Highly heterogeneous temperature sensitivity of 2009 pandemic influenza A(H1N1) viral isolates, northern France

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We assayed the temperature sensitivity of 2009 pandemic influenza A(H1N1) viral isolates (n=23) and seasonal influenza A(H1N1) viruses (n=18) isolated in northern France in 2007/08 and 2008/09. All isolates replicated with a similar efficiency at 34 °C and 37 °C, and with a lower efficiency at 40 °C. The pandemic viral isolates showed a stronger heterogeneity in their ability to grow at the highest temperature, as compared with the seasonal isolates. No statistically significant difference in temperature sensitivity was observed between the pandemic viral isolates from severe and mild cases of influenza. Our data point to the impact of temperature sensitivity on the genetic evolution and diversification of the pandemic influenza A(H1N1) virus since its introduction into the human population in April 2009, and call for close surveillance of this phenotypic marker related to host and tissue tropism.

Introduction

A novel influenza A(H1N1) virus emerged in April 2009 [1-3] and rapidly spread all over the world. In France, the first cases were identified in early May 2009. The 2009 pandemic A(H1N1) virus presented a unique combination of genomic segments that had not been reported previously [4]. The segments coding for the neuraminidase (NA) and the matrix (M) proteins of the virus were related to the Eurasian lineage of swine influenza A(H1N1) viruses, whereas the six remaining gene segments were related to triple swine–human–avian influenza A(H1N1) reassortants that have been isolated from humans in contact with pigs in North America since 1998 [5,6]. Although the properties of isolates of the 2009 pandemic influenza A(H1N1) virus have already been largely examined in vitro and in vivo (for a review, see [7]), sensitivity to elevated temperature has not been characterised precisely. Temperature sensitivity is an important viral phenotypic marker, as it may be involved in host species restriction, tissue specificity and/or virulence [8-11]. In humans and pigs, influenza A viruses initially replicate in the upper respiratory tract at temperatures close to 33 °C and 37 °C, respectively, whereas in aquatic birds, influenza A viruses with low pathogenicity preferentially replicate in the intestinal tract at a temperature close to 40 °C [12-14]. The sensitivity of avian influenza A viruses to low temperature (33 °C) has been clearly demonstrated [15,16]. In contrast, no reduction in viral multiplication at 33 °C was observed for the swine viruses, and it has been proposed that temperature sensitivity might represent a specific, host-dependent signature of influenza A viruses [17]. Depending on the optimal temperature for viral multiplication, fever in infected patients may either limit or facilitate viral multiplication and consequently the administration of anti-pyretic drugs may or not be beneficial. Treatment of ferrets infected with influenza virus with sodium salicylate (an anti-pyretic) resulted in increased viral loads in nasal washes [18].

In order to characterise and compare the temperature sensitivity of both pandemic influenza A(H1N1) viral isolates and seasonal viruses isolated in northern France in 2007/08 and 2008/09 before the emergence of the pandemic virus, we developed a test to compare viral multiplication at 34 °C, 37 °C and 40 °C.

Methods

Virus samples and reference isolates

We included 23 isolates of 2009 pandemic influenza A(H1N1) virus and 18 seasonal influenza A(H1N1) viral isolates in our study. The pandemic isolates were collected in northern France between weeks 39 and 51 (24 September to 16 December) in 2009; pandemic activity in this area started at week 42 in 2009, peaked at week 49 and ended at week 2 in 2010 [19]. The isolates for the 2007/08 season were collected in northern France between week 44 (29 October) in 2007 and week 3 (14

January) in 2008 and those for the 2008/09 season between week 45 (3 November) in 2008 and week 4 (19 January) in 2009.

One of the 2007/08 seasonal influenza A(H1N1) viral isolates, (A/Paris/1149/2008), was included in most experiments (12/13, due to a technical problem in one) as a control to assess the reproducibility of our experimental conditions. A further 12 seasonal influenza A(H1N1) viral isolates from 2007/08, either susceptible or resistant to oseltamivir, and five seasonal influenza A(H1N1) viral isolates from 2008/09, all resistant to oseltamivir, were also included. These seasonal viral isolates were chosen at the beginning and peak of the influenza seasons in northern France, as for the pandemic isolates, and, for the 2007/08 seasonal isolates, we also took into account the co-circulation of viruses sensitive or naturally resistant to oseltamivir.

