiagen EZI virus mini kit on the automated EZI Advanced XL instrument (Qiagen, Hilden, Germany). Partial NA gene was amplified using the OneStep RT-PCR system (Qiagen, Hilden, Germany) with primers 5' AGACACTATCAAGAGTTGGAGAAACA 3' and 5' TGTGATTTCACTAGAATCAGG 3' according to the manufacturer's instructions. PCR products were purified and served as template for padlock probe recognition, followed by Rolling Circle Amplification (RCA) of probe signal as previously described [5,13,14].

All samples showing a positive signal for the NA H275Y mutation, together with randomly selected samples with wild-type influenza A(H1N1)pdm09 virus, underwent full-length NA and haemagglutinin (HA) gene amplification with the NA primers (NA Ext: 5' GATAATAACCATTGGTTCGG 3', 5' AAATGGCAACTCAGCACC 3', Int: 5' GGTCTGTATGACAATTGGAAT 3', 5' CACCGTCTGGCCAAGACC 3'), and HA primers (HA Ext: 5' GGCAATACTAGTAGTTCTGCTATAT 3', 5' CATATTCTACACTGTAGAGACCC 3', Int: 5' CTATATACATTTGCAACCG 3' and 5' CCATTAGAGCACATCCAGAAAC 3'). PCR products were purified and sequenced (Applied Biosystems, Foster City, CA, USA).

Chromatograms, together with their sequences, were aligned with the influenza A(H1N1)pdm09 consensus sequence derived from Australian sequences submitted to the National Center for Biotechnology Information (NCBI) Influenza Virus Sequence Database (http://www.ncbi.nlm.nih.gov/genomes/FLU/) using Sequencher software (Gene Codes Corporation, Ann Arbor, USA), and were carefully examined at the location where resistance mutations have been reported. Sequences generated in this study were deposited in the GenBank database with the accession numbers: JQ624635–JQ624655. Neuraminidase sequences carrying the NA H275Y mutation are represented by GenBank accession numbers JQ624645–JQ624648 and JQ624650, while their correspondent HA sequences accession numbers are JQ624635–JQ624639. Near

Table

Influenza A(H1N1)pdm09 viral sequences from Newcastle, Australia, 2011, retrieved from Global Initiative on Sharing All Influenza Data, and used for the phylogenetic analysis in this study

Segment ID	Segment	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI334766	HA	Australia	2011-07-10	A/NEWCASTLE/132/2011	John Hunter Hospital, Virology Unit, Clinical Microbiology	World Health Organization Collaborating Centre for Reference and Research on Influenza	Deng YM, Iannello P, Caldwell N, Leang SK
EPI334768	HA	Australia	2011-07-11	A/NEWCASTLE/151/2011			
EPI334770	HA	Australia	2011-06-20	A/NEWCASTLE/17/2011			
EPI334772	HA	Australia	2011-05-31	A/NEWCASTLE/2/2011			
EPI334780	HA	Australia	2011-07-01	A/NEWCASTLE/82/2011			
EPI334782	HA	Australia	2011-07-04	A/NEWCASTLE/85/2011			
EPI334765	NA	Australia	2011-07-11	A/NEWCASTLE/151/2011			
EPI334767	NA	Australia	2011-07-10	A/NEWCASTLE/132/2011			
EPI334769	NA	Australia	2011-07-01	A/NEWCASTLE/82/2011			
EPI334773	NA	Australia	2011-06-23	A/NEWCASTLE/37/2011			
EPI334781	NA	Australia	2011-07-04	A/NEWCASTLE/85/2011			
EPI334783	NA	Australia	2011-07-04	A/NEWCASTLE/89/2011			

GISAID: Global Initiative on Sharing All Influenza Data.

We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu Database on which this research is based. All submitters of data may be contacted directly via the GISAID website: www.gisaid.org.

full-length NA and HA gene alignment was carried out by using the Clustal W program available from biomanager (https://biomanager.info/); Amino acids were numbered starting after the signal peptide (DTLC) and/or the first methionine. Phylogenetic trees were constructed using the neighbour-joining distance matrix algorithm with the Kimura 2 parameter as an evolutionary model and tested using the bootstrap method with 100 replicates. The trees were rooted with the reference strain A/California/7/2009 HA (GenBank accession number: FJ969540) and NA (GenBank accession number: FJ984386) sequences respectively, representing earlier pandemic viral sequences. Sequences from 2009 influenza A(H1N1)pdm09 viruses for HA (GenBank accession numbers: CY055700, CY055756, CY055534, CY055558 and GQ160611) and NA (GenBank accession numbers: CY055798, CY055544, CY055910, CY055678 and CY055567) from Australia, together with 2011 influenza A(H1N1)pdm09 isolates from Australia and other parts of the world (GenBank accession numbers for HA: JN561789, CY099996, CY092864, CY092094, JN714527, CY092856, CY089463, and NA: JN716363, CY092866, JN714545, CY092858, CY089465, CY111288) were retrieved from the NCBI Influenza Virus Sequence Database and six NA H275Y-bearing strains derived from Newcastle, Australia were also included for comparison (Global Initiative on Sharing All Influenza Data (GISAID) (www.gisaid.org) with accession numbers: HA: EPI334766, EPI334768, EPI334770, EPI334772, EPI334780, EPI334782 and NA: EPI334765, EPI334767, EPI334769, EPI334773, EPI334781, EPI334783).

Results

Two of the 143 (1.4%) individuals who had not been treated with oseltamivir had viruses containing the NA H275Y mutation, as did three of 23 (13%) individuals post-treatment with oseltamivir. Statistical analyses of the difference in the frequency of oseltamivir-resistance between treated and untreated patients, by chi-squared test, indicated that the frequency of oseltamivir-resistance was significantly higher ($P<0.001$) in treated patients.

Full-length NA gene sequencing of two community-derived (GenBank accession number JQ624645 and JQ624646) and three post-treatment influenza samples (GenBank accession number JQ624647, JQ624648 and JQ624650) showing positive signal for the NA 275Y probe further confirmed the presence of the NA H275Y mutation in viruses that infected these five individuals. A comparison of full-length HA and NA sequences derived from influenza A(H1N1)pdm09 viruses carrying NA H275Y mutation and five randomly selected wild-type influenza A(H1N1)pdm09 viruses collected during the same time period in Sydney showed closely related virus (99.65–100% HA nucleotide similarity and 99.22–100% NA nucleotide similarity), although two additional NA amino acid substitutions, NA V83A, and NA E128G, were observed from two distinct strains (GenBank accession number: JQ624645 and JQ624650) (Figure 1A) in the NA H275Y-bearing influenza A(H1N1)

Figure 1

Alignments of haemagglutinin (n=10) and neuraminidase (n=10) amino acid sequences from influenza A(H1N1) pdm09 viruses in Sydney, Australia, in 2011, to respective haemagglutinin and neuraminidase consensus sequences from influenza A(H1N1)pdm09 viruses in Australia in 2009

A. Neuraminidase amino acid sequence alignment

Amino acid number[a]	44	62	83	128	241	275	369	386
CON_NA2009:	N	V	V	E	V	H	N	N
JQ624645:	S	I	A	.	I	Y	K	S
JQ624646:	S	I	.	.	I	Y	K	S
JQ624647:	S	I	.	.	I	Y	K	S
JQ624648:	S	I	.	.	I	Y	K	.
JQ624650:	S	I	.	G	I	Y	K	S
JQ624649:	S	I	.	.	I	.	K	S
JQ624651:	S	I	.	.	I	.	K	S
JQ624652:	S	I	.	.	I	.	K	S
JQ624653:	S	I	.	.	I	.	K	S
JQ624654:	.	.	.	.	I	.	K	.

