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Oseltamivir-resistant influenza A(H1N1) viruses in south of France, 2007/2009

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ABSTRACT

Influenza A(H1N1) viruses resistant to oseltamivir carboxylate (OC) emerged in 2007/2008 in the absence of antiviral pressure. These OC-resistant A(H1N1) viruses had a better fitness than the sensitive ones as they were 100% prevalent in 2008/2009.

To better understand the role of the neuraminidase (NA) affinity in the emergence of these OC-resistant A(H1N1) viruses we compared the NA properties among A(H1N1) clinical isolates in south of France between 2005 and 2009 and reference strains from 1977 to 2007, using NA inhibition assays, kinetic analyses of NA activities, and sequence analysis of viral NA and hemagglutinin (HA).

In 2007/2008, among 374 A(H1N1) isolates tested, 38% were resistant to OC with a mean IC_{50} of 564 ± 357 nM. The mean Km of OC-sensitive isolates (H275) was significantly lower (22.6 ± 4.7 μM) than the Km of previous reference strains (44.9 ± 5 μM) and the mean Km of the OC-resistant isolates (Y275) (37.2 ± 7.7 μM). The combination of different amino acid mutations in N1 particularly the D344N could explain the higher NA affinity of A/Brisbane/59/2007 related variants compared to the previous A(H1N1) strains and the H275Y mutation allowed to retrieve Km values near 40 μM.

1. Introduction

Influenza viruses epidemics cause important morbidity and mortality annually. Vaccination is the first line strategy to prevent influenza infection. However, antiviral drugs are essential to prevent and control the spread of influenza outbreaks. The surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) share the same target: the sialic acid (SA). HA allows the attachment of the virus to the cell by specific linkage between the receptor binding site and SA, whereas the NA cleaves the link between SA and HA to allow virus release from the infected cells. As a consequence, an optimal equilibrium between HA and NA activities is needed for an efficient viral multiplication (Wagner et al., 2002). The neuraminidase inhibitors (NAI) oseltamivir and zanamivir block the active site of the NA and subsequently prevent the release of new virions.

Until 2007, influenza viruses resistant to NAI were rare, representing less than 1% of the total circulating viruses (Monto et al., 2006; Escuret et al., 2008). It was postulated that resistant influenza viruses to NAI could not emerge because of a lower fitness (Ives et al., 2002; Herlocher et al., 2004). Unexpectedly, in 2007/2008 a significant proportion of influenza A(H1N1) viruses resistant to OC was detected in several countries. Norway gave the alert in December 2007 and at the end of winter 2007/2008 the mean percent of resistant isolates to OC was 25% in Europe (Lackenby et al., 2008; Meijer et al., 2009), 12,3% in USA (Dharan et al., 2009), 26% in Canada, 17% in China and only 3% in Japan (WHO, 2008). In the southern hemisphere, the resistant variants continued to be transmitted and reached up to 100% in South Africa in July 2008. The following winter (2008/2009) in the USA and Canada 99% of A(H1N1) isolates were resistant to OC (Dharan et al., 2009; WHO, 2009). The emergence of these OC-resistant strains was not related to any antiviral selective pressure. Indeed, these resistant viruses were detected mostly in non-treated patients and only 3% were reported in Japan albeit it is the country where NAI are largely used (Kramarz et al., 2009).

In a previous study, Rameix-Welti et al. (2008) suggested that there could be a link between the higher NA affinity of A/Brisbane/59/2007 related viruses and the emergence of the related OC-resistant strains.

The objective of our study was to better understand the role of the NA affinity in the emergence of these OC-resistant A(H1N1)

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Table 1
Global mean IC_{50} for oseltamivir and zanamivir for the A(H1N1) viruses isolated in 2005/2006, 2007/2008 and 2008/2009 winter seasons.