Among the 23 pandemic influenza A(H1N1) viral isolates included in our study, we defined two distinct groups of viruses according to the disease severity of the patients (Table1). Information about the existence of underlying conditions prone to increase disease severity was noted when available (Table 1). Severe influenza cases were those who were hospitalised in an intensive care unit or died as a result of their infection. Patients with mild disease were matched as much as possible by the week and geographical area of collection.

Two representative isolates from the human North American triple reassortant influenza A(H1N1) viruses (A/Illinois/09/2007 and A/Ohio/02/2007) and from the swine Eurasian influenza A(H1N1) and Hong Kong triple reassortant internal gene (TRIG) influenza A(H1N2) lineages (A/Swine/Cotes d'Armor/0231/2006 and A/

Table 1

Origin and characteristics of 2009 pandemic influenza A(H1N1) viral isolates from mild and severe influenza cases, northern France, 24 September–16 December 2009 (weeks 39–51) (n=23)

Viral isolate[a]	Sample type	Week of sampling	Type of patient[b]	Age of patient (years)	Disease severity	Additional information	Haemagglutinin residue 222[c]	Neuraminidase residue 275[c]
20097639	Nasal and pharyngeal	51	Outpatient	40	Mild	NA	E	H
20097214	Nasal and pharyngeal	49	Outpatient	47	Mild	NA	D	Y
20096074	Nasal and pharyngeal	45	Outpatient	16	Mild	NA	D	H
20095771	Nasal and pharyngeal	44	Outpatient	24	Mild	NA	D	H
20095509	Nasal and pharyngeal	42	Outpatient	45	Mild	NA	D	H
20095501	Nasal and pharyngeal	43	Outpatient	8	Mild	NA	D	H
20095383	Nasal and pharyngeal	42	Outpatient	29	Mild	NA	D	H
20095016	Nasal and pharyngeal	41	Outpatient	14	Mild	NA	D	H
20097391	Nasal and pharyngeal	49	Inpatient	44	Severe	Deceased	D	H
20097367	Nasal and pharyngeal	48	Inpatient	26	Severe	NA	D	H
20097155	Nasal and pharyngeal	48	Inpatient	2.5	Severe	NA	D	H
20097097[d]	Lung	49	Inpatient	6	Severe	Deceased	D	H
20097101[d]	Brain	49	Inpatient	6	Severe	Deceased	G[e]	H
20096934	Nasal and pharyngeal	47	Inpatient	63	Severe	Haemopathy	D	H
20095911	Nasal and pharyngeal	43	Inpatient	10	Severe	Chronic respiratory insufficiency	D	H
20096365	Nasal	45	Inpatient	55	Severe	Chronic obstructive bronchopneumopathy	E	H
20094517	Nasal and pharyngeal	39	Inpatient	20	Severe	NA	D	H
20094518	Nasal and pharyngeal	39	Inpatient	45	Severe	NA	E	H
20094785	Nasal and pharyngeal	40	Inpatient	29	Severe	NA	E	H
20096928	Nasal and pharyngeal	45	Inpatient	22	Severe	Acute respiratory distress syndrome	D	H
20097105	Lung	48	Inpatient	46	Severe	Deceased	G	H
20097208	Nasal and pharyngeal	48	Inpatient	51	Severe	Deceased	D	H
20097388	Nasal and pharyngeal	49	Outpatient	19	Severe	Deceased (at home)	D	H

NA: not available.

[a] 2009XXXX stands for A/Paris/XXXX/2009.

[b] Samples from outpatients are from the Groupes Régionaux d'Observation de la Grippe (GROG), the national network of sentinel general practitioners and paediatricians and from the Réseau National des Laboratoires (RENAL), a network of hospital laboratories.

[c] Sequence information refers to the viruses isolated after one passage in Madin-Darby canine kidney (MDCK) cells.

[d] Viruses 20097097 and 20097101 were isolated from the lung and brain, respectively, of the same patient.

[e] The sequence of the virus present in the original specimen was also determined, and a D was found at residue 222 of the haemagglutinin.

Swine/Hong Kong/1578/2003) [20], respectively, were tested in parallel.