B. Haemagglutinin amino acid sequence alignment

Amino acid number[a]	129	154	160	202	214	391	468
Antigenic site number[b]	112	137	143	185	197	374	451
CON_HA2009:	E	P	S	S	A	E	S
JQ624635:	K	.	G	T	T	K	N
JQ624636:	.	.	G	T	T	K	N
JQ624637:	K	H	G	T	T	K	N
JQ624638:	K	.	G	T	T	K	N
JQ624639:	K	.	G	T	T	K	N
JQ624641:	K	.	G	T	T	K	N
JQ624642:	K	.	G	T	T	K	N
JQ624643:	K	.	G	T	T	K	N
JQ624644:	.	.	G	T	T	K	N
JQ624655:	K	.	G	T	T	K	N

CON_HA2009: consensus haemagglutinin amino acid sequence of influenza A(H1N1)pdm09 viruses in 2009; CON_NA2009: consensus neuraminidase amino acid sequence of influenza A(H1N1)pdm09 viruses in 2009.

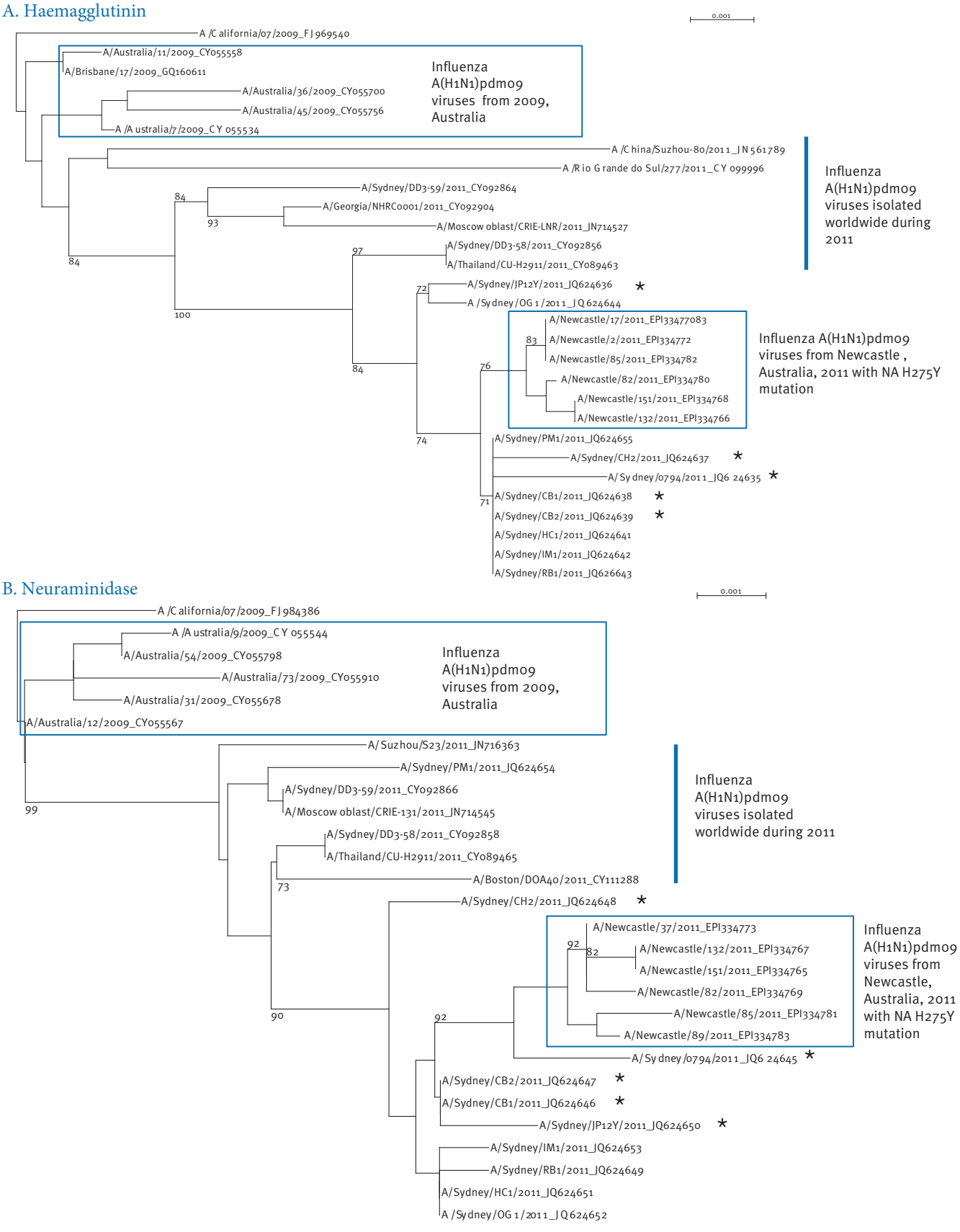
In each of the alignments in panel A and B, a dot indicates an amino acid identical to the respective 2009 consensus sequence. Except for the consensus sequences, the Genbank database accession number figures for each sequence.

[a] The amino acid numbering starts at the first methionine.

[b] The antigenic site amino acid numbering starts after the signal peptide.

Figure 2

Phylogenetic analysis of (A) haemagglutinin and (B) neuraminidase genes nucleotide sequences from influenza A(H1N1) pdm09 viruses isolated in Sydney, Australia, 2011



GISAID: Global Initiative on Sharing All Influenza Data; NA: Neuraminidase.

Nucleotide sequences from influenza A(H1N1)pdm09 viruses isolated in Sydney, Australia, 2011 were compared against influenza A(H1N1)pdm09 viruses sequences from Australia and other parts of the world. GenBank or GISAID accession numbers are shown. Bootstrap values figure on the branches adjacent to the tree nodes. The 2011 influenza A(H1N1)pdm09 virus sequences from GenBank are highlighted by vertical lines. Sequences derived from viruses isolated in Sydney bearing the NA H275N mutation are indicated by an asterisk.

The authors gratefully acknowledge the originating and submitting laboratories who contributed sequences used in the phylogenetic analysis to GISAID.

pdm09 virus. When compared with influenza A(H1N1) pdm09 sequences derived in 2009 during the first pandemic wave in Australia, the 2011 sequences showed five additional mutations (N44S, V62I, V241I, N369K and N386S) in NA and six additional amino acid substitutions (E130K, S160G, S202T, A214T, E391K and S468N) in HA (Figure 1). These were commonly present in most of the 2011 sequences and independent of their resistance profile.