A(H1N1) from seasons	*N*	IC_{50} (nM)			
		OC[a]		Zana[b]	
		Mean ± SD	Max	Mean ± SD	Max
2005/2006 sensitive to OC	150	1.7 ± 0.7	4.6	1.2 ± 0.5	3.2
2007/2008 sensitive to OC	232	1.4 ± 0.7	4.3	1.5 ± 0.7	5.1
2007/2008 resistant to OC	142	564 ± 357	2730	1.4 ± 1.2	11.9
2008/2009 resistant to OC	16	649 ± 269	1411	1.5 ± 0.9	2.7

[a] OC, oseltamivir carboxylate.
[b] Zana, zanamivir.

viruses. We analysed isolates collected in the community or from hospitalized patients and reference strains. We examined the *in vitro* susceptibility to oseltamivir and zanamivir for all the viruses isolated in culture. The NA and HA genomic sequences were performed for some isolates for phylogenetic analysis. Ultimately neuraminidase affinity (Km) was performed for some OC-sensitive and resistant isolates and for the A(H1N1) vaccine reference strains from 1977 to 2007.

2. Material and methods

2.1. Cells and viruses

The influenza A(H1N1) viruses described in this study were collected during winter seasons from 2005 to 2009, in the community (National Influenza Centre of the South of France) and also came from hospitalized patients with an influenza syndrome. Viruses were isolated on 3-day old confluent monolayers Madin-Darby Canine Kidney (MDCK) cells. The A(H1N1) reference strains from 1977 studied are listed in Table 3. In 2005/2006, the A(H1N1) isolates were related to the A/New Caledonia/20/1999 and in 2007/2008 and 2008/2009 they were related to the A/Brisbane/59/2007 reference strain.

2.2. Compounds

Zanamivir and Oseltamivir carboxylate (abbreviated OC) (GS4071) were kindly provided by GlaxoSmithKline and Roche, respectively and were stored at −20 °C.

2.3. Fluorometric neuraminidase activity assay and IC_{50} determination for OC and zanamivir

A fluorescent inhibition assay was performed as described in a previous study by Ferraris et al. (2005). This assay was done in duplicate for each isolate. The IC_{50} is the antiviral agent concentration able to inhibit 50% of the neuraminidase activity.

2.4. Kinetic analyses of neuraminidase activities

The Michaelis–Menten constant (Km) which reflects the affinity of NA for the substrate was evaluated on viral suspensions using the MUNANA substrate (reference M8639, Sigma) as already described by Rameix-Welti et al. (2006). The substrate was used at concentrations: 5 μM, 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, 70 μM and 100 μM. The 4-methylumbelliferone fluorescence was measured every minute during 1 h with the FLUOstar OPTIMA fluorometer (BMG LABTECH) at 37 °C ($\lambda_{excitation}$ = 330 nm, $\lambda_{emission}$ = 450 nm). Vi (initial velocity) was calculated for each substrate concentration and integrated in a non-linear Michaelis and Menten equation by the MARS program (BMG) for Km calculation. Every isolate was tested at least in duplicate and each reference strain was tested in triplicate in different experiments each time. The results are means of the values obtained for each experiment. The Km was determined for a selection of isolates sensitive or resistant to OC from 2005 to 2009 (Table 2) and for the reference vaccine strains from 1977 to 2007 (Table 3).

2.5. Phylogenetic analysis for the NA and the HA1 genes for a selection of isolates found sensitive or resistant to OC

Sequences of primers were kindly given by the WHO Collaborating Centre, MRC National Institute of Medical Research in London. Amplifications were done with the Isis DNA Polymerase (MB Biologicals).

The sequencing was performed by the "Institut Pasteur, Genotyping of Pathogens and Public Health Platform" (Dr Valérie Caro) and by MWG/Eurofins.

The phylogenetic analysis of the N1 and HA1 sequences were performed on the alignment of sequences (numbering from ATG) from nucleotides 82 to 1330 (amino acids 28–443) for N1 and from nucleotides 76 to 1041 (amino acids 9–330 numbering after the signal peptide) for HA1. Comparison of sequences was performed with the BioEdit (7.1) program. The phylogenetic trees were constructed with Mega4 (4.0) by genetic distance matrix and calculated using the Kimura-2 parameters model with transition-to-transversion ratio of 2.0 and neighbor-joining analysis. Significant bootstrap values of 1000 replicas are given as percentages at the nodes.

