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Increased detection in Australia and Singapore of a novel influenza A(H1N1)2009 variant with reduced oseltamivir and zanamivir sensitivity due to a S247N neuraminidase mutation

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A novel influenza A(H1N1)2009 variant with mildly reduced oseltamivir and zanamivir sensitivity has been detected in more than 10% of community specimens in Singapore and more than 30% of samples from northern Australia during the early months of 2011. The variant, which has also been detected in other regions of the Asia-Pacific, contains a S247N neuraminidase mutation. When combined with the H275Y mutation, as detected in an oseltamivir-treated patient, the dual S247N+H275Y mutant had extremely high oseltamivir resistance.

Introduction

The emergence and global spread in 2007/08 of an oseltamivir-resistant seasonal influenza A(H1N1) variant containing a histidine to tyrosine substitution (H275Y) in the neuraminidase (NA), demonstrated the potential for drug-resistant influenza viruses to arise and spread within the community in the absence of drug-selective pressure [1]. Since the start of the 2009 pandemic, the oseltamivir-resistant H275Y variant has only been detected on rare occasions in pandemic influenza A(H1N1)2009 community specimens (‹1%), although recent reports have suggested that this frequency may be increasing [2]. Other NA mutations in influenza A(H1N1)2009 viruses have been reported to confer mildly reduced oseltamivir and/or zanamivir sensitivity, such as substitutions at the isoleucine residue at position 223 (N1 numbering), but the detection of these mutants has been very rare and has occurred mostly in isolated cases of immunocompromised individuals under long-term NA inhibitor (NAI) treatment [3,4]. Here we report the identification and increased rate of detection in community samples of a novel influenza A(H1N1)2009 variant with reduced oseltamivir and zanamivir sensitivity. The variant contained a serine to asparagine mutation at residue 247 (S247N) of the NA, and has been detected in recent community specimens from Australia, Brunei and Singapore. Although the mutation has been described before in a small number of seasonal influenza A(H1N1) and highly pathogenic influenza A(H5N1) viruses with reduced NAI sensitivity [5,6], it has not previously been reported in influenza A(H1N1)2009 viruses, and has not occurred in any strains at the frequencies that are described here.

Materials and methods

Specimens and isolates from the Asia-Pacific region were submitted to the World Health Organization (WHO) Collaborating Centre for Reference and Research on Influenza, Melbourne, as part of the WHO Global Influenza Surveillance Network. Isolates cultured in Madin-Darby canine kidney (MDCK) cells were analysed for oseltamivir, zanamivir and peramivir sensitivity using a fluorescence-based neuraminidase inhibition assay [7]. Oseltamivir carboxylate, the active form of the ethyl ester prodrug oseltamivir phosphate, was kindly provided by Hoffmann-La Roche Ltd, Switzerland; zanamivir was kindly provided by GlaxoSmithKline, Australia; peramivir was kindly provided by BioCryst, United States. Isolates and specimens were sequenced using standard techniques at the WHO Collaborating Centre, Melbourne, except for Singaporean strains which were sequenced at the National Public Health Laboratory, Singapore. Haemagglutinin (HA) and NA sequences reported here have been deposited on GISAID (www.gisaid.org; accession numbers EPI319165 to EPI319183) or GenBank (accession numbers CY091664 to CY091724 and CY063853 to CY063854). Other HA and NA sequences were analysed from GISAID or Genbank sequence databases (see supplementary* for details: http://mendel.bii.a-star.edu.sg/SEQUENCES/H1N1/S247N/Hurt_et_al_NA-S247N_suppl.

pdf). A concatenated HA and NA nucleotide alignment was created with MAFFT (FFT-NS-2) (http://mafft.cbrc.jp/alignment/software/) and a maximum likelihood phylogenetic tree was generated with PhyML (http://www.atgc-montpellier.fr/phyml/) using the HKY85 substitution model, 4 gamma-distributed categories (shape parameter 0.46) and the approximate likelihood ratio test. The trees were visualised and annotated in MEGA5 (http://www.megasoftware.net/).

The S247N and dual S247N+H275Y NA mutations were investigated further by site-directed mutagenesis and reverse genetics using the eight-plasmid system. Mutations were engineered into the NA of influenza A/Auckland/1/2009(H1N1) and transfected together with plasmids containing the seven remaining segments from the influenza A/Puerto Rico/8/34 strain, as described previously [8].

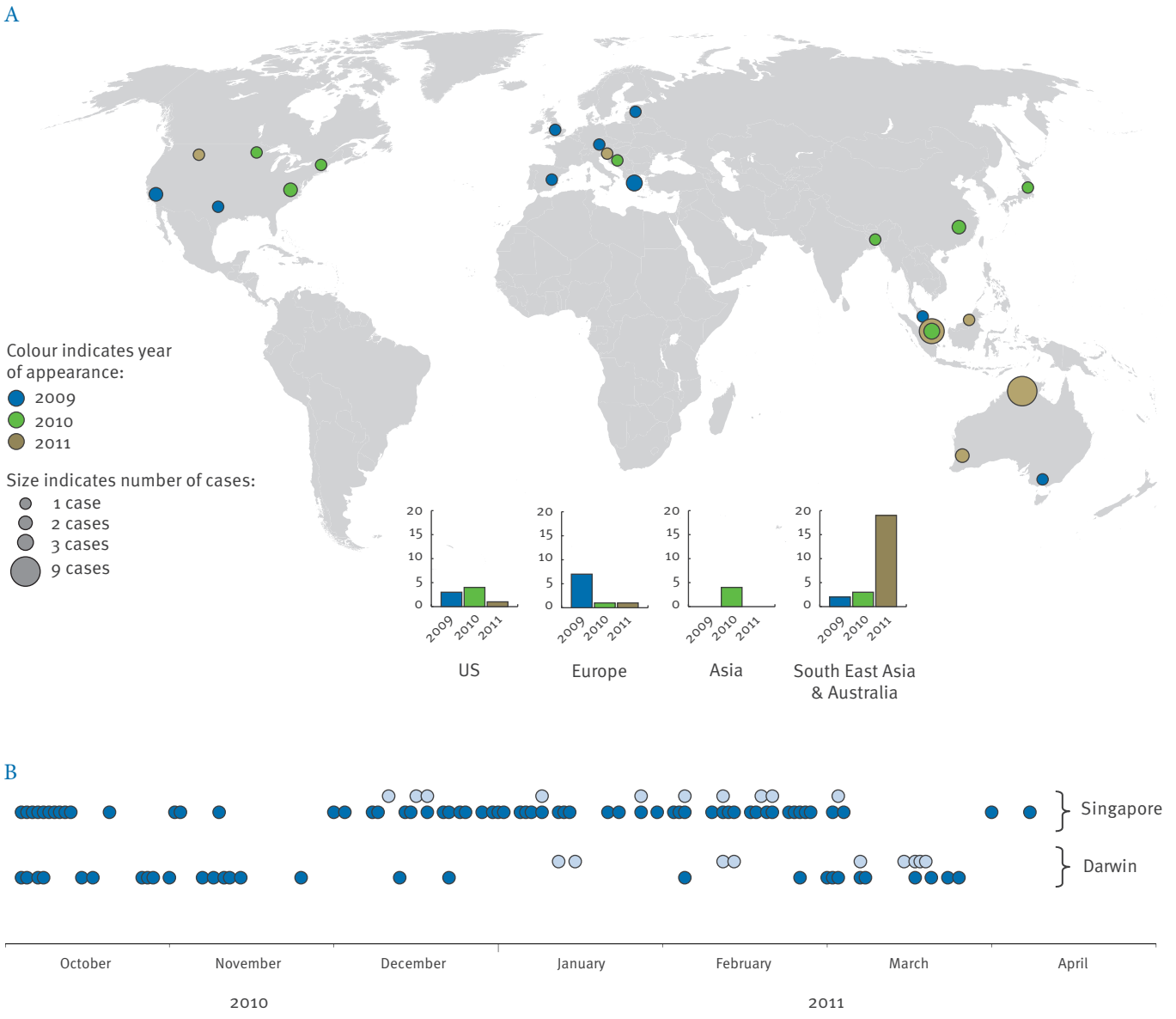
The mutations S247N and H275Y were modelled into the influenza A(H1N1)2009 neuraminidase crystal structure and the effects of the mutations on oseltamivir binding were visualised and examined using FoldX (http://foldx.crg.es) and Yasara (http://www.yasara.org).

Results

From April 2009 to December 2010, over 2,900 influenza A(H1N1)2009 influenza viruses from the Asia-Pacific region were analysed for NAI sensitivity with only 23 H275Y oseltamivir-resistant viruses detected (0.8%), and virtually all of the remaining strains being fully sensitive to both oseltamivir and zanamivir. However

Figure 1

Detection of influenza A(H1N1)2009 S247N variants since 2009**



A. Year and frequency of detection of S247N mutants globally since 2009 (n=49) . Dots are overlaid on top of each other for Singapore.