Phylogenetic analyses based on the full-length NA and HA genes confirmed our observation that most of the circulating influenza A(H1N1)pdm09 viruses during the 2011 season in Sydney were distinct from those collected during the 2009 season (Figure 2). The circulating influenza A(H1N1)pdm09 viruses during the 2011 season in Sydney were also clearly associated with influenza A(H1N1)pdm09 viruses collected from Newcastle, Australia at the same period when the outbreak of community transmitted oseltamivir-resistant influenza A(H1N1)pdm09 virus had occurred. By including six full-length HA and NA sequences characterised by NA H275Y mutation from Newcastle [11] (Figure 2), a close phylogenetic relationship of viruses was observed between Sydney and Newcastle. The presence of different influenza A(H1N1)pdm09 viruses in 2011 was also supported by phylogenetic analysis that included influenza A(H1N1)pdm09 viruses isolated worldwide during 2011: these sequences (GenBank accession numbers: JN561789, CY099996, CY092864, CY092940, JN714527, CY092856, CY089463 for HA, and JN716363, CY092866, JN714545, CY092858, CY089465, CY111288 for NA) formed a single cluster with our sequences (Figure 2). Also, a close phylogenetic relationship was observed in both NA and HA of the NA H275Y-bearing oseltamivir-resistant influenza A(H1N1)pdm09 viruses with wild-type variants collected at same time (Figure 2). The presence of NA H275Y-bearing oseltamivir-resistant influenza A(H1N1)pdm09 viruses at various locations in the phylogenetic tree further confirms that the NA H275Y viruses emerged several times in Sydney rather than as a clonal expansion of a single resistant mutant (Figure 2).

Discussion

Influenza A(H1N1)pdm09 strains remained the predominant influenza virus circulating in the southern hemisphere in 2011 [1]. Although oseltamivir resistance amongst influenza A(H1N1)pdm09 viruses worldwide has been low, the recent occurrence in Newcastle, Australia, of the first significant community outbreak of NA H275Y-bearing oseltamivir resistant influenza A(H1N1)pdm09 virus has raised concerns about transmission elsewhere [11]. To determine the frequency and the extent of the spread of these oseltamivir-resistant influenza A(H1N1)pdm09 viruses in Sydney, Australia, the adjacent major city and transport hub, respiratory tract samples collected contemporaneously from influenza NAT positive individuals were examined for the presence of the NA H275Y mutation. Of 166 samples collected from June to August 2011, 1.4% of samples collected from untreated patients and 13% of samples collected after five days of oseltamivir treatment contained the NA H275Y mutation. These rates approximate previous studies of oseltamivir resistance in influenza A(H1N1)pdm09 viruses [3,5,9], although more viral strains would need to be analysed before this conclusion could be confirmed. It is worth noting that the frequency of oseltamivir-resistance is significantly higher ($P<0.001$) for treated rather than untreated patients, confirming that resistance usually emerges in response to antiviral drug selection pressure. As only 1.4% of untreated patients carried oseltamivir resistance, there is no convincing evidence of significant community transmission of NA H275Y-bearing influenza A(H1N1)pdm09 virus within Sydney at the same time or following the Newcastle outbreak. Geography and the high degree of population travel between these two cities highlights that rapid responses and testing of large numbers of viruses is important following the first identification of clusters of resistance to determine if community transmission is occurring.

Genetic characterisation of influenza A(H1N1)pdm09 viral strains derived from Sydney suggested close relatedness of viruses isolated in 2011, regardless of their resistance profile. This relationship, evidenced by sharing of signature changes different to 2009 variants, provides evidence of continued viral evolution as well as suggesting recent emergence and limited spread of oseltamivir-resistant variants. This evolutionary process of influenza A(H1N1)pdm09 virus after its introduction to human population and its impact on the effectiveness of current vaccine remains to be clarified. The presence of additional amino acid substitutions in two of the NA H275Y-bearing strains' NA genes also raise the possibility that these changes may be needed for oseltamivir-resistant influenza A(H1N1) pdm09 virus to sustain its replication and transmissibility. Whether the changes are as important as the NA R222Q and NA V234M substitutions in the pre-2009 oseltamivir-resistant seasonal influenza A(H1N1) viruses that are required for sustained transmissibility remains to be investigated [15]. The close association between NA H275Y-bearing influenza A(H1N1)pdm09 viruses from Sydney and Newcastle support the possibility of further spread of such variants although simultaneous local emergence of such variants cannot be fully excluded. In the current situation, prudent use of the neuraminidase inhibitors remains necessary, as does continued monitoring for drug-resistant influenza viruses.

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Contribution of the Portuguese Laboratory Network for the Diagnosis of Influenza A(H1N1)pdm09 Infection during the 2009/10 and 2010/11 influenza seasons

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1. The members of the network are listed at the end of the article

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This article describes the data obtained by the Portuguese Laboratory Network, reactivated following the World Health Organization declaration of the 2009 influenza pandemic, on the diagnoses of influenza A(H1N1)pdm09 infection during the pandemic (2009/10) and post-pandemic (2010/11) influenza seasons. The laboratories analysed and reported cases of influenza-like illness (ILI) and severe acute respiratory infection (SARI) to the National Influenza Reference Laboratory, which performed more detailed antigenic and genetic characterisation of the virus isolates. In 2009/10, a total of 62,089 ILI cases, distributed in two peaks, were analysed, 25,985 of which were positive for influenza A(H1N1)pdm09. Children aged 5–14 years were the most affected. Viruses were both antigenically and genetically similar to the pandemic strain A/California/7/2009, included in the 2009/10 pandemic vaccine. During the post-pandemic season, 1,496 ILI cases were tested for influenza A(H1N1) pdm09, 572 of which were positive. Infection was mainly diagnosed in adolescent and adults. Although the 2010/11 viruses remained antigenically similar to A/California/7/2009, increased genetic variation was observed. During the two seasons, two viruses with the neuraminidase H275Y amino acid substitution, associated with oseltamivir resistance, were detected. The Laboratory Network made an important contribution to the description of the influenza activity in the two seasons.

Introduction

Although epidemics of influenza occur almost every year in temperate climates, the rates and severity of illness can vary substantially between seasons [1]. Every winter season influenza infection is associated with a considerable number of fatal cases especially in elderly people with chronic diseases [2]. Recent studies from different countries, including Portugal [3,4], estimate that 90% of excess deaths due to seasonal influenza occur in the elderly (≥65 years) who are at higher risk due to comorbidities. In Portugal, the seasons with higher excess mortality were associated with the predominant circulation of influenza A(H3) viruses, while predominant circulation of type B viruses had a lower impact in mortality [4].

In March 2009 a new influenza virus circulating in humans emerged in Mexico. The new influenza A(H1N1) pdm09, as it was later called, started disseminating throughout the world, giving rise to the first influenza pandemic of the 21st century, and appears to have been introduced in Europe by travellers returning from Mexico and by their contacts. Following the detection of the initial imported cases, there was a spring and summer wave of transmission which affected several European countries. Transmission accelerated again after the re-opening of schools, this time affecting all countries, and lead to an early autumn/winter wave [5].