The viruses selected for sequencing were a representative sampling of all the isolates and were from patients with various ages and various geographical origins in south of France.

2.6. Statistical analysis

Statistical tests were performed with the Epi info V3.5.1 (CDC) program. The significance of differences between mean values was evaluated by the *p*-value of Student's *t* tests or by the non-parametric Wilcoxon test. A *p*-value of <0.05 was regarded as statistically significant.

3. Results

3.1. Susceptibility of A(H1N1) isolates to NAI in NA inhibition assay (Table 1)

In 2005/2006 all 151 strains were sensitive to oseltamivir, but one, the A/Lyon/381/2006. In 2007/2008 among 374 A(H1N1) isolates, 38% were resistant to OC with a mean IC_{50} of 564 ± 357 nM. In the 2008/2009 season, most of the circulating influenza viruses were A(H3N2) related to A/Brisbane/10/2007(H3N2); we isolated 16 A(H1N1) viruses all related to A/Brisbane/59/2007(H1N1) and resistant to OC. All these A(H1N1) isolates remained sensitive to zanamivir.

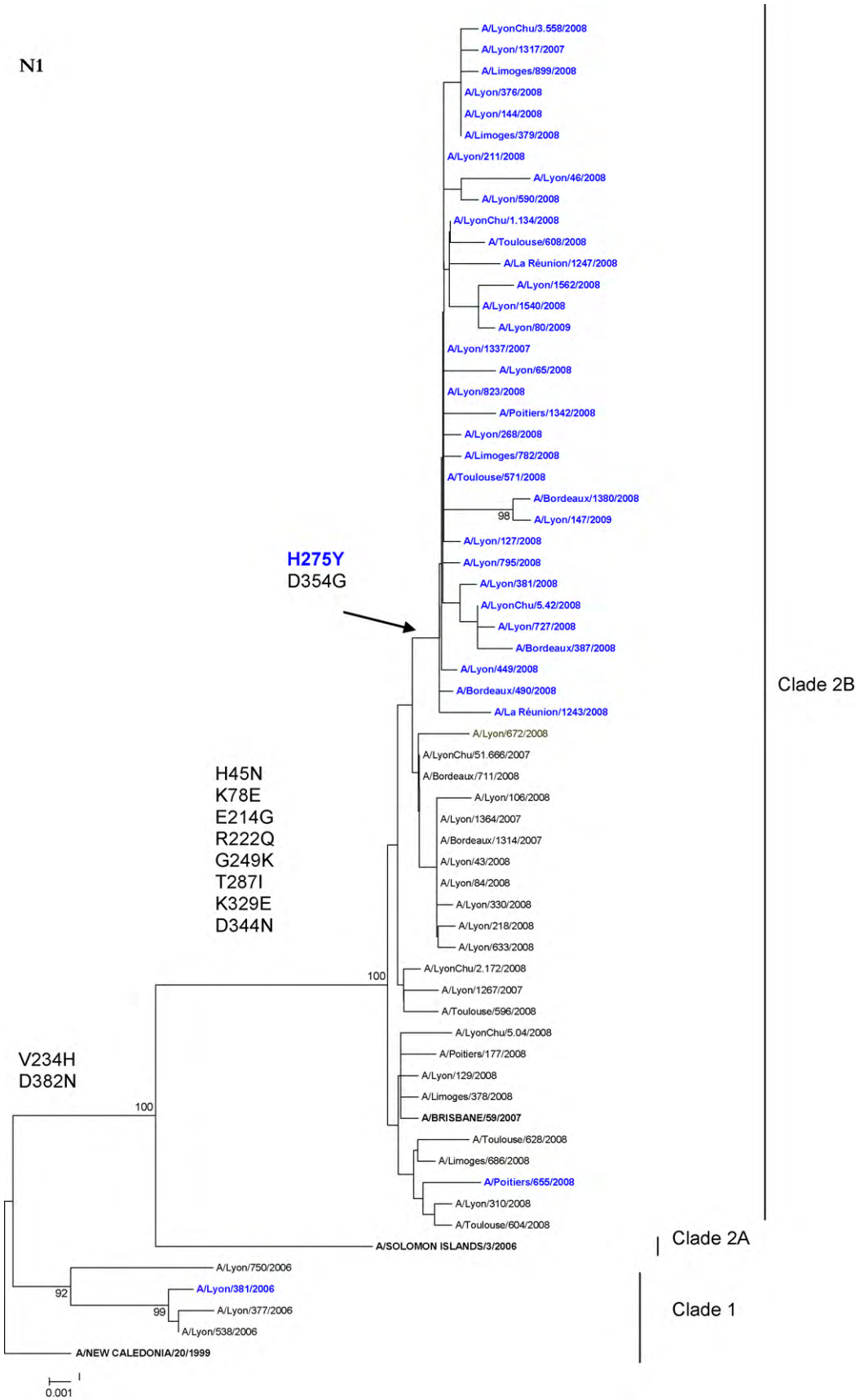

Fig. 1. Phylogenetic tree of the N1 and HA1 genes. The phylogenetic analysis was performed as explained in Section 2. Viruses mutated Y275 in N1 sequence are shown in bold and reference strains in capitals. The Genbank accession numbers for the reference strains are indicated for N1 (A/New Caledonia/20/1999 (CY033624), A/Solomon Islands/3/2006 (EU124136), A/Brisbane/59/2007 (CY030231)) and HA1 (A/New Caledonia/20/1999 (CY033622), A/Solomon Islands/3/2006 (EU124177) and A/Brisbane/59/2007 (CY030230)).