B. Detection of S247N mutants in Darwin and Singapore between October 2010 and April 2011 (n=19). S247N mutants are indicated by light blue circles and wildtype S247 viruses are indicated by dark blue circles.

since December 2010, 22 influenza A(H1N1)2009 viruses containing a novel S247N NA mutation (S246N based on N2 numbering), have been detected in both clinical specimens and isolates from the Asia-Pacific region. The majority of the S247N variants detected since December 2010 (n=19) have occurred in two clusters, one in the Darwin region in northern Australia and the other in Singapore (Figure 1A). The variant has also been detected in Western Australia and Brunei (in this study), and based on data from the public sequence databases has occurred, albeit rarely, in other locations such as the United States, Europe and Asia since 2009 (Figure 1A). During the first three months of 2011, 28 influenza A(H1N1)2009 strains were sampled from the Darwin region, of which nine contained the S247N mutation, while in Singapore 10 out of 80 viruses sampled since December 2010 had the S247N NA mutation (Figure 1B).

Of the 22 S247N variants detected, nine were cultured and in an NA inhibition assay showed a mean six-fold reduction in oseltamivir sensitivity, a three-fold reduction in zanamivir sensitivity, and no significant

Table

Neuraminidase inhibitor sensitivity of naturally occurring and recombinant S247N and S247N+H275Y influenza A(H1N1)2009 variants, December 2010–March 2011

			Zanamivir		Oseltamivir carboxylate		Peramivir	
Virus	Mutation	Specimen date	Mean IC_{50} ± SD (nM)	x-fold difference vs wildtype	Mean IC_{50} ± SD (nM)	x-fold difference vs wildtype	Mean IC_{50} ± SD (nM)	x-fold difference vs wildtype
Mean of sensitive influenza A(H1N1) 2009 viruses (n=3,169)	-	-	0.28±0.15	-	0.45±0.35	-	0.20±0.10[a]	-
Mean of S247N influenza A(H1N1) 2009 viruses[b] (n=9)	S247N	-	0.85±0.10	3	2.68±0.61	6	0.21±0.02	1
Naturally occurring strains								
A/Singapore/GP4565/2010	S247N	10 Dec 2010	0.96±0.06	3	3.21±0.50	7	0.21±0.02	1
A/Singapore/GP4588/2010	S247N	15 Dec 2010	0.81±0.08	3	3.02±0.54	7	0.21±0.02	1
A/Darwin/2/2001	S247N	13 Jan 2011	0.88±0.10	3	3.20±0.47	7	0.23±0.01	1
A/Brunei/1/2011	S247N	13 Jan 2011	0.86±0.08	3	1.62±0.11	4	0.26±0.03	1
A/Darwin/10/2011	S247N	9 Feb 2011	0.79±0.08	3	2.41±0.47	5	0.19±0.01	1
A/Perth/30/2011[c]	S247N	1 Mar 2011	0.80±0.01	3	2.34±0.21	5	0.22±0.01	1
A/Perth/29/2011[c]	S247N+ H275Y	8 Mar 2011	1.30±0.08	5	2,646.81±293.55	5,880	66.88±4.19	334
A/Darwin/70/2011	S247N	14 Mar 2011	1.04±0.10	4	3.49±0.45	8	0.21±0.01	1
A/Darwin/74/2011	S247N	16 Mar 2011	0.68±0.03	2	2.15±0.23	5	0.19±0.01	1
A/Darwin/75/2011	S247N	17 Mar 2011	0.82±0.04	3	2.69±0.11	6	0.21±0.02	1
Recombinant strains[d]								
RG-wildtype	-	-	0.24±0.05	-	0.30±0.20	-	0.09±0.01	-
RG-S247N	S247N	-	0.57±0.10	2	0.96±0.48	3	0.18±0.02	2
RG-S247N+ H275Y	S247N+ H275Y	-	1.25±0.17	5	2,149.96±309.02	7,073	60.91±5.88	704
RG-H275Y	H275Y	-	0.26±0.03	1	195.02±21.05	642	19.72±1.42	228

RG: reverse genetics; SD: standard deviation.

[a] Mean and standard deviation of peramivir IC_{50} values based on analysis of n=273 isolates.

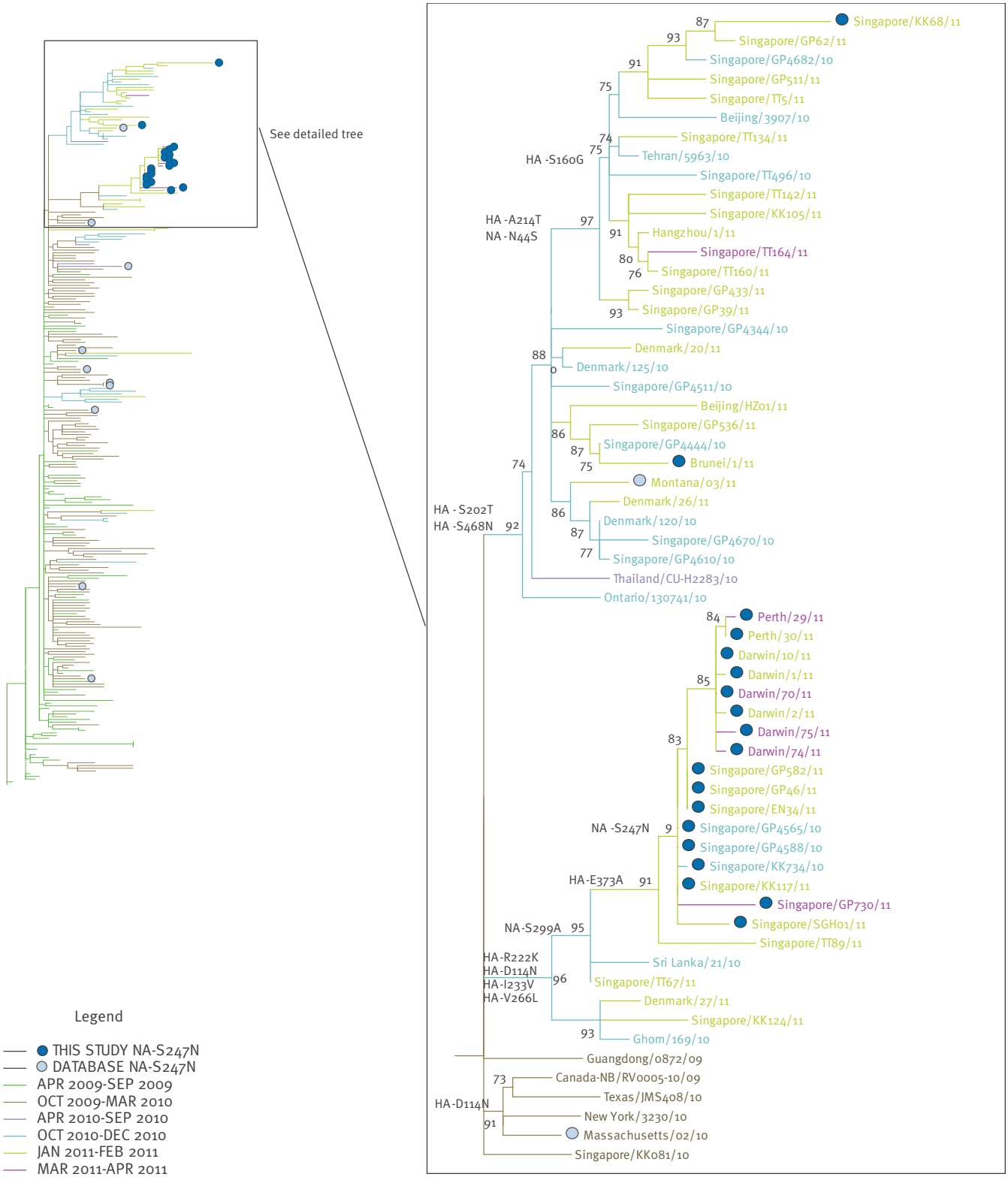
[b] Mean does not include the dual S247N+H275Y mutant.

[c] Viruses were from the same patient before and after oseltamivir treatment (refer to the text for more details).

[d] Recombinant strains were derived using site-directed mutagenesis and reverse genetics.

Figure 2

Phylogenetic trees of concatenated haemagglutinin and neuraminidase sequences from influenza A(H1N1)2009 S247N variants detected December 2010-March 2011



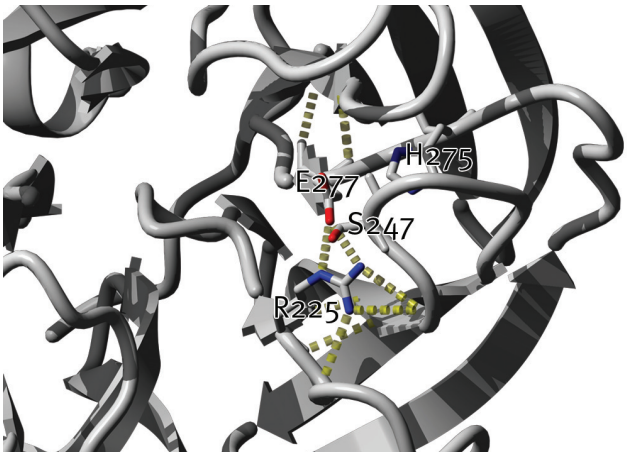
Dark blue dots: S247N variants identified in this study (n=22); light blue dots: S247N variants previously reported on Genbank or GISAID sequence databases.

Branches and designations are coloured according to their sample collection date. The nucleotide sequences for these strains were concatenated such that a single sequence contains nucleotide sequences encoding both the haemagglutinin and neuraminidase proteins in order to increase the evolutionary signal for strain differences of these almost identical sequences.