Since 1990 and until the 2009/10 winter season, influenza surveillance in Portugal had been carried out in the context of a National Influenza Surveillance Programme, coordinated by the National Influenza Reference Laboratory in cooperation with the Department of Epidemiology of the National Institute of Health and the General-Directorate of Health [6]. Activities within the Programme included collecting, analysing and reporting clinical, epidemiological and virological data on influenza-like illness (ILI), integrated from a sentinel network of general practitioners and a non-sentinel network of emergency units located at hospitals and health centres from the National Health System. As it was well established, the Programme proved essential for monitoring the pandemic in Portugal [7], particularly in the pre-pandemic phases when all suspected cases were referred to reference hospitals and the laboratory analysis was carried out centrally at the National Influenza Reference Laboratory, as defined in the National Contingency Plan for an Influenza Pandemic [8].

After the beginning of the pandemic, and to cope with the increasing number of diagnoses requested by the National Health System, the Portuguese Laboratory Network for the Diagnosis of Influenza was activated [9]. This Network had originally been established in 2006 with eight laboratories involved [10], after the emergence of human cases of influenza A(H5) virus infection, with the purpose of supporting, at the national level, the laboratory diagnosis of infection by a new influenza pandemic virus, but was subsequentially de-activated in the absence of zoonotic transmission of new emergent influenza viruses. In May 2009, professionals from these laboratories began training in molecular methodologies for detection of the new influenza A(H1N1)pdm09 virus. In the following two months, additional laboratories were involved and by the end of July 2009, all 13 members of the Network were able to perform those methods. Since then, the information generated, particularly that concerning the detection and molecular characterisation of influenza viruses associated with severe disease, has been integrated into the National Influenza Surveillance Programme and improved the understanding of the influenza activity in Portugal.

The National Influenza Reference Laboratory, which coordinates the Portuguese Laboratory Network for the Diagnosis of Influenza, also analysed and confirmed suspected cases of antiviral resistance. The majority of globally circulating influenza A(H1N1)pdm09 viruses during the two seasons were still sensitive to oseltamivir and zanamivir; oseltamivir-resistant influenza A(H1N1)pdm09 strains were found only sporadically and were associated with the substitution H275Y in neuraminidase gene [11].

This study describes the influenza activity during the 2009/10 and 2010/11 influenza seasons, based on the information collected through the Portuguese Laboratory Network for the Diagnosis of Influenza.

Methods

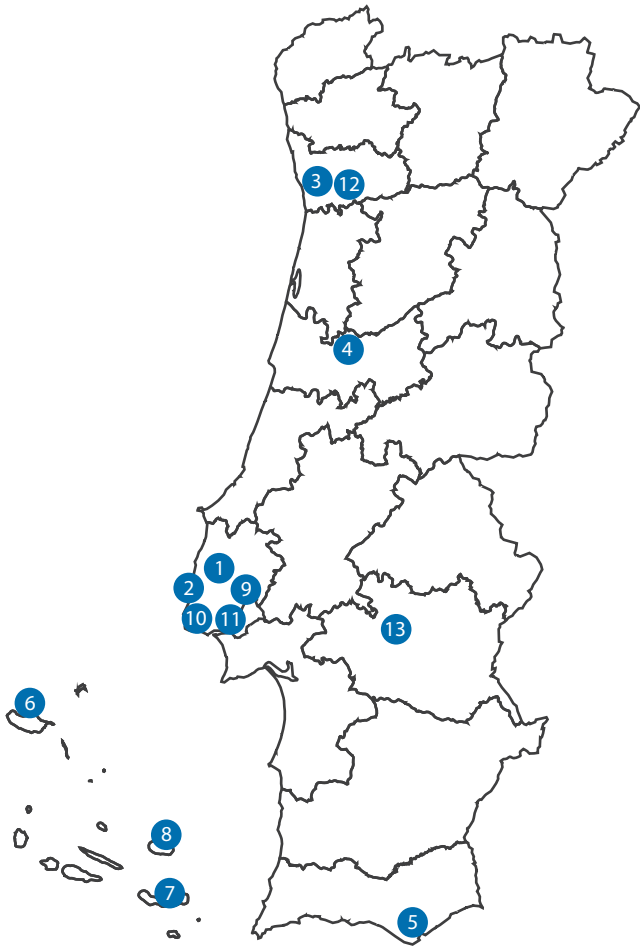
Surveillance period

The influenza period 2009/10 was epidemiologically different from a normal influenza winter season. For this reason, it was decided to consider as the 2009/10 pandemic season the period from week 17, 2009 to week 20, 2010. This particular period was chosen because the first influenza-positive case attributed to influenza A(H1N1)pdm09 infection was observed in week 17, 2009. During the 2010/11 season the laboratory Network was active during the usual surveillance period between October and May (weeks 40, 2010 through 20, 2011).

All 13 laboratories reported cases during the pandemic 2009/10 season. During the 2010/11 winter, only nine participated in the reporting of cases with respiratory disease.

Figure 1

Laboratories of the Portuguese Laboratory Network for the Diagnosis of Influenza Infection



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The Portuguese Laboratory Network for the Diagnosis of Influenza Infection

The Portuguese Laboratory Network for the Diagnosis of Influenza Infection is currently composed of 13 laboratories from reference hospitals on mainland Portugal and the islands (Figure 1).

The main objective of this Network during the 2009/10 and 2010/11 influenza seasons was to carry out the laboratory diagnosis of influenza A(H1N1)pdm09 infection requested by the National Health Service. Currently, diagnostic capabilities include detection not only of influenza A(H1N1)pdm09 virus but also influenza B virus (six laboratories), influenza A(H1) and

A(H3) seasonal viruses (four laboratories), and other infectious agents associated with respiratory disease: adenovirus, coronavirus, rhinovirus, respiratory syncytial virus, metapneumovirus, parainfluenza virus, *Haemophilus influenzae* and *Streptococcus pneumoniae* (three laboratories).

Cases and variables studied

During the two seasons, ILI cases from hospital emergency units as well as from other primary healthcare units, and hospitalised cases of severe acute respiratory infection (SARI), were reported through a common Internet-based database or by an Excel spreadsheet sent to the National Influenza Reference Laboratory. Published case definitions were applied [12]. The notification form included clinical and epidemiological information, such as demographic data, signs and symptoms, underlying conditions and information on vaccination and antiviral therapy.

Laboratory analysis

For laboratory diagnosis and further virological characterisation, nasopharyngeal swabs, or combined nasopharyngeal/oropharyngeal swabs, were collected into a suitable transport medium. Different real-time RT-PCR methodologies and platforms were used within the Network for the laboratory diagnosis of influenza infection.