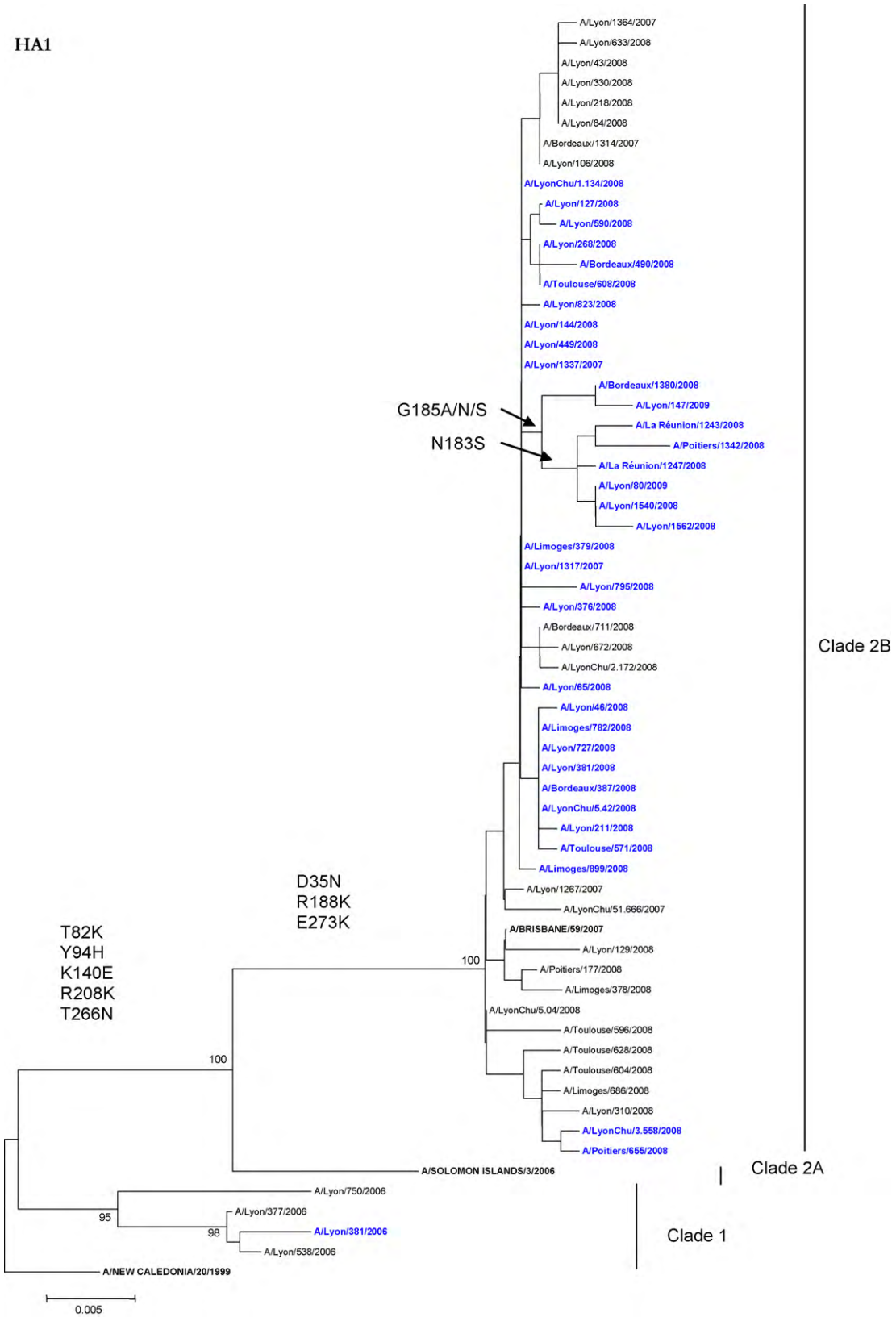

Fig. 1. (*Continued*).

3.2. Sequence and phylogenic analysis of viral N1 and HA1 proteins (Fig. 1)

The analysis of N1 and HA1 sequences showed key positions that permit to distinguish the A(H1N1) viruses related to A/Brisbane/59/2007 (clade 2B) and those related to A/New Caledonia/20/99 (clade 1) or A/Solomon Islands/3/2006 (clade 2A). For N1, these mutations are H45N, K78E, E214G, R222Q, G249K, T287I, K329E and D344N. For HA1, only three key mutations D35N, R188K, E273K

Table 2
Correlation between Km, IC_{50} for oseltamivir and zanamivir and the presence of different amino acid in N1, for a selection of A(H1N1) viruses isolated in 2005/2006, 2007/2008 and 2008/2009 winter season.

Clade	A(H1N1) from seasons	IC_{50} (nM)		Mean Km (μM)	NA amino acid position				
		OC[a]	Zana[b]		222	249	275	344	354
1	2005/2006								
	A/Lyon/381/2006	524.0	0.7	89.3	R	G	Y	D	G
	A/Lyon/465/2006	2.1	0.7	50.6	–[c]	–	–	–	–
	A/Lyon/538/2006	2.5	0.9	56.3	R	G	H	D	G
	A/Lyon/750/2006	1.6	0.6	45.1	R	G	H	D	G
	A/Clermont Ferrand/27/2006	1.4	0.8	41.8	–	–	–	–	–
	A/St Etienne/948/2006	1.1	0.9	41.3	–	–	–	–	–
	Mean for isolates from 2005/2006 sensitive to OC	1.7 ± 0.7		47.0 ± 6.4					
2B	2007/2008								