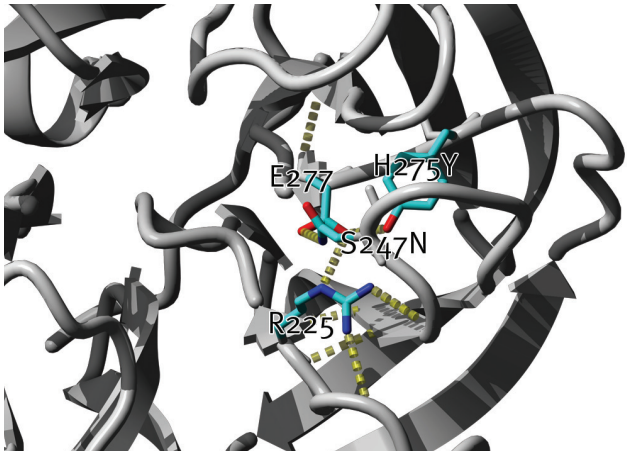
Figure 3

The structural effect of the S247N and S247N+H275Y mutations in the neuraminidase of influenza A(H1N1)

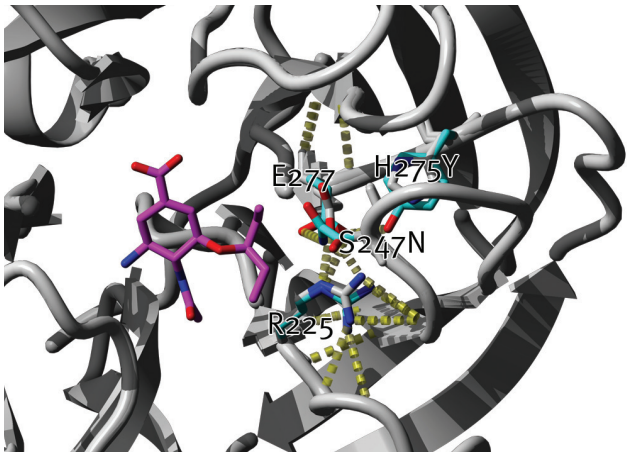
A



B



C



A. Wildtype.

B. Dual S247N+H275Y mutant.

C. The merged image shows the superimposition of the wildtype and the double mutant relative to bound oseltamivir (pink, from PDB:3clO). The crystal structure of influenza A(H1N1)2009 neuraminidase (PDB:3nss, chain A) was used to model the double mutations S247N and H275Y using Yasara.

Hydrogen bonds among the important residues are shown in yellow. Side chains of residues undergoing conformational changes upon the double mutation are shown in cyan.

reduction in peramivir sensitivity compared to the mean IC_{50} (concentration required to inhibit 50% of NA activity) of sensitive influenza A(H1N1)2009 viruses (Table).

In Perth, Western Australia, an immunocompromised patient was found on 1 March 2011 to be infected with an influenza virus that contained the S247N NA mutation (A/Perth/30/2011) (Table). Oseltamivir treatment was commenced two days after detecting the initial S247N variant (3 March). A sputum specimen collected five days later (8 March) contained an influenza virus with both the S247N and H275Y NA mutations (A/Perth/29/2011) (Table). Despite commencement of intravenous zanamivir the patient died on 16 March from multiple organ failure. The isolate with the dual S247N and H275Y mutations had an oseltamivir IC_{50} nearly 6,000-fold higher than sensitive viruses and 10-fold higher than seen for influenza A(H1N1)2009 viruses with the H275Y mutation alone (Table). Clonal analysis of the virus population from the sputum specimen collected after oseltamivir treatment confirmed that both the S247N and H275Y mutations occurred together in the NA. The effect of the S247N and dual S247N and H275Y mutations on NAI sensitivity was confirmed by reverse genetics experiments (Table).

Sequence analysis of the S247N variants from the recent Darwin and Singapore clusters revealed that the majority belonged to the same genetic lineage (Figure 2). The two strains from the immunocompromised patient in Perth were also genetically similar to the Darwin and Singapore strains, but the virus from Brunei and one strain from Singapore were placed in a separate clade together with one other S247N strain from the United States (Figure 2).

Structural analyses indicate that the S247N mutation could change the hydrogen bonding network and side chain conformation of residue E277 (E276 by N2 numbering) reflecting similar but weaker alterations known from the H275Y mutation. When adding H275Y, E277 gets pushed further into the drug binding pocket which is believed to weaken oseltamivir binding (Figure 3).

Discussion

Previous studies have reported the effect of the S247N mutation on NAI sensitivity in N1 neuraminidases. The levels of oseltamivir and zanamivir sensitivity reported for the S247N mutation in a pre-pandemic seasonal influenza A(H1N1) virus were similar to those reported here for the mutation in influenza A(H1N1)2009 strains [5]. However a greater reduction in oseltamivir sensitivity was reported for a highly pathogenic influenza A(H5N1) strain with a S247N mutation compared to the influenza A(H1N1)2009 strains reported here (24-fold vs 6-fold reduction compared to their respective wildtypes) [6]. The same study also reported on an

influenza A(H5N1) virus with the S247N and two additional mutations, I223L and K150N (N1 numbering). This triple mutant virus had a greater reduction in oseltamivir sensitivity compared to the S247N mutation alone (77-fold vs 24-fold reduction), further demonstrating that mutations at other key residues such as I223, in combination with S247N, can cumulatively decrease NAI sensitivity. Given that a number of studies have recently reported I223 mutations conferring NAI resistance in influenza A(H1N1)2009 strains [3,4,9,10], and that data from the United Kingdom show an increased frequency of H275Y mutants in community samples [2], the likelihood of mutation combinations S247N+I223X and S247N+H275Y is considerably increased if the S247N influenza A(H1N1)2009 variant continues to spread.

Pharmacokinetic data would suggest that the maximum drug levels achieved via the recommended dose easily exceed the observed IC_{50} values of the S247N mutant [11], and therefore the variant is unlikely to be clinically resistant. However, it is noteworthy that recent clinical studies have demonstrated a reduced oseltamivir efficacy for normal influenza B viruses [12] which have IC_{50} values only 6-fold higher than that of the S247N influenza A(H1N1)2009 variants (mean oseltamivir IC_{50}±SD for influenza B viruses from 2010 and 2011: 15.5 nM±11.3 (n=557)). Data from Singapore and Darwin demonstrate that the S247N variant is able to circulate widely and therefore does not appear to have compromised viral fitness or transmissibility. If the S247N variant spreads globally, the greatest concern is that other NA mutations which may have previously caused only mild reductions in NAI susceptibility (e.g. mutations at the I223 residue) could instead cumulatively decrease NAI sensitivity to levels that may be clinically significant and affect treatment efficacy. Laboratories should consider screening currently circulating specimens and isolates for the S247N NA mutation to determine whether the variant is spreading into other regions.

***Note:** Supplementary information made available by the authors on an independent website is not edited by *Eurosurveillance*, and *Eurosurveillance* is not responsible for the content.

****Erratum:** The map in Figure 1 was misaligned with the dots. This was corrected on 10 June 2011.

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Genetic characterisation of the emerging invasive *Neisseria meningitidis* serogroup Y in Sweden, 2000 to 2010

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Neisseria meningitidis **serogroups B and C have been responsible for the majority of invasive meningococcal disease in Europe. Recently, an increase of *N. meningitidis* disease due to serogroup Y has been noted in Sweden (in 2010, the proportion was 39%, with an incidence of 0.23 per 100,000 population), as well as in other northern European countries. We aimed to investigate the clonal pattern of the emerging serogroup Y in Sweden during 2000 to 2010. The serogroup Y isolates identified during this time (n=85) were characterised by multilocus sequence typing and sequencing of the *fetA*, *fHbp*, *penA*, *porA* and *porB* genes. The most frequent clone (comprising 28 isolates) with identical allele combinations of the investigated genes, was partly responsible for the observed increased number of *N. meningitidis* serogroup Y isolates. It was sulfadiazine resistant, with genosubtype P1.5-2,10-1,36-2, sequence type 23, clonal complex 23, *porB* allele 3-36, *fetA* allele F4-1, *fHbp* allele 25 and *penA* allele 22. The first case with disease due to this clone was identified in 2002: there was a further case in 2004, six during 2006 to 2007, eight during 2008 to 2009, with a peak of 12 cases in 2010. An unusual increase of invasive disease in young adults (aged 20–29 years) caused by this clone was shown, but no increase in mortality rate was observed.**

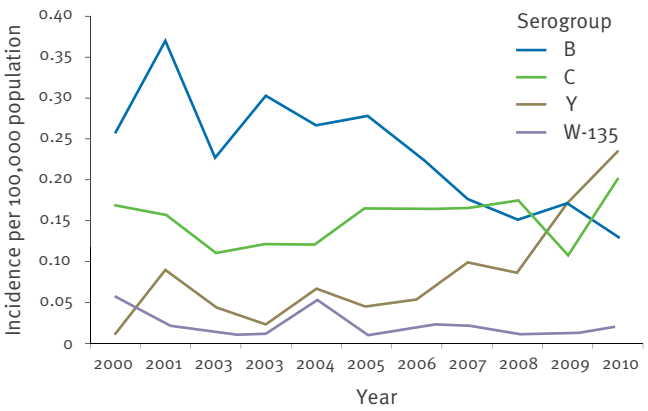
Introduction

Neisseria meningitidis is a Gram-negative bacterium that is an obligate commensal of humans: it is carried without symptoms in the pharynx by about 10% of the general population. It can occasionally cause severe infection, mainly septicaemia and meningitis, when it crosses the epithelial barrier to invade the bloodstream and the meninges [1]. Meningococcal disease is most common in infants but the carriage rate is highest in young adults. Despite treatment and modern intensive care, the disease is still fatal in about 10% of cases [2].