Preparation and analysis of viral isolates

In order to produce suitable viral stocks, all isolates were amplified by two serial passages at a multiplicity of infection of 10^{-3} plaque forming units per cell at 35 °C in MDCK cells in serum-free minimal essential medium (MEM) containing 1µg/ml trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK). We assumed that the pandemic viral isolates would be able to grow efficiently at 35 °C (as the temperature of the human upper respiratory tract is about 33 °C) and we therefore chose to amplify the virus at 35 °C rather than 37°C in order to avoid the preselection of variants that grow preferentially at high temperature. Viral stocks were clarified and aliquots for single use were kept frozen at –80 °C.

Viral RNA was prepared using the QIAamp Viral RNA Mini Kit (Qiagen). Reverse transcription PCR was carried out using the SuperScript One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen) and oligonucleotides specific for the haemagglutinin (HA) and NA segments. The amplicons were sequenced using a Big Dye terminator sequencing kit and an automated sequencer (Applied Biosystems). In some cases, pyrosequencing was used to determine specifically the sequence at residue 222 of the HA or at residue 275 of the NA. For the H275Y mutation, the primers GRswN1-780Fw/090206 (5'-GGGGAAGATTGTYAAATCAGTYGA-3') and GRswN1-1273Rv/090207 (5'-biotin-CWACCCAGAAR-CAAGGYCTTATG-3') were used for amplification, and GRswN1-804Fw/090208 (5'-GYTGAATGCMCCTAATT-3') for sequencing, as previously described [21]. For the D222G mutation, the primers GRswH1-672Fw (5'-CAAGAAGTTCAAGCCGGAAATAGC-3') and GRswH1-821 Rv (5'-biotin-ATTGCGAATGCATATCTCGGTAC-3') were used for amplification, and GRswH1-693Fw (5'-AGCAATAAGACCCAAAG-3') for sequencing. Primers were designed using the Pyrosequencing Assay Design Software (Biotage). Pyrosequencing reactions were performed on purified biotinylated amplicons as previously described [22].

Temperature-sensitivity assays

Confluent three-day-old cultures of MDCK cells in 96-well plates, prepared in MEM containing 5% foetal calf serum and 50 µg/ml gentamycin, were washed twice with serum-free MEM before infection. Serum-free MEM (170 µl/well) containing trypsin-TPCK (1µg/ml) and gentamycin (50 µg/ml) were added to cultures. Ten-fold dilutions of each virus sample in MEM (30 µl/well, 10 wells/dilution, 3 plates/virus sample) were added to cells. Plates were sealed with an adhesive membrane and covered with lids and incubated at 34 °C, 37 °C and 40 °C. Incubators were used for incubation at 34 °C and 37 °C, whereas a water bath was used to incubate plates at exactly 40 °C.

Cytopathic effects were observed under the microscope three days after infection and virus titres as 50% tissue culture infectious doses ($TCID_{50}$) per mL were determined as previously described by Reed and Muench [23]. The reproductive capacity at the high, potentially restrictive temperature of 40 °C (RCT_{40} value) is the difference, in log values, between the titres at 40 °C and at 37 °C for each viral isolate. Similarly, the reproductive capacity at 34 °C (RCT_{34} value) is the difference in log values between the viral titres at 34 °C and at 37 °C. Both RCT values are expressed as the mean ± standard deviation (SD) from at least three independent experiments.

Results

For all isolates tested, viral titres were similar at 34°C and 37°C; the RCT_{34} values varied between –0.63±0.53 and +0.50±0.16 (Figure). In contrast, significant differences were observed between isolates grown at 40 °C, since the RCT_{40} values varied between 0.00±0.16 and –4.23±0.42. The RCT_{40} value of the pool of 2007/08 and 2008/09 seasonal viruses varied between –2.40±0.29 and –3.97±0.12, indicating that the titres of these viruses were about 250- to 9,300-fold lower at 40 °C than at 37 °C. The pandemic viruses showed RCT_{40} values ranging from –1.30±0.29 to –4.23±0.42, indicating that their titres were about 20- to 17,000-fold lower at 40 °C than at 37 °C.