	A/Lyon/1267/2007	1.1	1.5	21.9	Q	K	H	N	D
	A/Lyon/1364/2007	0.7	0.6	21.2	Q	K	H	N	D
	A/Lyon/106/2008	2.3	1.6	25.5	Q	K	H	N	D
	A/Lyon/129/2008	0.7	1.9	21.9	Q	K	H	N	D
	A/LyonChu/5.04/2008	0.9	0.3	15.5	Q	K	H	N	D
	A/Poitiers/177/2008	3.0	3.0	29.6	Q	K	H	N	D
	A/Limoges/378/2008	2.3	0.5	20.4	Q	K	H	N	D
	Mean for isolates from 2007/2008 sensitive to OC	1.4 ± 0.7		22.6 ± 4.7					
	A/Lyon/1337/2007	759.2	1.2	42.2	Q	K	Y	N	G
	A/Lyon/144/2008	832.0	1.0	29.9	Q	K	Y	N	G
	A/Lyon/268/2008	521.8	0.3	30.8	Q	K	Y	N	G
	A/Lyon/376/2008	367.9	1.8	41.6	Q	K	Y	N	G
	A/Lyon/449/2008	539.6	2.1	30.9	Q	K	Y	N	G
	A/Lyon/795/2008	424.4	0.9	33.2	Q	K	Y	N	G
	A/Poitiers/655/2008	405.1	0.7	35.9	Q	K	Y	N	D
	Mean for isolates from 2007/2008 resistant to OC	564 ± 357		33.0 ± 7.0					
2B	2008/2009								
	A/Lyon/1540/2008	549.5	0.5	33.4	Q	K	Y	N	G
	A/Lyon/1562/2008	598.6	2.7	43.1	Q	K	Y	N	G
	A/Lyon/80/2009	586.9	0.8	32.4	Q	K	Y	N	G
	A/Lyon/147/2009	572.5	1.8	33.9	Q	K	Y	N	G
	A/Lyon/226/2009	724.8	0.9	52.5	Q	K	Y	N	G
	A/Poitiers/1342/2008	799.2	1.0	30.9	Q	K	Y	N	G
	A/Bordeaux/1380/2008	441.7	0.8	31.0	Q	K	Y	N	G
	A/Toulon/228/2009	1411.2	2.1	54.6	Q	K	Y	N	G
	A/Limoges/474/2009	630.1	0.6	38.5	–	–	–	–	–
	Mean for isolates from 2008/2009 resistant to OC	649 ± 269		38.9 ± 9.2					