N. meningitidis is divided into different serogroups, depending on the biochemical composition of the capsule surrounding the bacterium, but only isolates belonging to the A, B, C, W-135, X and Y serogroups have a major role in causing disease [1]. Different hyperinvasive lineages of *N. meningitidis* cause disease with a unique epidemiology and the distribution of the serogroups is highly regional [2]. In Africa, especially in the so-called meningitis belt, the disease is mostly caused by serogroup A, but serogroups C, W-135 and X may also be involved. In Asia, serogroup A is also the most common serogroup. In Europe, North and South America and Australia, serogroups B and C have for decades been the dominating serogroups [3]. In the mid-1990s, however, the incidence of disease due to serogroup Y increased substantially in the United States (US) and today one third of the *N. meningitidis* infections in that country are caused by this serogroup [4,5]. During the last decade, there has also been an increase of meningococcal disease caused by serogroup Y in Canada and Colombia [6,7]. In addition, some northern European countries, displayed higher proportions of disease caused by this serogroup: for example, in Norway in 2009 and 2010, the proportion was 31%; in Finland, it was 38% in 2010 [8].

FIGURE 1

Incidence of invasive *Neisseria meningitidis* caused by serogroups B, C, Y and W-135, Sweden, 2000–2010[a]



[a] In 2010, the number of *N. meningitidis* isolates were: 12 (serogroup B), 19 (serogroup C), 22 (serogroup Y) and 2 (serogroup W-135).

Until recently, meningococcal disease in Sweden has followed the European serogroup distribution pattern, with the disease being caused mainly by serogroups B and C. However, the incidence of invasive *N. meningitidis* caused by serogroup Y started to increase in Sweden in the mid-2000s, rising from 0.04 per 100,000 population in 2005 to 0.23 per 100,000 population in 2010 (Figure 1). The reasons for the dramatic shift in serogroup distribution are unknown. One explanation could be that a new serogroup Y clone has been introduced in Sweden. To investigate this, extensive genetic characterisation must be conducted.

The European Meningococcal Disease Society has published a designation scheme for genetic characterisation of *N. meningitidis* based on serogroup, genosubtype based on analysis of the variable regions of the *porA* gene (encoding porin A, an outer membrane protein), *fetA* (encoding FetA, an iron-regulated outer membrane protein), sequence type (ST) and clonal complex (cc), the last two being determined by multilocus sequence typing (MLST) [9]. In addition, other gene targets such as *porB* (encoding porin B, another outer membrane protein), ***penA*** (encoding penicillin-binding protein 2, involved in penicillin susceptibility) and *fHbp* (encoding factor H-binding protein, which is a promising novel vaccine antigen) can be used for more discriminatory characterisation.

The aim of this study was to investigate by genetic characterisation the clonal pattern of the emerging invasive *N. meningitidis* serogroup Y isolates in Sweden between 2000 and 2010.

Methods

Bacterial isolates

The Swedish Institute for Infectious Disease Control (SMI) is mandatorily notified of all invasive cases of meningococcal disease by clinicians, using the European Union

Table 1

Primers for all target genes used in real-time PCR to characterise invasive *Neisseria meningitidis* serogroup Y isolates, Sweden, 2000–2010 (n=85)

Target gene	Primer	Primer sequence (5'→3')	Length of amplicon[a] (base pairs)	Reference
abcZ	P2C	TCCCCGTCGTAAAAAACAATC	856	[14]
	P1C	TGTTCCGCTTCGACTGCCAAC		
adk	P1B	CCAAGCCGTGTAGAATCGTAAACC	697	
	S2	CAATACTTCGGCTTTCACGG		
aroE	P1B	TTTGAAACAGGCGGTTGCGG	835	
	P2B	CAGCGGTAATCCAGTGCGAC		
fumC	S1	TCCGGCTTGCCGTTTGTCAG	530	
	S2	TTGTAGGCGGTTTTGGCGAC		
gdh	P1B	CTGCCCCCGGGGTTTTCATCT	677	
	P2B	TGTTGCGCGTTATTTCAAAGAAGG		
pdhC	P1B	CCGGCCGTACGACGCTGAAC	818	
	P2B	GATGTCGGAATGGGGCAAACA		
pgm	P1	CTTCAAAGCCTACGACATCCG	1,186	
	S2A	GGTGATGATTTCGGTYGCRCC		
penA	1F	ATCGAACAGGCGACGATGTCb	697	[15]
	ModGcDown3-R	CGGGGATATAACTGCGGCCGTC[c]		[16]
	1R[d]	GATTAAGACGGTGTTTTGACGG[c]	512	[15]
fetA	s12	TTCAACTTCGACAGCCGCCTT[b]	429	[17]
	s15	TTGCAGCGCGTCRTACAGGCG[c]		
fHbp	F	TGACCTGCCTCATTGATGC	950	[18]
	R	CGGTAAATTATCGTGTTCGGACGGC		
	5CE2086 mod[d]	TATGACTAGGAGYAAACCTG	882	Modified from [19]
porB	S1	GCAGCCCTTCCTGTTGCAGC	973	[17]
	S2	TTGCAGATTAGAATTTGTG		

[a] Amplicon lengths in MC58 [13].

[b] Universal forward sequence adaptor (5′-GTTTTCCCAGTCACGACGTTGTA-3′) added to the 5′-end of the primer [15].

[c] Universal reverse sequence adaptor (5′-TTGTGAGCGGATAACAATTTC-3′) added to the 5′-end of the primer [15].

[d] The penA1R and fHbp 5CE2086mod primers were used to complement the penA ModGcDown3-R and fHbp F primers, respectively, due to polymorphism in the annealing sites.

case definition [10] and the corresponding isolates are sent to the Swedish National Reference Laboratory for Pathogenic Neisseria. Basic epidemiological data (age, sex, area of residence and clinical site of isolation) are gathered routinely for all isolates from cases. We analysed all invasive *N. meningitidis* serogroup Y isolates in Sweden between 2000 and 2010 (n=85) from a total of 637 invasive isolates collected during this time period. The isolates were from clinical specimens of cerebrospinal fluid (n=11), blood (n=73) and joint fluid (n=1) and were cultured on chocolate agar.

Serogrouping and genosubtyping

The isolates were routinely serogrouped by co-agglutination [11] and subsequently genosubtyped (analysis of *porA* variable regions), as previously described [12]. Antibiotic susceptibility was determined using Etest (AB Biodisk, Sweden). As a reference, a serogroup B strain MC58 [13] was included.

Real-time PCR

The DNA used for amplification and sequencing was prepared from bacterial colonies by boiling or using the Bullet BUGS'n BEADS kit (NorDiag ASA, Norway). The genes targeted in MLST (*abcZ, adk, aroE, fumC, gdh, pdhC* and *pgm*), together with *fetA*, *fHbp*, *penA* and *porB*, were amplified by real-time PCR using the PCR primers shown in Table 1. In each PCR run, MC58 was used as positive control.

DNA sequencing and sequence alignment

The PCR products were purified by vacuum filtration and subsequently cycle sequenced. The primers used for sequencing of the genes used for MLST were those recommended [14], except for *pdhC*, for which the PCR primer P2B used to amplify *pdhC* was used for sequencing. The *fHbp* gene was sequenced with the respective PCR primers as well as gna1870 s2 and gna1870 s3 [18]. The *fetA* and *penA* genes were sequenced with the oligonucleotide sequences of the adaptors (Table 1) attached to the respective PCR primer. When *penA*1R was used, it was used for both PCR and sequencing. The *porB* gene was sequenced with the PCR primers as well as 8U, 8L [17] and PB7f2 (5'-TYGGCAACGTAACGC-3'), where Y is C or T.

The sequence alignments were assembled using ChromasPro software version 1.33 (Technelysium Pty Ltd, Australia). The different sequences were assigned allele numbers using the *N. meningitidis* sequence query database [20]. A clone was defined by all isolates sharing the same genosubtype, ST, sulfadiazine susceptibility and combination of *penA*, *fetA*, *fHbp* and *porB* alleles.

Data analysis

We evaluated whether each sequenced gene could be used to discriminate between strains, on the basis of Simpson's index of diversity [21]. The discrimination (D)-index determines the probability that two randomly picked strains will be separated into different typing groups. A high D-index (close to 1) divides the isolates into many small groups (high discriminatory capacity), whereas a low D-index (close to 0) indicates that the typing target only divides the isolates into a few large groups (low discriminatory capacity). Confidence intervals of 95% were calculated [22].

Minimum-spanning trees of the MLST profile data were created to investigate the ST clusters. The links were determined with Prim's algorithm and the clustering was created with the BURST (based on related STs) algorithm [23]. The *porA* genosubtypes and *porB*, *penA*, *fetA* and *fHbp* alleles were subsequently distributed over the different STs in the minimum-spanning trees, to describe the variation of different genes within each ST.