Upon request from the Network, the National Influenza Reference Laboratory performed additional diagnoses (typing, subtyping and lineage determination by real-time PCR of other influenza viruses), viral isolation [13] and antigenic and genetic characterisation of viral isolates, according to the procedures adopted from the manual for the laboratory diagnosis and virological surveillance of influenza published by the World Health Organisation (WHO) [1] and adapted from the United States Centers for Disease Prevention and Control (CDC) [14].

Influenza virus isolates were characterised antigenically by haemagglutination inhibition tests (HAI) using antisera and reference virus strains distributed by the WHO Collaborating Centre in Atlanta. Selected isolates were sent to the WHO Collaborating Centre in London for further study. Genetic analysis of isolates was carried out by sequencing the haemagglutinin (HA) gene encoding the HA1 subunit, using primer sequences available from CDC [14], for a subset of approximately 25% of isolates encompassing the beginning, middle and end of each season. Sequences are available from the GenBank database. Phylogenetic analysis was performed with MEGA Software [15]. HA1 sequences from reference strains used in the genetic analysis were obtained from the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID) (Table 1).
The detection of antiviral resistance was performed by phenotypical assays, such as the neuraminidase inhibition assay, and by neuraminidase gene sequencing [1,14].

Statistical analysis

The probability of occurrence of an influenza case in the presence of the signs/symptoms considered for definition of ILI was estimated by calculating the individual crude odds ratios, and the respective 95% confidence intervals, of reporting each sign/symptom in both influenza cases (laboratory-confirmed) and ILI cases (influenza-negative result).

Results

2009/10 season

In Portugal, a total of 62,089 ILI cases were reported through the Laboratory Network during the 2009/10 pandemic season. Two peaks were observed, one occurring towards the end of the summer 2009 and another during November and December of the same year (Figure 2).

The first case of influenza A(H1N1)pdm09 infection in Portugal was detected during week 17, 2009, when seasonal influenza viruses, linked to the previous winter season, were still circulating. A total of 25,985 laboratory-confirmed influenza cases were found, over 99% of which were associated with the new pandemic strain A(H1N1)pdm09. Other influenza viruses were also found: 190 non-subtyped influenza A viruses, 15 seasonal influenza A(H1) viruses, 10 influenza A(H3) and 31 influenza B viruses. A first peak in influenza cases was observed during week 33 with 1,145 influenza A(H1N1)pdm09 cases (45.9% of all ILI cases notified in week 33). Most of the cases during the first wave were detected in the south of Portugal and in the main urban areas (Lisbon and Oporto). The first wave was followed by a second wave with cases all over the country (mainland and islands) and with a peak during week 46 with 3,266 influenza A(H1N1)pdm09 cases (60.2% of all ILI cases notified in week 46).

Although the majority of notifications occurred in the population with 15 to 44 years of age (n=21,267; 34.3% of all ILI cases), the highest proportion of influenza-positive results was observed in children aged between five and 14 years (n=10,093; 38.8% of all influenza A(H1N1)pdm09-positive cases) (Table 2). The lowest number of reported cases (n=1,095; 1.8%) and influenza-positive results (n=515; 2.0%) were reported in the elderly. The highest proportion of influenza A(H1N1)pdm09-positive results within any one age group (64.0%) was observed in children aged 5–14 years.

Eighty influenza strains were isolated and antigenically characterised, 43 of which were also genetically analysed (HA gene). The 80 influenza A(H1N1) pdm09 strains isolated were antigenically homogeneous and showed high similarity with influenza A/California/7/2009 (strain included in the pandemic

TABLE 1

Origin of the haemagglutinin sequences of influenza A(H1N1)pdm09 isolates used for the phylogenetic analysis

Segmetn ID	Segment	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI215957	HA	Ukraine	2009-Oct-27	A/Lviv/N6/2009	Ministry of Health of Ukraine	National Institute for Medical Research	
EPI301779	HA	Sweden	2010-Dec-12	A/Stockholm/14/2010	Swedish Institute for Infectious Disease Control	Swedish Institute for Infectious Disease Control	
EPI319590	HA		2011-Feb-28	A/Astrakhan/1/2011	WHO National Influenza Centre	National Institute for Medical Research	
EPI319459	HA	Estonia	2011-Feb-11	A/Estonia/54375/11	Health Protection Inspectorate	National Institute for Medical Research	
EPI319524	HA	Italy	2011-Jan-11	A/Trieste/11/2011	Istituto Superiore di Sanità	National Institute for Medical Research	
EPI319434	HA	Belgium	2011-Jan-01	A/Brussels/S0004/2011	Scientific Institute of Public Health	National Institute for Medical Research	
EPI309671	HA	France	2010-Dec-30	A/Paris/2301/2010	Institut Pasteur	National Institute for Medical Research	
EPI319527	HA	Russia	2011-Feb-14	A/St. Petersburg/27/2011	WHO National Influenza Centre	National Institute for Medical Research	
EPI320141	HA	Russia	2011-Mar-14	A/St. Petersburg/100/2011	Russian Academy of Medical Sciences	Centers for Disease Control and Prevention	
EPI301900	HA	Germany	2010-Dec-27	A/Baden-Wurttemberg/14/2010	Robert Koch Institute	National Institute for Medical Research	
EPI309643	HA	United Kingdom	2010-Dec-31	A/England/375/2010	Centre for Infections, Health Protection Agency	National Institute for Medical Research	
EPI319607	HA	United Kingdom	2010-Dec-16	A/England/676/2010	Centre for Infections, Health Protection Agency	National Institute for Medical Research	
EPI309683	HA	Slovenia	2011-Jan-10	A/Slovenia/167/2011	Laboratory for Virology, National Institute of Public Health	National Institute for Medical Research	
EPI278607	HA	New Zealand	2010-Jul-12	A/Christchurch/16/2010	Canterbury Health Services	WHO Collaborating Centre for Reference and Research on Influenza	Deng Y-M, Iannello P, Caldwell N, Leang S-K, Komadina N
EPI279895	HA	Hong Kong	2010-Jul-16	A/Hong Kong/2212/2010	Government Virus Unit	National Institute for Medical Research	
EPI279897	HA	Hong Kong	2010-Jul-16	A/Hong Kong/2213/2010	Government Virus Unit	National Institute for Medical Research	
EPI301399	HA		2010-Dec-01	A/Alborz/5607/2010	Tehran University of Medical Sciences	National Institute for Medical Research	
EPI319503	HA	Moldova	2011-Feb-07	A/Moldova/448/2011	National Centre for Preventive Medicine	National Institute for Medical Research	
EPI331048	HA	France	2011-Feb-22	A/Toulon/1173/2011	CRR virus Influenza region Sud	National Institute for Medical Research	
EPI319447	HA	Czech Republic	2011-Jan-18	A/Czech Republic/32/2011	National Institute of Public Health	National Institute for Medical Research	
EPI319430	HA	Algeria	2010-Dec-21	A/Algeria/G388/2010	Institut Pasteur d'Algerie	National Institute for Medical Research	
EPI178507	HA	United Kingdom	2009-Apr-28	A/England/195/2009		WHO National Influenza Centre, National Institute of Medical Research (NIMR)	
EPI253705	HA	Germany	2009-Jan-01	A/Bayern/69/2009	Robert Koch Institute	Robert Koch-Institute	Biere B; Schweiger B
EPI179605	HA	New Zealand	2009-Apr-25	A/Auckland/3/2009	Auckland Hospital	WHO Collaborating Centre for Reference and Research on Influenza	
EPI247245	HA	United States	2010-Feb-11	A/California/04/2010	California Department of Health Services	Centers for Disease Control and Prevention	
EPI176620	HA	United States	2009-Apr-09	A/California/07/2009		Centers for Disease Control and Prevention	