[a] OC, oseltamivir carboxylate.
[b] Zana, zanamivir.
[c] –, not done.

are responsible for the evolution from the clade 2A to B (Fig. 1).

The phylogenetic tree of N1 sequences showed that the mutation in position 275 in N1 is the only constant difference between sensitive (H275) and resistant (Y275) strains. Most of the 2008 OC-resistant isolates belonged to the same sub-clade (top of the N1 phylogenetic tree) except the A/Poitiers/655/2008 located in a different sub-clade. There was a marked tendency for the presence of an Asp in 354 for sensitive strains and a Gly in 354 for resistant strains, except for the A/Poitiers/655/2008, resistant to OC,

Table 3
IC_{50} for oseltamivir and zanamivir, kinetic neuraminidase affinity and the presence of different amino acid in N1 for A(H1N1) reference vaccine strains from 1977.

Reference strains	IC_{50} (nM)		Mean Km (μM)	NA amino acid position				
	OC[a]	Zana[b]		222	249	275	344	354
A/USSR/90/1977	0.6	0.2	49.8	Q	G	H	D	G
A/Brazil/11/1978	0.7	0.2	52.7	Q	G	H	D	G
A/Chile/1/1983	0.3	0.7	39.8	R	G	H	D	G
A/Singapore/6/1986	2.0	0.8	46.2	R	G	H	D	G
A/Beijing/262/1995	1.0	0.5	45.4	R	G	H	D	G
A/Johannesburg/82/1996	1.0	0.4	37.4	R	G	H	D	G
A/New Caledonia/20/1999	0.6	0.5	44.7	R	G	H	D	G
A/Solomon Islands/3/2006	0.6	0.2	43.4	R	G	H	D	G
Mean for reference strains from 1977 to 2006			44.9 ± 5.0					
A/Brisbane/59/2007	0.3	0.7	21.6	Q	K	H	N	D

[a] OC, oseltamivir carboxylate.
[b] Zana, zanamivir.

which had an Asp in 354. However, all the reference strains except A/Brisbane/59/2007, sensitive to OC, had a Gly in position 354.

The phylogenetic tree of HA1 sequences showed that sensitive and resistant viruses belonged to the same clade and were related to A/Brisbane/59/2007. However, 2008 isolates resistant or sensitive to OC were not separated in two distinct groups like for the N1, but formed some little clusters. Notably, the isolates of the 2008/2009 season formed a particular cluster visible on the HA1 phylogenetic tree (Fig. 1). When analysing the HA1 sequences, we did not find any specific mutations that could distinguish sensitive or resistant strains to OC.