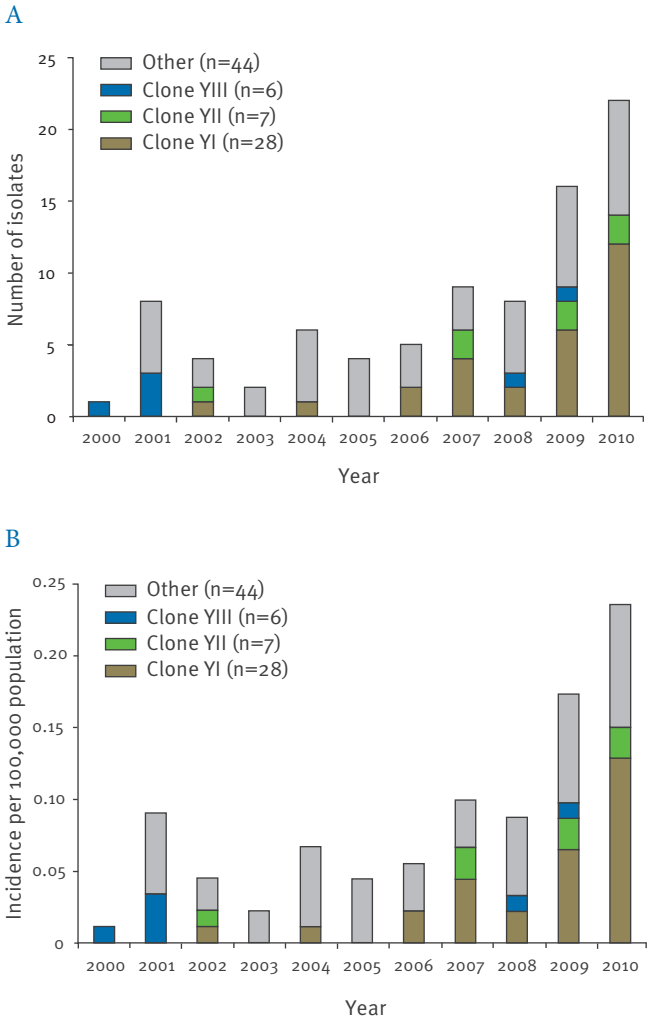
Results

Genetic characterisation

Using genosubtyping, MLST, and *porB*, *fetA*, *penA* and *fHbp* analysis, we found three distinctive clones comprising more than five isolates each. The most frequent clone (n=28), referred to as Clone YI, was sulfadiazine

Figure 2

Distribution over time of the three most common invasive *Neisseria meningitidis* serogroup Y clones (YI, YII and YIII) and all other invasive serogroup Y isolates, shown by (A) number of isolates and (B) incidence, Sweden, 2000–2010 (n=85)



resistant, with genosubtype P1.5-2,10-1,36-2, ST 23 (cc23), *porB* allele 3-36, *fetA* allele F4-1, *fHbp* allele 25 and *penA* allele 22. The second most frequent (Clone YII; n=7) was sulfadiazine susceptible, with genosubtype P1.5-1,2-2,36-2, ST 23 (cc 23), *porB* allele 2-55, *fetA* allele F5-8, *fHbp* allele 25 and *penA* allele 22. The third most frequent (Clone YIII; n=6) was sulfadiazine susceptible, with genosubtype P1.5-1,2-2,36-2, ST23 (cc23), *porB* allele 3-36, *fetA* allele F5-8, *fHbp* allele 25 and *penA* allele 1. The remaining 44 isolates included clones of two to five identical isolates (n=21) and 23 isolates with individual genetic profiles.

Distribution of clones and other isolates

Compilation of the epidemiological data on the isolates and genetic characterisation data generated a pattern of the distribution of the clones over time (Figure 2). There was no indication of an epidemiological link between the notified cases. The age distribution of patients with invasive disease caused by the three most common clones and all other serogroup Y isolates is shown in Figure 3. An increase of invasive disease caused by Clone YI was seen in young adults (aged 20–29 years). Overall, this clone was significantly more prone ($p<0.05$ Mann–Whitney U test) to cause disease in a younger age group (¬median age: 47 years; interquartile range: 20–70), compared with the isolates with other genetic profiles. These other clones and isolates had the usual pattern of serogroup Y regarding age groups affected, i.e. they affected mainly elderly people (median age: 72 years; interquartile range: 36–84).

We also studied the geographical distribution of the three most common serogroup Y clones and all other serogroup Y isolates, which showed a higher incidence of Clone YI in young adults (aged 15–25 years) in the central parts of Sweden (data not shown). No such geographical pattern was seen for elderly cases with invasive isolates of Clone YI or cases with Clone YII, Clone YIII or any of the other isolates.

The mortality rate among patients with disease caused by serogroup Y was 13% (11 of 85). Two of the 11 deceased patients were infected with Clone YI.

The genes that displayed the highest variability in the minimum-spanning trees (*porA*, *porB* and *fetA*) are shown in Figure 4; the *penA* and *fHbp* genes were found to be fairly conserved (data not shown). These findings were further confirmed by the D-indices calculated for the genes used in MLST, and *porA*, *porB*, *fetA*, *fHbp* and *penA* genes (Table 2).

Discussion and conclusion

Our results show that the most common invasive *N. meningitidis* serogroup Y isolate, clone YI, was partly responsible for the increase of meningococcal disease caused by this serogroup in Sweden in recent years. This clone was prevalent at the beginning of the 2000s, with one isolate being identified in both 2002 and 2004 (incidence of 0.01 per 100,000 population in each year). The number of isolates gradually increased, from two isolates in 2006 to a peak of 12 in 2010 (incidence of 0.02 and 0.13, respectively, per 1000,000 population). No outbreaks or clusters of cases due to serogroup Y infection were noted in Sweden during 2000 to 2010.

The reasons for the increased incidence of invasive disease caused by Clone YI, compared with the other clones and isolates, among younger people are unclear. However, a similar pattern for disease due to serogroup Y has been observed in Canada, where the proportion of cases with this serogroup in the age group 10–19 years increased from 11.8% in 1999 to 26.3% in 2003, with a peak of 41.4% in 2001 [6]. In the US, however, no such increase has been observed among persons aged 15–24 years [25].

Figure 3

Age distribution of patients with invasive meningococcal disease caused by the three most common *Neisseria meningitidis* serogroup Y clones (YI, YII and YIII) and all other invasive serogroup Y isolates, Sweden, 2000–2010 (n=85)