On average, pandemic viral isolates were about three-fold less sensitive at 40 °C than the pool of the 2007/08 and 2008/09 seasonal viruses (RCT_{40} values of –2.55±0.82 and –3.06±0.46, respectively; $p<0.05$, Student's *t*-test) and showed a significantly higher variability in temperature sensitivity (variance ratio: 3.18; $p<0.025$, Fisher's exact test). No statistically significant differences were seen in RCT_{40} values regardless of whether the pandemic viral isolates had been isolated from severe cases with or without underlying condition (n=15) or from mild cases (n=8) (Table 2).

Interestingly, two human isolates representative of the North American triple reassortant influenza A(H1N1) viruses (A/Illinois/09/2007 and A/Ohio/02/2007) grew similarly at 40 °C and 37 °C (Table 2). Their growth was thus clearly more resistant to high temperature than that of the pandemic viral isolates. The Hong Kong TRIG swine influenza A(H1N2) and Eurasian swine influenza A(H1N1) viruses included in our study showed an intermediate phenotype between the triple reassortant and pandemic viruses (Table 2 and Figure).

A D222G substitution in the receptor binding site of HA was seen in two of the viral isolates included in our study (isolates 20097101 and 20097105). This substitution has been detected sporadically, with some degree of correlation between the presence of the substitution and the severity of the disease [24-27]. Isolates 20097101 and 20097105 showed RCT_{40} values of –2.25±0.57 and –1.33±0.12, respectively (data not shown). The 20097101 virus was isolated from the

brain of a young patient who died after infection and showed a G residue at position 222 of the HA (Table 1). The viruses detected in the initial brain specimen showed a D at this position, but probably contained a low, undetectable fraction of viruses of the HA-222G genotype upon amplification in MDCK cells.

The 20097097 virus isolated from the lung of the same patient showed a D residue at position 222 of the HA (Table 1). No statistically significant difference in temperature sensitivity was observed between the 20097101 and 20097097 isolates.

One of the pandemic viral isolates (20097214) included in our study had the H275Y substitution in the NA (Table 1) that is associated with oseltamivir resistance [28,29] and was characterised by a marked sensitivity to high temperature, with an RCT_{40} value of –3.90±0.57. However, the two panels of oseltamivir-resistant and -sensitive seasonal isolates from 2007/08 showed no statistically significant difference in temperature sensitivity (Table 2 and Figure). Overall, our results suggest that neither the D222G substitution in the HA nor the H275Y substitution in the NA have a major impact on the viral sensitivity to high temperature.

The NA and M gene sequences of the 23 pandemic viral isolates included in our study were determined: the Global Initiative on Sharing Avian Influenza Data (GISAID) accession numbers are shown in Table 3. The NA and M1 amino acid sequences of the 23 pandemic viral isolates included in our study were were aligned with the corresponding sequences of the swine and triple reassortant viruses. The pandemic virus-derived sequences showed very few variations: their NA and M1 sequence shared about 91% and 94% identity with the respective Eurasian swine virus-derived sequences and 81% and 88% identity with the respective triple reassortant virus-derived sequences.