3.3. Kinetic analyses of neuraminidase activities for isolates and reference strains

The Km was determined for a selection of isolates sensitive or resistant to OC from 2005 to 2009 (Table 2). We selected a representative subset of the isolates based on the NA and HA phylogenetic tree. The Km was also determined for the reference vaccine strains from 1977 to 2007 (Table 3).

The mean Km for the A/New Caledonia/20/1999-like sensitive isolates (2005/2006) is $47.0 \pm 6.4\,\mu M$. This Km is similar to the mean Km found for the reference strains from 1977 to 2006 ($44.9 \pm 5.0\,\mu M$). On the contrary, the Km for the A/Brisbane/59/2007 ($21.6\,\mu M$) and for the 2007/2008 related isolates sensitive to OC ($22.6 \pm 4.7\,\mu M$) are lower than the Km of the previous strains ($p < 0.01$). The A/Lyon/381/2006 isolate resistant to OC and related to A/New Caledonia/20/1999 has a Km ($89.3\,\mu M$) higher than the Km of the related isolates sensitive to OC ($47.0 \pm 6.4\,\mu M$). Similarly but with lower absolute values, the mean Km of A/Brisbane/59/2007-like isolates sensitive to OC ($22.6 \pm 4.7\,\mu M$) is lower ($p < 0.01$) than the Km of the related isolates resistant to OC in 2007/2008 ($33.0 \pm 7.0\,\mu M$) and 2008/2009 ($38.9 \pm 9.2\,\mu M$).

In order to better understand the impact of different key mutations on the NA affinity, we have correlated the Km to the most important key positions for isolates and reference strains in Tables 2 and 3. The presence of an Asp or a Gly in position 354 does not seem to be related to the NA affinity as we did not find significant difference between the Km of A/Poitiers/655/2008 and the Km of the other clade 2B OC-resistant isolates. The residues 222, 249 and 344 are structurally close to the active site and could influence the NA affinity. However, the presence of a Gln in 222 does not seem to be responsible for the Km diminution as the reference strains A/USSR/90/1977 and A/Brazil/11/1978 have a Gln in 222 and have relatively high Km. With the isolates and reference strains studied here, we observed a constant correlation between a decreased Km and the presence of both a Lys in 249 and an Asn in 344 (Tables 2 and 3). These data could suggest that the G249K and the D344N substitutions could be responsible for the increase in NA affinity.

4. Discussion

Globally during the 2007/2008 winter, the prevalence of A(H1N1) viruses resistant to OC was around 38% in south of France. There was an increase in the prevalence as one goes along the weeks and the prevalence varied between countries in Europe with a mean of 25% (Meijer et al., 2009). All the viruses resistant to OC had a H275Y mutation in N1. The crystal structures of wild-type and mutant N1 from H5N1 viruses allowed a good visualisation of the molecular basis of resistance (Collins et al., 2008). As a consequence the H275Y mutation is able by itself to prevent the link of OC to the active site without disrupting the link of sialic acid and zanamivir. We did not find any other mutation neither in N1 nor in HA1 sequences that distinguished sensitive and resistant isolates to OC. There was a preferential association between the H275Y and D354G mutations as it was observed for other European clade 2B isolates (Meijer et al., 2009; Rameix-Welti et al., 2008). However, the amino acid residue at 354 was a Gly for the sensitive reference strains other than A/Brisbane/59/2007 and an Asp for one OC-resistant isolate (A/Poitiers/655/2008). This residue 354 is located on the external side of neuraminidase tetramer and would be too far from the active site to interfere with the active site linking (Rameix-Welti et al., 2008).