Figure 2

Figure 2. Weekly distribution of influenza-like illness and influenza A(H1N1)pdm09-positive cases, Portugal, 2009/10 and 2010/2011 seasons (n=63,585)

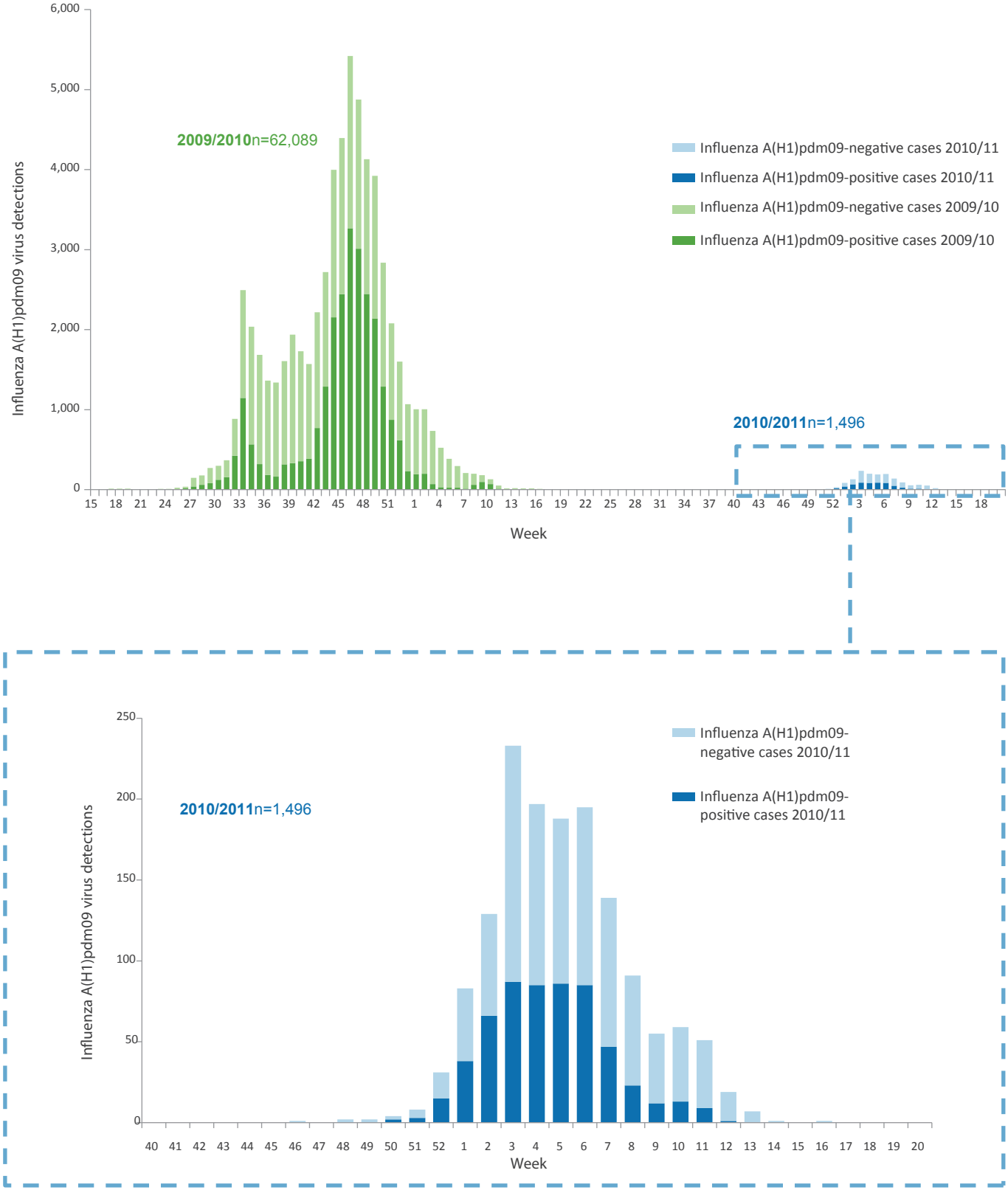


TABLE 2

Distribution of influenza A(H1N1)pdm09 cases, by age group, Portugal, 2009/10 and 2010/11 seasons (n=63,585)

Age group (years)	2009/10		2010/11	
	Number of cases	Influenza A(H1N1)pdm09 (% positive within the age group)	Number of cases[a]	Influenza A(H1N1)pdm09 (% positive within the age group)
0-4	15,189	4,042 (26.6%)	192	48 (25.0%)
5-14	15,765	10,093 (64.0%)	47	19 (40.4%)
15-44	21,267	9,216 (43.3%)	637	286 (44.9%)
45-64	6,132	1,831 (29.9%)	402	173 (43.0%)
≥65	2,641	288 (10.9%)	207	41 (19.8%)
Information unavailable	1,095	515 (47.0%)	11	5 (45.5%)
Total	**62,089**	**25,985 (41.9%)**	**1,496**	**572 (38.2%)**

[a] Although 1,512 cases were reported in 2010/11, only 1,496 were tested for influenza A(H1N1)pdm09. h.

vaccine of the season) and with the later pandemic influenza A(H1N1) viruses A/Bayern/69/2009 and A/Lviv/N6/2009 (data not shown).

All sequenced HA1 segments from 2009/10 isolates were similar to influenza A/California/7/2009 (Figure 3). Compared to the vaccine strain, with the exception of influenza A/Lisboa/31/2009 and A/Lisboa/35/2009, all isolates contained the P83S amino acid substitution and grouped into clade 7 (S203T substitution in the Ca antibody binding site of HA1), described in the literature early in the pandemic [16]. Additional mutations were found in the HA1 antigenic sites: G170R (A/Lisboa/65/2009), R205K (A/Lisboa/73/2009), D222E (observed in 18 isolated viruses) and S74G (A/Lisboa/171/2009). The sequences are available from GenBank with the following accession numbers: CY067801, CY067804, CY067807, CY067810, CY067819, CY067822, CY067825, CY067828, CY067831, CY067834, CY067837, CY067840, CY067843, CY067846, CY067849, CY067852, CY067855, CY067858, CY067861, CY067864, CY067867, CY067870, CY067873, CY067876, CY067879, CY067882, CY067885, CY067888, CY067891, CY067894, CY067897, CY067900, CY067903, CY067906, CY067909, CY067912, CY067915, CY067918, CY067765, CY067789, CY067792, CY067795, CY067798.