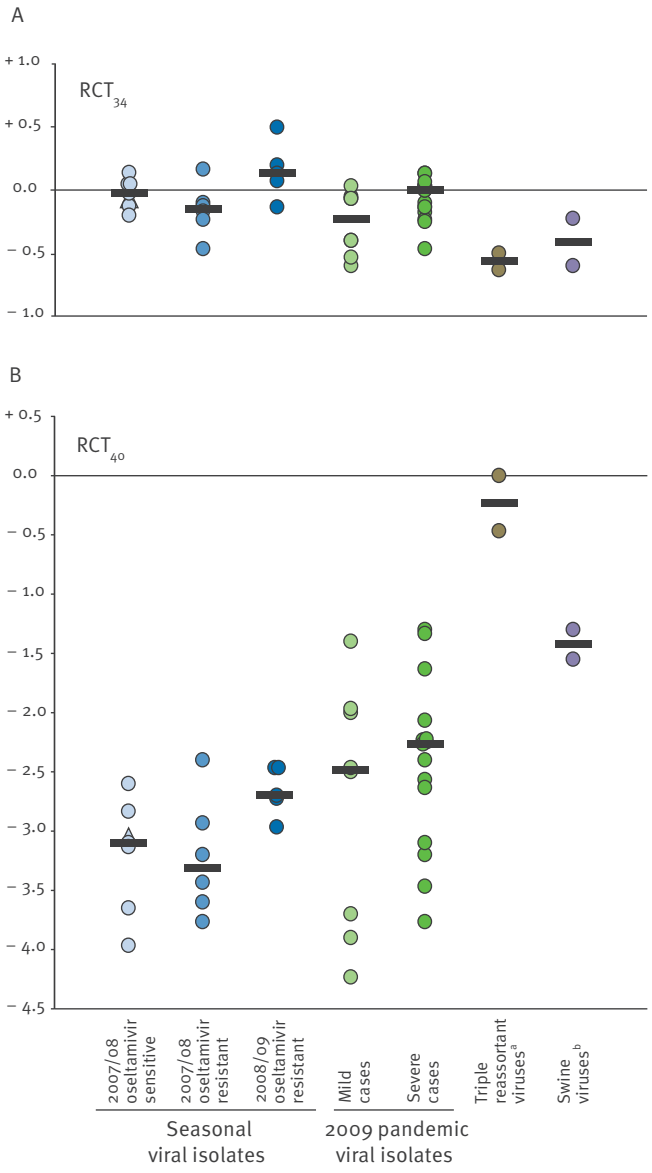
Discussion and conclusion

A panel of seasonal and pandemic influenza A(H1N1) viral isolates from northern France in 2007/08 to 2008/09 grew with similar efficiency at 34 °C and 37 °C, suggesting that these viruses are well adapted to the physiological temperatures of the upper and lower respiratory tract. In contrast, they replicated less efficiently at 40 °C than at 37 °C. As compared with seasonal isolates, the pandemic viral isolates showed a marked heterogeneity in temperature sensitivity as indicated by a significantly higher variability in the corresponding RCT_{40} values. This heterogeneity probably reflects ongoing evolution and genetic diversification of the virus since its introduction in the human population in April 2009.

The sensitivity to high temperature of isolates of the pandemic virus from severe cases of influenza was not statistically significantly different from that of isolates from mild cases, but the numbers were small. These results suggest that there was little or no correlation between temperature sensitivity of pandemic viruses and clinical severity. However, this finding should be confirmed by analysing a larger panel of viruses, given

Figure

Reproductive capacity of 2009 pandemic influenza A(H1N1) viral isolates (n=23) and 2007/08 and 2008/09 seasonal influenza A(H1N1) viral isolates (n=18) at 34 °C and 40 °C, relative to 37 °C, northern France



RCT: reproductive capacity at a given temperature.

RCT34 (panel A) and RCT40 (panel B) values are shown for 2008 and 2009 seasonal influenza A(H1N1) viral isolates (blue symbols) and 2009 pandemic influenza A(H1N1) viral isolates (green symbols). Two North American influenza A(H1N1) triple reassortant viruses (brown symbols) and two swine influenza viruses (purple symbols) were included for comparison. The 2008 seasonal influenza A(H1N1) viral isolate used as reference in most experiments is indicated with a triangle, whereas other influenza strains are indicated with circles. The line at 0.0 separates viral isolates that replicate more efficiently at 34 °C or 40 °C than at 37 °C (RCT values >0) from those that replicate less efficiently at 34 °C or 40 °C than at 37 °C (RCT values <0). Median values are indicated by horizontal black bars.

[a] Swine–human–avian triple reassortant influenza A(H1N1) viruses isolated from humans.

[b] A Eurasian swine influenza A(H1N1) virus and a Hong Kong triple reassortant internal gene (TRIG) swine influenza A(H1N2) virus.

Table 2

Reproductive capacity of 2009 pandemic influenza A(H1N1) viral isolates (n=23) and 2007/08 and 2008/09 seasonal influenza A(H1N1) viral isolates (n=18) at 34 °C and 40 °C, northern France