The Km values obtained with the reference A(H1N1) strains from 1977 to 2006 were similar between them and to the ones of viruses isolated in 2005/2006 season and tended to a mean of $45\,\mu M$ and $47\,\mu M$, respectively in our technical conditions (Tables 2 and 3). The OC-sensitive A(H1N1) variants related to A/Brisbane/59/2007 had a significantly lower Km than the previous strains, and the H275Y mutation responsible for the OC-resistance allowed to retrieve Km to higher values. These results and the Km values we obtained are in accordance with previous studies (Rameix-Welti et al., 2008; Collins et al., 2009). Our report enlarges these observations as it gives enzymatic characteristics for A(H1N1) reference strains from 1977. Regarding the position of the substitutions that differentiate clade 2B from clade 1 or 2A isolates, the residues 45 and 78 located in the stalk of the NA and residues 287, 329 located distant to the NA active site should not influence the NA affinity (Russell et al., 2006; Rameix-Welti et al., 2008; Collins et al., 2009). The residue 214 does not seem to influence the NA affinity as the Km value was similar whatever the presence of a Glu or a Gly in 214 in a previous study (Rameix-Welti et al., 2008). Other residues 222, 249 and 344 are close to the NA active site (Russell et al., 2006). The presence of a Gln in 222 does not induce Km decrease as some reference strains with a Gln residue in 222 have a relatively high Km. On the contrary, both the G249K and D344N substitutions were associated with a lower Km in our results and may have contributed to the NA increased affinity. The recent study of Collins et al. (2009) pointed the D344N substitution, located in the head of the molecule, to be responsible for this NA enhanced affinity. Indeed, some clade 2C and 2B isolates both bearing an Asn in 344 but differing only by the presence of a Gly or a Lys for clade 2C and B respectively, presented similar low Km values. These results suggest that the D344N mutation by itself is sufficient to induce an increased NA affinity (Collins et al., 2009). Km values are related to the NA affinity and this value should be correlated to the HA/NA balance and to the affinity of HA for the sialic acid substrate. It would be interesting to measure the HA affinity of the different strains studied here to better understand the HA/NA balance and its potential impact in viral fitness and in the emergence of isolates resistant to OC.

In MDCK cells, replicative capacities were similar for A/Brisbane/59/2007, some related OC-sensitive or resistant viruses, A/New Caledonia/20/1999 and an OC-sensitive related virus (data not shown). Previous studies found similar viral titers for sensitive or resistant viruses related to A/Brisbane/59/2007 (Rameix-Welti et al., 2008; Baz et al., 2010). However, *in vitro* replicative assays in MDCK cells cannot represent the global viral fitness of viruses and the emergence of OC-resistant viruses is a complex phenomenon difficult to limit to *in vitro* laboratories assays. Both OC-sensitive and resistant A/Brisbane/59/2007-like isolates were transmitted efficiently in the population but the OC-resistant ones were finally predominant and *in vivo* models are better appropriate to understand that phenomenon. Indeed, *in vivo* assays in ferrets showed higher nasal wash titers for OC-resistant than OC-sensitive A/Brisbane/59/2007-like variants (Baz et al., 2010).

Kinetic parameters of the neuraminidase could provide insights to explain the emergence of the 2007/2008 isolates resistant to OC.

However other factors had been studied: the genetic background could be linked to the better fitness of OC-resistant isolates. The mutated H275Y isolates were more likely than the OC-sensitive isolates to have the mutations A209T and R224G in NS1 (Eshaghi et al., 2009) and the substitution P453S in PB2 associated to the D354G mutation in N1, were found to be preferentially correlated with the H275Y mutation in N1 (Gerloff et al., 2009). However, no explanation was provided on how these mutations could have influenced viral fitness.

In conclusion, the combination of different amino acid mutations in N1 particularly the D344N could explain the higher NA affinity of A/Brisbane/59/2007 variants compared to the previous A(H1N1) strains and the H275Y mutation allowed to retrieve NA affinity near 40 μM. The emergence of the oseltamivir-resistant A(H1N1) variants argues with the fact that the monitoring of the susceptibility of influenza viruses to NAIs and the search for new antiviral strategies against influenza viruses must continue.

Conflict of interest

BL declares conflict of interest with Biocryst, Merck and Roche.

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Ethical approval

None required.

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