The amino acid substitution H275Y associated with oseltamivir resistance was found in the neuraminidase of one strain (A/Lisboa/171/2009), which was proved to be resistant by phenotypic assays.

2010/11 season

Nine laboratories reported a total of 1,512 ILI cases, from week 40, 2010 to week 16, 2011, with a peak of 236 cases (15.6%) observed in week 3, 2011. Influenza A(H1N1)pdm09 was tested for in 1,496 cases, 572 (38.2%) of which were positive. The peak of laboratory-confirmed A(H1N1)pdm09-associated cases was also registered in week 3 of 2011 (n=87; 37.3% of all ILI cases notified in week 3).

The majority of notifications (n=637; 42.6% of all ILI cases) and the higher proportion of influenza A(H1N1) pdm09 cases (n=286, 50.0% of all positive results) occurred in the population between 15 and 44 years of age (Table 2). The lowest number of reported cases (n=47; 3.1%) and pandemic influenza-positive results (n=19; 3.3%) were reported in children aged 5 to 14 years. The higher proportion of influenza A(H1N1) pdm09-positive results within an age group was observed in older children and adults (15–44 years: 44.9%; 45–64 years: 43.0%).

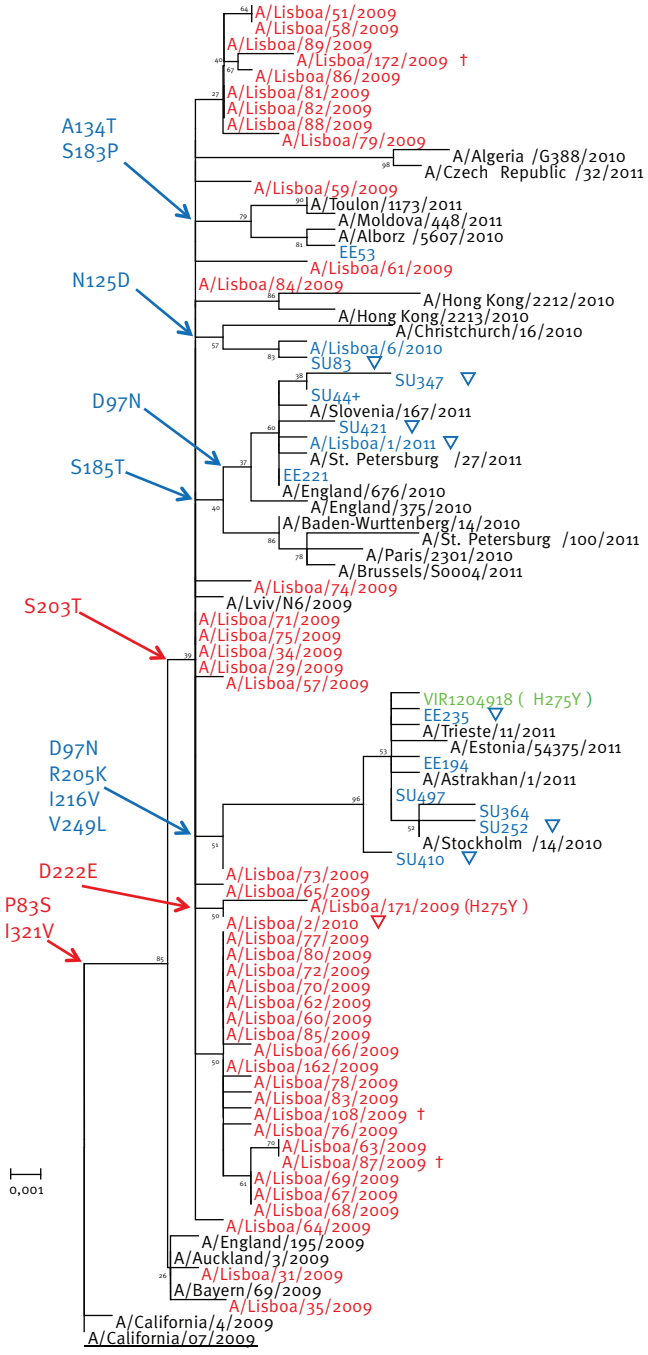
Following a single request from the Network in that season for additional characterisation at the National Influenza Reference Laboratory, the HA1 nucleotide sequence of one influenza A(H1N1)pdm09 detected in a clinical specimen was determined (Figure 3). This virus clustered in the genetic clade of A/Astrakhan/1/2011, one of four genetic clades circulating in Portugal during the 2010/11 season (data not shown), and contained the P83S, I321V and S203T amino acid substitutions common to all 14 viruses analysed in that season, and the substitutions D97N, R205K, I216V and V249L characteristic of the A/Astrakhan/1/2011 group. This virus also presented the H275Y mutation in the neuraminidase, associated with oseltamivir resistance.

From the clinical symptoms analysed, fever was the one that revealed a higher risk of being observed in an influenza case (crude OR: 3.3; 95% CI: 1.3–8.0) (Table 3).

Three laboratories also performed the differential diagnosis of respiratory infection in 284 cases, 12.7% of which were positive for other infectious agents (one adenovirus, three coronaviruses, one human metapneumovirus, 12 rhinoviruses, nine respiratory syncytial

Figure 3

Maximum likelihood phylogenetic tree of HA1 nucleotide sequences from influenza A(H1N1)pdm09 viruses, Portugal, 2009/10 and 2010/2011 seasons (n=44).



The 43 strains and amino acid substitutions from the 2009/10 pandemic season are represented in red. The virus detected in a clinical specimen from the 2010/11 season is represented in green. The other 14 viruses detected in clinical specimens, which were circulating in Portugal during the 2010/11 season (data from the National Influenza Surveillance Programme), and amino acid substitutions found are represented in blue. Reference strains (obtained from GISAID`s EpiFlu database) are in black and the vaccine strain is underlined; † indicates cases with fatal outcome. ▽ indicates cases vaccinated at least 14 days before onset of symptoms. Bootstrap values above 50 are shown (500 replicates).

virus, one *H. influenzae*, one *S. pneumoniae* and seven mixed infections).

Discussion

Facing the circulation of a new influenza A(H1N1)pdm09 virus and the potential threat to the population and the healthcare system, and the resulting increased awareness and diagnostic requirements, the total number of ILI cases reported and analysed in our country during the 2009/10 winter was much higher than in previous influenza seasons. Cases with laboratory-confirmed diagnosis for influenza A(H1N1)pdm09 increased mainly due to wide sampling criteria and to rapid diagnostic tests.

Given the considerable number of diagnoses performed in different settings, both in primary care and hospitals, the Laboratory Network has made an important contribution to improve the surveillance of influenza activity in Portugal. Regarding the pattern of disease, the data collected through the Laboratory Network support the data obtained from the National Influenza Surveillance Programme [7] and suggest that the 2009/10 pandemic was similar to the previous influenza season in intensity and geographical distribution. Seasonal viruses were replaced by the new influenza A(H1N1)pdm09 strain, which caused disease particularly in young children, consistent with the data from all European countries where children under the age of 14 years were affected the most [5].