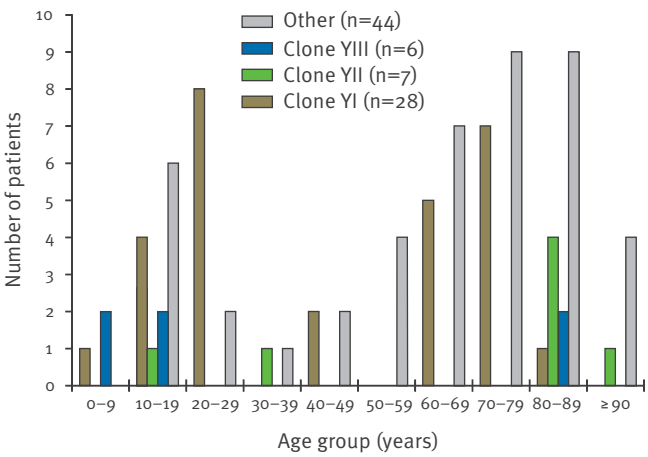


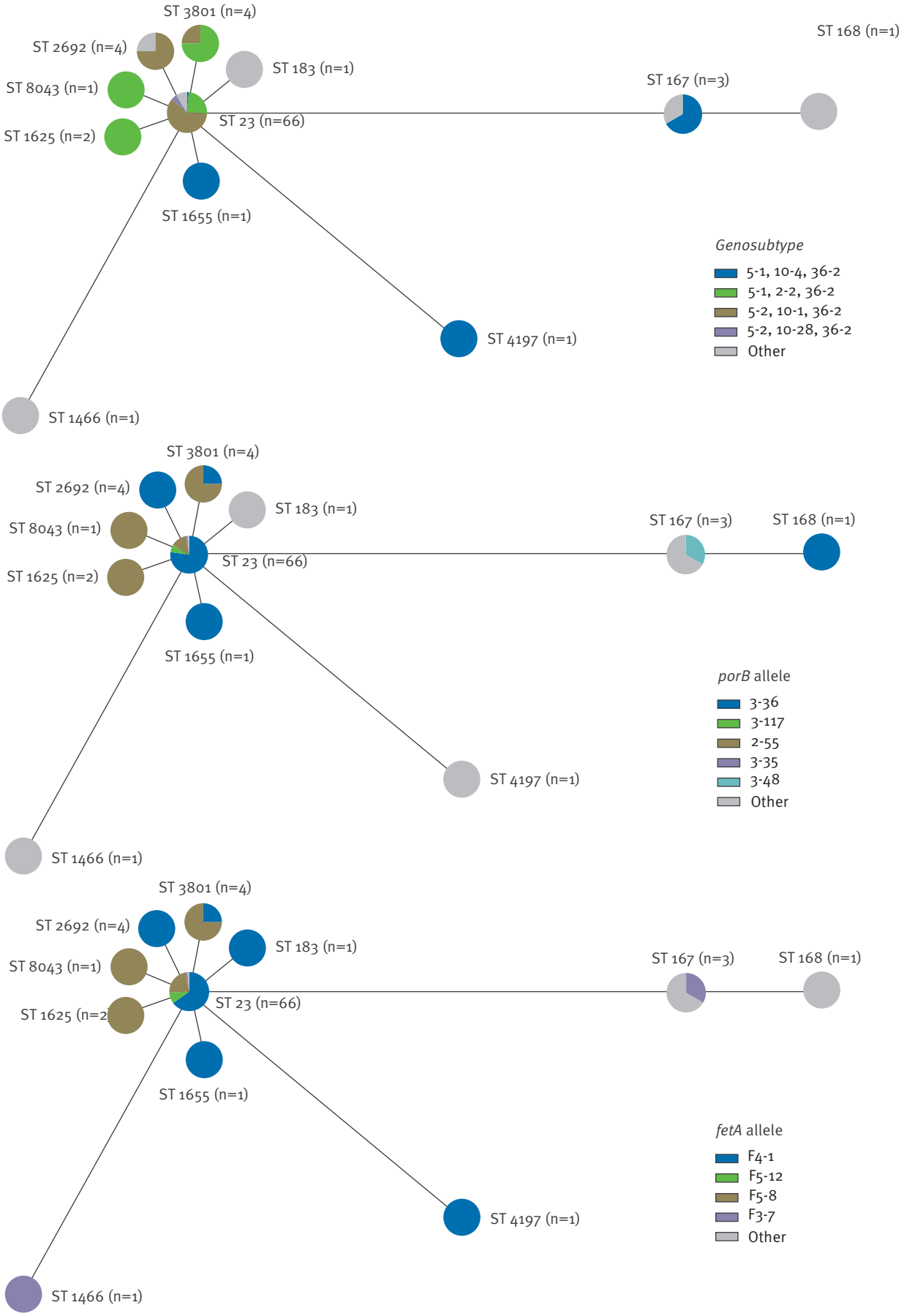
Table 2

Discrimination indices for sequence types and *porA*, *porB*, *fetA*, *fHbp*, *penA* genes, invasive *Neisseria meningitidis* serogroup Y isolates, Sweden, 2000–2010 (n=85)

Target	Number of types	Number (%) of isolates with the most common type	Discrimination index (95% CI)
porA	15	45 (53)	0.65 (0.57–0.74)
fetA	7	53 (62)	0.55 (0.45–0.65)
porB	9	58 (68)	0.51 (0.39–0.62)
Sequence types	12	66 (78)	0.39 (0.26–0.53)
penA	4	72 (85)	0.27 (0.15–0.39)
fHbp	6	78 (92)	0.16 (0.5–0.26)

Figure 4

Minimum-spanning trees based on multilocus sequence typing of all invasive *Neisseria meningitidis* serogroup Y isolates, Sweden, 2000–2010 (n=85)



The circles denote different sequence types. The genosubtype (*porA*) and *porB* and *fetA* alleles are distributed over the different sequence types.

The second and third most common clones in Sweden, YII and YIII, consisted of few isolates per clone, which made the age distribution for cases with these clones difficult to interpret. It is interesting to note that in Maryland, US, two clones with different antigenic profiles seemed to be responsible for the increased number of invasive *N. meningitidis* serogroup Y isolates in the 1990s and that the clones are differently distributed over time [25]. The first, which shares parts of the genetic profile of clones YII and YIII in our study (cc23; P1.5-1,2-2; F5-8), was mainly responsible for early cases (before 1998), while the other clone, with the same genetic profile as the dominant Clone YI in our study (cc23; P1.5-2,10-1; F4-1), has dominated since 1998. In the Czech Republic, the most common clone (cc23; P1.5-2,10-1,36-2; F4-1) and second most common clone (cc23; P1.5-1,2-2,36-2; F5-8) present indistinguishable *porA* and *fetA* profiles and ST as the most common clones in Sweden, in the same order of frequency [8].

Clone YI does not seem to be related to increased mortality, although a difference would be difficult to detect because of the low incidence of meningococcal disease in general. The mortality rate of all the studied invasive serogroup Y isolates (13%) is within the range of the overall global mortality rate for meningococcal disease caused by all serogroups (about 10%) [2], but is considerably lower than the mortality rate for disease due to serogroup Y in Sweden in 1995 to 2005 (17%) [26]. The decreased mortality rate may be the result of the increased incidence of Clone YI in a younger age group (median age: 47 years).

The emergence of serogroup Y Clone YI is presumably due to multiple underlying factors, but the most probable is that it is an epidemiologically competent clone with increased pathogenicity. It is less likely that host adaptive immunity has decreased in different geographical areas concurrently. It is also possible that Clone YI possesses characteristics that lead to increased transmission efficiency. This possibility is difficult to investigate and no dynamic carriage studies have been performed in Sweden. However, in some carriage studies from elsewhere in Europe and the US, it was found that serogroup Y was one of the most common serogroups among carrier isolates [27-30].

The results from the minimum-spanning tree and D-indices indicate that in the case of serogroup Y, targeting the *penA* and *fHbp* genes yields a less discriminatory pattern than MLST or targeting the *porA*, *porB* and *fetA* genes. Although *penA* and *fHbp* seem to be too conserved to be of use for further differentiation of clones, these genes are still of interest for following penicillin resistance and the effect of promising novel vaccines that include the factor H-binding protein. Although none of the individual genes in our study reached the recommended discrimination level of 90% [21], a combination of target genes and inclusion of other serogroups probably could. However, using only typing tools with high D-indices may result in missing isolates belonging to the same clone. Similarly, the apparent frequency of the clone would increase if only stable genes are targeted, as in MLST. Therefore, Clone YI is defined by what we think is an informative and appropriate number of target genes, which is also somewhat supported by the minimum-spanning trees and D-indices.

Further research could be carried out using characterisation tools that would increase the discrimination, such as variable number tandem repeat (VNTR) analysis, to provide more information about the clone. However, it will also be a challenge to customise the characterisation tools, to achieve an appropriate balance between the information required for the particular investigation and the time and cost.

In conclusion, the clone responsible for the recent emergence of *N. meningitidis* serogroup Y isolates was identified and described in this study. To achieve a complete epidemiological profile of the clone, it would be useful to examine invasive serogroup Y isolates from before 2000 and carry out additional typing. The general increase of meningococcal disease due to *N. meningitidis* serogroup Y has an important public health implication because this is one of the serogroups that is covered by available vaccines for meningococcal disease.

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Pandemic influenza A(H1N1)2009 in Morocco: experience of the Mohammed V Military Teaching Hospital, Rabat, 12 June to 24 December 2009

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On 12 June 2009, Morocco was the first country in North Africa to report a laboratory-confirmed case of influenza A(H1N1)2009 virus infection. This study describes the epidemiological and clinical characteristics of 240 laboratory-confirmed cases among 594 outpatients with influenza-like illness at the Mohammed V Military Teaching Hospital, Rabat, from 12 June to 24 December 2009. Real-time reverse transcription-PCR was used to confirm the infection. The epidemic peaked in weeks 47 to 49 (16 November to 6 December 2009). The mean age of cases was 23 years (standard deviation: 14 years). Cough was the most common symptom in 200 cases (83%), followed by fever (≥38 °C) in 195 (81%). Diarrhoea or vomiting was reported in 12 (5%) patients. None of the cases developed any complications and no deaths occurred during the study period.

Introduction

Following its identification in humans in Mexico and in the United States in April 2009 [1], the pandemic influenza A(H1N1)2009 virus spread worldwide [2]. On 11 June 2009, the World Health Organization (WHO) raised the pandemic alert level from phase 5 to phase 6, officially marking the beginning of the 2009 influenza pandemic [3]. On 12 June 2009, the Division of Epidemiology and Disease Control of the Moroccan Ministry of Health reported the first laboratory-confirmed case of influenza A(H1N1)2009 virus infection in north Africa, in a traveller returning from Canada [4]. Subsequently, the number of laboratory confirmed cases in Morocco rose continuously, and reached a total of 2,890 including 64 deaths by 10 March 2010 [5].

The burden of influenza on the African continent is unclear. This is in part due to a lack of systematic surveillance across the continent, limited testing facilities and the prioritisation of other infectious diseases. Factors, such as health care availability, prevalence of co-morbidities or co-infections, or population age structure could affect the influenza burden specifically for this continent. Data from Africa on the pandemic influenza A(H1N1)2009 is also scarce [6,7]. A comprehensive surveillance from all parts of the world is nevertheless important.

The aim of this study, performed in the Mohammed V Military Teaching Hospital (MVMTH) in Rabat, Morocco, was to investigate the epidemiological and clinical characteristics of the cases of influenza A(H1N1)2009 and to report the laboratory diagnosis data in our hospital during the pandemic from June 2009 to December 2009.

The MVMTH in Rabat, Morocco, is a 1,000 bed university hospital with about 80,000 inpatient admissions and 200,000 outpatients per year. It is intended for active or retired military personnel as well as their families in priority, but it also treats civilians thereby serving about 5 million people living in the north of the country. At the time the pandemic began (12 June 2009), the MVMTH was opened to all the population for influenza diagnosis and treatment. This resulted in a small, yet insignificant, increase in out- and inpatients at the hospital.

Methods

Patients and samples

The study, conducted from 12 June to 24 December 2009, involved 594 outpatients with influenza-like illness (ILI) characterised by at least two of the following symptoms including fever (≥38 °C), cough, muscular pain, headache, rhinorrhoea, dyspnoea, diarrhoea and vomiting. Demographic, clinical and epidemiological data of patients were collected by the medical staff using a questionnaire that was completed when

samples were taken. The items collected on the questionnaire were: age, sex, clinical symptoms, co-morbidities, recent travel history in an epidemic country and vaccination against seasonal influenza.