Type of viral isolate	Viral isolates[a]	Mean RCT_{34} ± SD	Mean RCT_{40} ± SD
2007/08 seasonal isolates, oseltamivir sensitive n=7	20081149	−0.03±0.11	−3.19±0.43
	20081207		
	20081129		
	20080730		
	20080658		
	20080552		
	20080286		
2007/08 seasonal isolates, oseltamivir resistant n=6	20081093	−0.15±0.19	−3.22±0.46
	20081019		
	20080749		
	20080577		
	20081170		
	20081166		
2008/09 seasonal isolates, oseltamivir resistant n=5	20090244	+0.16±0.21	−2.67±0.19
	20091401		
	20091349		
	20090639		
	20090445		
2009 pandemic isolates from mild cases n=8	20097639	−0.26±0.23	−2.77±0.97
	20097214		
	20096074		
	20095771		
	20095509		
	20095501		
	20095383		
	20095016		
2009 pandemic isolates from severe cases among inpatients and/or deceased patients n=15	20097391	−0.07±0.16	−2.43±0.70
	20097367		
	20097155		
	20097097		
	20097101		
	20096934		
	20095911		
	20096365		
	20094517		
	20094518		
	20094785		
	20096928		
	20097105		
	20097208		
	20097388		
Swine–human–avian triple reassortant influenza A(H1N1) viruses isolated from humans	A/Illinois/09/2007	−0.57±0.07	−0.23±0.23
	A/Ohio/02/2007		
Swine viruses: a Eurasian swine influenza A(H1N1) virus and a Hong Kong TRIG swine influenza A(H1N2) virus	A/Swine/Cotes d'Armor/0231/2006	−0.41±0.19	−1.43±0.13
	A/Swine/Hong Kong /1578/2003		

RCT: reproductive capacity at a given temperature; SD: standard deviation.

[a] 2009XXXX stands for A/Paris/XXXX/2009.

the strong heterogeneity in temperature sensitivity, the possible bias due to the fact that the severity of the disease in up to 25% of severe cases during the pandemic was due to bacterial secondary infections rather than the characteristics of the pandemic virus [30,31] and the fact that host factors, such as underlying conditions identified as risk factors, seem to have contributed substantially to the clinical course of severe cases with 2009 pandemic influenza A(H1N1) [32,33].

The Eurasian swine influenza A(H1N1) virus, a Hong Kong TRIG swine influenza A(H1N2) virus and two A(H1N1) triple reassortant viruses included in our study showed a lower sensitivity to elevated temperature (40 °C) than the pandemic and seasonal viral isolates on average, in agreement with the fact that the normal body temperature of pigs varies between 38.5 °C and 39.2 °C [34]. All the pandemic viral isolates included in our study replicated less efficiently at 40 °C than did the triple reassortant viruses although their genomic segments, except for the NA and matrix (M) segments, are phylogenetically related to the triple reassortants.

No specific sequence signature was observed for the viruses that showed the highest RCT_{40} (data not shown). Overall, our observations suggest that the sensitivity to high temperature of the pandemic viral isolates is determined by complex gene constellation and/or mutation effects.

In conclusion, our small dataset shows that the pandemic viruses that circulated in northern France in 2009 were more heterogeneous with respect to their ability to grow at high temperature (40 °C) than the seasonal viruses that circulated there in 2007/08 and 2008/09. They point to the impact of viral temperature sensitivity on the genetic evolution and diversification of the pandemic virus during the first year after its introduction into the human population and they call for a close monitoring of this phenotypic marker related to host and tissue tropism during the coming years.

Table 3

GISAID accession numbers of 2009 pandemic influenza A(H1N1) viral isolates, northern France (n=23)

Viral isolate[a]	NA sequence	M1 sequence
20094517	EPI320178	EPI320177
20094518	EPI320180	EPI320179
20094785	EPI320181	EPI320182
20095016	EPI320184	EPI320183
20095383	EPI320186	EPI320185
20095501	EPI320188	EPI320187
20095509	EPI320190	EPI320189
20095771	EPI320192	EPI320191
20095911	EPI320194	EPI320193
20096074	EPI320196	EPI320195
20096365	EPI320198	EPI320197
20096928	EPI320200	EPI320199
20096934	EPI320202	EPI320201
20097097	EPI320204	EPI320203
20097101	EPI320206	EPI320205
20097105	EPI320208	EPI320207
20097155	EPI320210	EPI320209
20097208	EPI320212	EPI320211
20097214	EPI320214	EPI320213
20097367	EPI320216	EPI320215
20097388	EPI320218	EPI320217
20097391	EPI320220	EPI320219
20097639	EPI320222	EPI320221

GISAID: Global Initiative on Sharing Avian Influenza Data; M: matrix; NA: neuraminidase.

[a] 2009XXXX stands for A/Paris/XXXX/2009.

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