Using the data generated from the Laboratory Network it was possible to better characterise the beginning of the pandemic season, as the early cases were mainly reported to the reference hospitals, dedicated to the isolation of patients in an attempt to contain the virus and contribute to the management of the infection, as defined in the National Contingency Plan for an Influenza Pandemic [8]. During the mitigation phase of the pandemic, when the new pandemic strain had disseminated throughout the world, both the Laboratory Network and the National Influenza Surveillance Programme contributed to the clinical, epidemiological and virological characterisation of the infection.

The pandemic season was characterised by two waves, one in the summer and another during the winter. A very similar pattern was observed in Spain and other European countries [5,17]. In Portugal the pandemic influenza A(H1N1)pdm09 virus was first detected on 4 May, soon after the first case confirmed in Spain (27 April 2009) and in Europe (13 April 2009). Data from Spain and combined data for Europe show the first peak of influenza cases in 2009 during weeks 31 and 30, and the second peak during weeks 47 and 46, respectively [5,17]. The 2009 pandemic was considered a mild one for Europe, with little impact on services outside the health sector [5].

In the 2010/11 season, the first post-pandemic season, the number of laboratory diagnosis requests

TABLE 3

Correlation between presence of symptoms/signs considered for clinical definition of influenza-like illness and influenza cases, Portugal, 2009/10 and 2010/11 seasons (n=63,585)

Sign/symptom	2009/10			2010/11		
	Positive cases (%)	OR (crude)	CI (95%)	Positive cases (%)	OR (crude)	CI (95%)
Fever (≥38°C)	44.1	**1.3**	**1.3–1.4**	43.3	**3.3**	**1.3–8.0**
Cough	47.4	**2.4**	**2.3–2.5**	39.6	1.0	0.4–2.5
Sore throat	42.1	1.0	0.4–2.4	44.7	1.4	0.7–3.0
Respiratory difficulty	29.4	0.5	0.4–0.5	50.0	1.0	0.3–2.8
Contact with another influenza patient	63.6	**3.6**	**3.1–4.1**	NA	NA	NA
Myalgia	47.2	**1.4**	**1.4–1.5**	NA	NA	NA
Headache	50.4	**1.8**	**1.8–1.9**	NA	NA	NA
Sudden onset	NA	NA	NA	40.0	1.1	0.6–2.3

CI: confidence interval; NA: not applicable; OR: odds ratio.

The symptoms/signs that revealed an elevated risk of being observed in an influenza case are represented in bold. Number of ILI cases reporting information differed for each sign/symptom; only valid records (reporting presence or absence of signs/symptoms and with a valid laboratory result) were considered in the calculation of individual crude odds ratios. The presence of a sign/symptom is compared between an ILI case and an influenza case.

dramatically decreased due to tighter criteria for sampling, although the actual ILI incidence rates were in the same order of magnitude as in the two previous seasons. The highest incidence rates for the seasons 2008/09, 2009/10 and 2010/11 were 199.5, 133.7 and 121.1 cases per 100,000 inhabitants, respectively [7]. Hence, the 41-fold difference in reporting cases between the pandemic and post-pandemic season, as seen in the data from the Laboratory Network, is due to the dramatic reduction in requests for diagnostics. The Network being a non-sentinel component of the National Influenza Surveillance Programme and inactive before the start of the 2009 pandemic, 2008/09 data for comparison are not available.

According to the National Influenza Surveillance Programme, the influenza A (H1N1)pdm09 continued to circulate in the 2010/11 season as the predominant virus, but co-circulating with influenza B viruses [18,19]. In Portugal, influenza B viruses were detected earlier in that season, in contrast to what was observed in Europe [18]. The circulation of influenza A(H1N1)pdm09 virus occurred during the expected period without off-season peaks, but the peak of the 2010/11 season was registered by the Laboratory Network three weeks later than the peak observed by the sentinel surveillance system within the National Influenza Surveillance Programme (week 52, 2010, data not shown), which was in agreement with what was observed in Spain and the rest of Europe at that time [20].

The age group of 5–14 year-old children was most affected by A(H1N1)pdm09-associated influenza in 2009/10, as was also observed in Europe [21,22]. However, in the 2010/11 season, the highest percentage of A(H1N1)pdm09-influenza positive cases was found in a broader age range, from 15 to 64 years. This was in agreement with what was observed in Austria and Norway, but in contrast to other European countries where the most affected age group were children under 15 years of age [19].

In the 2009/10 pandemic season, cough and contact with another influenza patients were strongly associated with confirmed A(H1N1)pdm09 influenza, whereas in 2010/11, fever was the symptom that showed a higher association with a positive case. These results were consistent with other findings for seasonal [23] and pandemic influenza [24], where fever and cough were in strong association with influenza disease. This information can be useful for an earlier and more accurate diagnosis of influenza in order to maximise the effectiveness of antiviral therapy and to avoid unnecessary antibiotic use [23].

During the pandemic season, a genetically similarity of the strains in circulation with the A/California/7/2009 vaccine strain was observed. Since the 2010/11 season, increased genetic variation has occurred among the pandemic A(H1N1)pdm09 viruses circulating in Portugal and in Europe [25]. The Portuguese viruses clustered into four different genetic clades from the eight genetic groups so far identified [26,27]. The majority of them clustered in genetic group 5 represented by the A/Astrakhan/1/2011 strain.

Despite this genetic variation among the circulating A(H1N1)pdm09, very little antigenic drift was observed over the two seasons, and isolates were closely related to the strain A/California/7/2009 included in the pandemic vaccine of the 2009/10 season and of the seasonal influenza vaccine for the 2010/11 season.

During the two winter seasons, the Laboratory Network found two viruses with the H275Y amino acid substitution in neuraminidase that has been associated with

oseltamivir resistance. Globally, a limited number of sporadic oseltamivir-resistant cases of A(H1N1)pdm09 have been reported [11,28], but the majority of circulating influenza A(H1N1)pdm09 viruses were sensitive to neuraminidase inhibitors [11]. The two Portuguese H275Y viruses were detected in immunocompromised patients under oseltamivir treatment: one was confirmed resistant to oseltamivir by phenotypic assays (2009/10 season); the other came from a case of severe influenza with fatal outcome (2010/11 season), but confirmation by phenotypic assays could not be done since the virus was not isolated. The monitoring of antiviral resistance was, and continues to be, essential since antiviral treatment is an important tool in the clinical management of severe or complicated influenza cases [7, 11].

During 2010/11 the Network also increased the diagnostic capability by implementing methodologies for the detection of other respiratory pathogens, having found several other agents associated with ILI co-circulating with influenza. This information can be a major contribution to a better understanding of the epidemiology of the disease.

Since September 2011 the Portuguese national health authorities have been developing and implementing an integrated surveillance system for SARI in five hospitals members of the Portuguese Laboratory Network. In the near future, this surveillance system will hopefully be extended to other hospitals and we expect that the established Laboratory Network will play a major role. Continuous laboratory participation and further advances in diagnostic capacity are essential to improve the surveillance of respiratory infections in Portugal.

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