In the period from 12 June to 28 August (referred to as the pre-epidemic period for this study), in which all the confirmed cases of A(H1N1)2009 were imported, swabs were taken from ILI outpatients and a total of 18 of their contacts. During the period from 29 August to 24 December (here referred to as the epidemic period), in which there were also autochtonous cases with no travel history in an epidemic country, the collection of specimens was restricted to ILI outpatients and for grouped cases, such as members of the same family or of the same school class or cases in the same military barracks, only a representative sample was tested for influenza A(H1N1)2009 virus infection.

Oseltamivir was given to all patients immediately after sampling. The treatment was stopped if patients tested negative for influenza A(H1N1)2009. Patients who were confirmed positive for influenza A(H1N1)2009 continued the treatment (oseltamivir 2x75mg/day for five days) and all their contacts received preventive treatment. The follow-up of patients was documented. At the beginning of the pandemic, a hospital ward with 20 beds was reserved for the treatment and quarantine of influenza patients. As the virulence of the strain was unknown, all suspected cases were maintained in quarantine until they were confirmed. For cases who were positive for influenza A(H1N1)2009, containment was maintained until they recovered. As ILI cases increased from mid-november, these measures were replaced by homecare.

Laboratory confirmation of infection with influenza A(H1N1)2009 virus

ILI patients were swabbed and nasopharyngeal swabs from the patients were transported to the biosafety level-3 laboratory in a standard, triple-packaging system following the United Nations (UN) 2814 class 6.2 specifications [8]. RNA was extracted from nasopharyngeal swabs using the High Pure Viral RNA Isolation Kit (Roche) and one-step real-time RT-PCR was performed using the RealTime ready Influenza A/H1N1 Detection Set and RealTime ready RNA Virus Master (Roche). A confirmed case was defined as an ILI case whose

Figure 1

Patients with influenza-like illness and laboratory-confirmed cases of influenza A(H1N1)2009 virus infections, Mohammed V Military Teaching Hospital, Rabat, Morocco, 12 June–24 December 2009 (n=240)

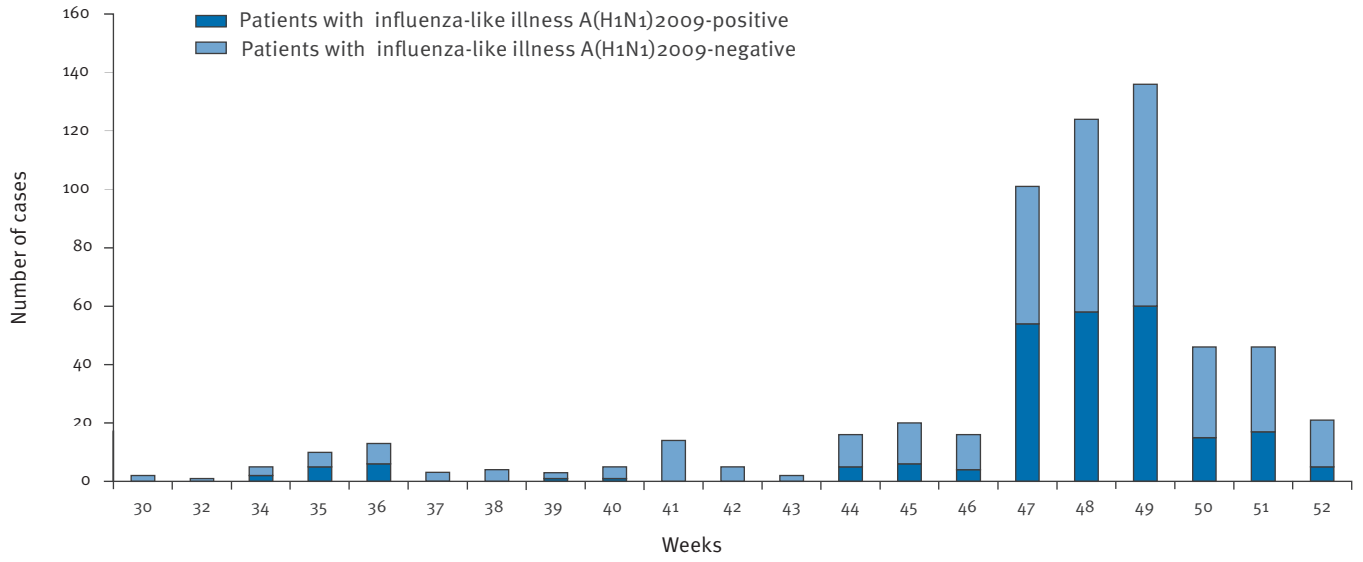


Table 1

Characteristics of patients with influenza-like illness and laboratory-confirmed cases of influenza A(H1N1)2009 virus infections, Mohammed V Military Teaching Hospital, Rabat, Morocco, 12 June–24 December 2009 (n=594)

Characteristic	**Total**	Patients with influenza-like illness A(H1N1)2009-negative n (%)	Patients with influenza-like illness A(H1N1)2009-positive n (%)	p value
Number of patients, N (%)	**594**	354 (60%)	240 (40%)	
Number of males, n/N (%)	**349 (59%)**	200/354 (56%)	149/240 (62%)	0.175
Mean Age and standard deviation	**28±16**	31±17	23±14	0.004

swab yielded a positive RT-PCR result. Between 12 June and 28 August 2009, in the pre-epidemic phase, 18 contacts of confirmed cases, who did not necessarily present with ILI, were also tested for pandemic influenza A(H1N1)2009 virus infection using the same procedure as above. All the patients considered for this study were tested. We had two inhibited tests: they were repeated using different concentrations (1/2, 1/10, 1/20) and were both positive at the dilution of 1/10.

Statistical analysis

All statistical analysis was carried out using SPSS (release 7.5.1). Normal data distributions were assessed with the Kolmogorov–Smirnov test. Skewed variables were natural log transformed. Patients' ages were categorised into six groups. Student's t-test and one-way analysis of variance procedures were used for the comparison of categorical variables between groups. Odds ratios (ORs) and 95% confidence interval (CI) were obtained using the logistic regression model with backward likelihood ratio method. Removal testing is based on the probability of the likelihood-ratio statistic, which is also based on the maximum partial likelihood estimates. For this purpose, all variables were entered and eliminated step by step according to default criteria with a probability for entry and removal of 0.05 and 0.10, respectively. Reference values were age greater than 40 years and an absence of symptoms for all clinical variables. A p value less than 0.05 was considered to be statistically significant.

Results

Of 594 ILI outpatients from 12 June to 24 December 2009, 240 (40%) were laboratory confirmed as cases of influenza A(H1N1)2009 virus infection and 354 (60%) were negative. Between 12 June and 28 August 2009, in the pre-epidemic phase, 18 contacts of confirmed cases, were also tested for pandemic influenza A(H1N1)2009. Ten contacts who were asymptomatic tested positive for pandemic influenza 2009. Contacts presenting with ILI were considered as ILI patients for our analysis.

At the beginning of the outbreak, 27 confirmed cases required a few days of hospitalisation because they had identifiable underlying conditions. These included pregnancy (n=13), asthma (n=7), obesity (n=2) and diabetes mellitus (n=5). All these cases were put under supervision until their full recovery.

A rapid increase in the number of confirmed cases was observed with a peak in weeks 47 to 49 (16 November to 6 December 2009): 172 cases were detected during this time (Figure 1).

At the beginning of the study period, from 12 June to 28 August all cases were imported (n=13, 100%), seven from Spain, two from France, and one respective case from Italy, the Netherlands, Brazil and Canada. In the epidemic period, the proportion of cases with no travel history increased from mid-November 2009, to reach 92% (n=222) by 24 December.

Of the total confirmed cases of influenza A(H1N1)2009 virus infection over the whole study period, 149 (62%) were men (Table 1). There were no differences in the mean age of cases by sex (women: 24±14 years, men: 22±14, p=0.57).

The ages of confirmed cases ranged between three months and 60 years, with a mean of 23 years (standard deviation: 14). The age followed a normal distribution according to the Kolmogorov–Smirnov test. A total of 207 (86%) cases were aged 40 years old and younger and only 5 cases (2%) were aged over 50 years. Compared to the reference group (›40 years), the risk of infection was greater in those aged 14 years old and under (OR: 3.38; 95% CI: 1.99–5.73, p‹0.001) and those aged from 14 to 27 years (OR: 2.62; 95% CI: 1.55–4.42, p‹0.05) (Table 2, Figure 2).

Table 2. Laboratory-confirmed cases of influenza A(H1N1)2009, and A(H1N1)2009 virus negative patients

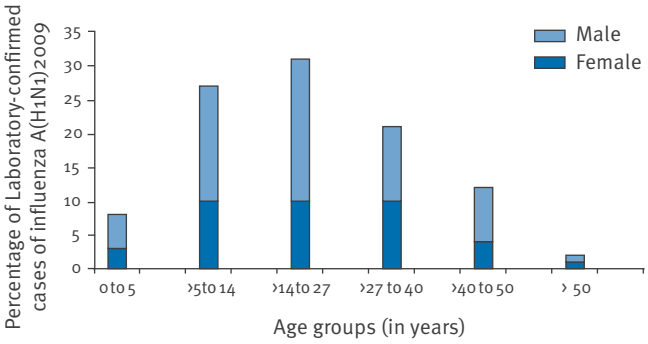
Table 2

Laboratory-confirmed cases of influenza A(H1N1)2009, and A(H1N1)2009 virus negative patients with influenza-like illness, by age group, Mohammed V Military Teaching Hospital, Rabat, Morocco, 12 June–24 December 2009 (n=594)

Age of patients in years	Number of A(H1N1)2009-negative cases	Number of A(H1N1)2009-positive cases	Total (%)
0 to 5	20	18	**38 (6)**
›5 to 14	48	65	**113 (19)**
›14 to 27	82	72	**154 (26)**
›27 to 40	97	52	**149 (25)**
›40 to 50	72	28	**100 (17)**
› 50	35	5	**40 (7)**
Total	**354**	**240**	**594 (100)**

Figure 2

Laboratory-confirmed cases of influenza A(H1N1)2009 by sex and age group, Mohammed V Military Teaching Hospital, Rabat, Morocco, 12 June–24 December 2009 (n=240)



with influenza-like illness, by age group, Mohammed V Military Teaching Hospital, Rabat, Morocco, 12 June–24 December 2009 (n=594)

Fever, cough, headache, muscular pain and rhinorrhoea were the main symptoms: cough was the most common (n=200, 82%) followed by fever (n=195, 80%). The ORs were 4.2 (95% CI: 2.51–7.04, p<0.001) and 5.58 (95% CI: 3.43–9.09, p<0.001) for cough and fever respectively. Diarrhoea or vomiting was reported in 12 cases (5%). None of the 240 cases developed any complications and there were no deaths during the study period. Variations of symptoms by age groups were not significant (Table 3).

A total of 238 (99%) cases received antiviral treatment with neuraminidase inhibitors (oseltamivir) immediately after laboratory confirmation of the infection.

Discussion

Morocco was the first country in North Africa to report a laboratory-confirmed case of influenza A(H1N1)2009 virus infection. Here we present a study from Morocco, thereby contributing data from North Africa to the global data and adding to the overview of the A(H1N1)2009 pandemic. The epidemic curve obtained for the hospital outpatients of this study was similar to the epidemic curve for the pandemic in Morocco [5]. In European countries, the pandemic started in weeks 30 to 32, 2009, and the number of confirmed-cases peaked in weeks 48 and 49 followed by a decline in weeks five to six, 2010 [9,10]. In contrast, the pre-epidemic period in Morocco began on week 34, 2009. The first positive case that we observed in our study was imported as well as all cases reported every week until week 40. This is in agreement with what was generally observed in Morocco where the first cases of A(H1N1)2009 virus infections were detected among Moroccans returning from a travel to a country where the virus was circulating actively and also from Moroccans residing abroad visiting their country. It is noteworthy that out of the first 13 cases we observed in our study, 11 were imported from Europe. This may reflect the impact of the population flows between Europe and Morocco: Almost 3.5 million Moroccan workers and their families live in Europe and return to Morocco for holidays every summer [11] and in addition approximately eight millions tourists from Europe, Asia and North America visit Morocco each year [11].

In weeks 41, 42, and 43, no confirmed case was reported. This can be explained by the fact that, by the end of summer holidays, there was a decline in arrivals of tourists and Moroccans who had spent their holidays abroad. Moreover, the measures taken for containment could also have contributed to the delay of the emergence of secondary cases until the week 44 marking the shift from imported to local cases. The peak of the epidemic was reported on week 49 and then the number of confirmed cases decreased from week 52. This difference with European countries is probably due to the fact that the decline in temperatures settles earlier in Europe than in Morocco and lasts for a longer period.
The distribution of the 240 cases of laboratory-confirmed A(H1N1)2009 virus infection by sex and age group was similar to that of cases observed in several European countries which may have a climate almost similar to that of Morocco like Spain [12] or with much cooler winters like Ireland [13]. There was an under-representation of infection in older people and the majority of cases were 40 years and under (86%). It has been shown that schoolchildren play an important role in the spread of influenza A(H1N1)2009 virus [12] and this predominance of infection in young people has been reported by other authors [15-16].

Moreover, several reports of the A(H1N1)2009 pandemic showed that attack rates were higher in children younger than 15 years [16,17]. In addition, a report from Mexico noted that most cases occurred in people younger than 50 years, with 89% of cases of pneumonia [17] and 85% incidence of mortality due to A(H1N1)2009 infection in this age group [18]. The lower frequency of influenza A(H1N1)2009 cases among those over 50 years of age is consistent with other investigations [17,18,19]. This can be explained by the fact that older people may have partial immunity from previous exposure to other influenza A(H1N1) strains [20] or that the

Table 3

Symptoms by age group of laboratory-confirmed cases of influenza A(H1N1)2009 virus infection, Mohammed V Military Teaching Hospital, Rabat, Morocco, 12 June–24 December 2009 (n=240)

Clinical symptoms	Total n (%)	Age of patients in years						p value
		0 to 5 n (%)	>5 to 14 n (%)	>14 to 27 n (%)	>27 to 40 n (%)	>40 to 50 n (%)	>50 n (%)	
Cough	**199 (83)**	14 (7)	58 (29)	59 (30)	41 (21)	23 (12)	4 (2)	0.168
Fever	**193 (80)**	15 (8)	56 (29)	57 (30)	37 (19)	25 (13)	3 (2)	0.060
Muscular pain	**122 (51)**	6 (5)	28 (23)	45 (37)	25 (20)	16 (13)	2 (2)	0.210
Headache	**54 (23)**	2 (4)	8 (15)	27 (50)	9 (17)	8 (15)	0	0.239
Rhinorrhoea	**85 (35)**	3 (4)	18 (21)	31 (36)	20 (24)	13 (15)	0	0.083
Dyspnoea	**5 (2)**	0	1 (20)	2 (40)	1 (20)	1 (20)	0	0.564
Diarrhoea, vomiting	**12 (5)**	2 (17)	2 (17)	4 (33)	0	4 (33)	0	0.894

influenza A(H1N1)2009 virus had not been widely introduced in this subpopulation.

The clinical manifestation of influenza A(H1N1)2009 virus infection in our investigation was similar to that observed for seasonal influenza [14,21,22]. All cases presented predominantly mild and self-limiting illness, with cough and fever being the most common symptoms. The ORs for fever and cough (3.38; 2.62 respectively) were consistent with a literature review by Petrosillo et al. with descriptions of A(H1N1)2009 cases across the world [23]. According to this study, A(H1N1)2009 patients complained mostly of the classical influenza symptoms. In the review from Petrosillo et al., fever was reported by a median of 87% (62–100%) of cases, cough by 82.5% (59–100%), sore throat by 57% (2–82%), diarrhoea by 13.5% (2–50%), and vomiting by 12.5% (2–50%). Other reported symptoms included myalgia, arthralgia, nasal-congestion headache, anorexia, nausea and conjunctivitis.

In our study, some cases required a few days of hospitalisation at the beginning of the outbreak because they had identifiable underlying conditions (such as pregnancy, asthma, obesity and diabetes mellitus). It was a precautionary measure given that during this period the pathogenicity of the A(H1N1)2009 virus was still poorly known. All these cases evolved favorably under treatment.

Almost all of the confirmed cases (98%) received early treatment (24 to 48 hours after onset of symptoms) with neuraminidase inhibitors, which may have had a favourable impact on the clinical expression of the infection. Indeed, most of them presented with mild illness and made good progress under specific antiviral treatment and no deaths were recorded. However, in another report from Morocco, the mortality rate was 3.5%. This difference may be explained by the fact that this study included hospitalised cases and severe infections [24] in contrast to the outpatients in this study.

The limitations of our study stem from the fact that it is derived from only one hospital. Also because of the workload of the clinicians during the pandemic period, the questionnaires were not always properly informed, in particular information on co-morbidities. Therefore some data were missing and not exploitable.

The study nevertheless contributed to the epidemiological surveillance of A(H1N1)2009 virus infections (number of cases, deaths, changes over time) during the pandemic and allowed to enrich national and international databases.

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Information resources and latest news about the Shiga toxin-producing *Escherichia coli* (STEC) outbreak in Germany available from ECDC

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On 22 May 2011, Germany reported a significant increase in the number of patients with haemolytic uremic syndrome (HUS) and bloody diarrhoea caused by Shiga toxin-producing *Escherichia coli* (STEC). ECDC has set up a special web page dedicated to the STEC outbreak [1]. This includes clinical reference information [2] which may be useful to those involved in the care of patients with STEC infection and with HUS as well as guidance on diagnostic investigations [3]. The Centre publishes epidemiological updates [4], which contain the most recent numbers of HUS and non-HUS cases. In these updates, Member States report cases according to the new European Union case definition.

In addition, public health advice and the ECDC risk assessment on the outbreak can be found on the ECDC *E. coli* health topic page [5].

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EFSA publishes scientific report on the public health risk of Shiga-toxin producing *Escherichia coli* (STEC) in fresh vegetables

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On 9 June 2011, the European Food Safety Authority (EFSA) published a report on the public health risk of STEC in fresh vegetables [1]. It aims to provide a fast-tracked risk assessment of the exposure of the consumer to Shiga-toxin producing *Escherichia coli* (STEC) through eating raw vegetables.

On exposure assessment, the report states that there is not much information available on the prevalence and quantity of STEC in vegetables, and that it is impossible to estimate the relative exposure to humans from pre-harvest or post-harvest contamination of vegetables by STEC at this time.

The report also provides advice on mitigation options pre-harvest, post-harvest and on good hygiene practices for caterers and in the home.

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