Antiviral Research 83 (2009) 290–297

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Dynamics of antiviral-resistant influenza viruses in the Netherlands, 2005–2008

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ARTICLE INFO

Article history:
Received 18 January 2009
Received in revised form 15 June 2009
Accepted 1 July 2009

Keywords:
Influenza
Surveillance
Antiviral resistance
Oseltamivir
Zanamivir
Amantadine

ABSTRACT

In the Netherlands, influenza specific antivirals are used for the therapy of influenza in nursing homes and hospitals, for prophylaxis in high risk groups and neuraminidase inhibitors are stockpiled as part of pandemic preparedness plans. To monitor the antiviral susceptibility profile, human influenza virus isolates derived from the Dutch influenza surveillance in 2005–2006 ($n = 87$), 2006–2007 ($n = 58$) and 2007–2008 ($n = 128$) were analyzed with phenotypic assays and sequencing. For adamantanes, a high proportion (>74%) of A(H3N2) viruses had the S31N mutation in M2 protein, while variation in the HA_1 region of adamantane-sensitive viruses suggested that adamantane-sensitive variants were reseeded into the Dutch population and re-emerged as drug-sensitive due to M-segment reassortment. For neuraminidase inhibitors oseltamivir and zanamivir, 98% of types A and B influenza viruses prior to 2007–2008 were sensitive for both, whereas 24% of the A(H1N1) viruses obtained in 2007–2008 were oseltamivir-resistant while retaining sensitivity to zanamivir and adamantanes. Furthermore, oseltamivir-resistant A(H1N1) or adamantane-resistant A(H3N2) virus infections were not associated with differences in clinical symptoms compared to infections with sensitive variants. Our data show the dynamic nature of emergence of drug-resistant influenza viruses, stressing the need for surveillance of resistance trends as part of influenza monitoring programs.

1. Introduction

Influenza A viruses of subtypes H1N1, H3N2 and influenza B virus are responsible for annual epidemics in different regions of the world. Between 7 and 20% of the human population is affected when the annual epidemic rapidly spreads around the world. Most infected individuals recover within 1–2 weeks without medical treatment, but infection may also lead to severe morbidity and mortality, especially in elderly people and very young children (Molinari et al., 2007). In addition to the burden of seasonal influenza virus infections, the introduction of a new influenza A virus subtype in the human population combined with lack of pre-existing immunity against this subtype could result in a pandemic.

The primary means to protect against influenza virus infection is by vaccination. However, due to the current 6-month vaccine production process it is impossible to respond timely to sudden antigenic drift variants or the introduction of a new pandemic virus in the human population (Gerdil, 2003). Therefore, antivirals offer a valuable addition to the use of vaccines. Current influenza antivirals are divided into two classes, targeting either the matrix (M2) ion-channel protein or the neuraminidase (NA) enzyme of the influenza virus. The adamantane derivatives amantadine and rimantadine, both M2 inhibitors, bind and irreversibly block the function of the viral M2 ion-channel protein. The conserved nature of the active site of influenza virus NA enzyme led to the development of two effective neuraminidase inhibitor (NAI) antivirals: oral oseltamivir and inhaled zanamivir.

Although adamantanes have been used for decades with <1% adamantane-resistant influenza virus isolates reported until the year 2003, worldwide influenza A(H3N2) viruses isolated since the year 2005 are characterized by a dramatic rise in the frequency of the emergence of adamantane-resistant viruses carrying S31N amino acid substitution in M2 (Ziegler et al., 1999; Bright et al., 2005). In contrast to adamantanes, influenza viruses were thought to be less prone to develop resistance to NAI because of structural constraints of the enzyme active site (Yen et al., 2006; Aoki et al., 2007). During clinical trials of oseltamivir, influenza viruses with reduced sensitivity to oseltamivir were isolated rarely (1.8%) at the end of the 5-day treatment period, while during adamantane therapy resistance emerged in about 30% of patients after 2–3 days of the treatment period (Hayden and Hay, 1992). Until 2008,

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doi:10.1016/j.antiviral.2009.07.003

NAI-resistant circulating influenza viruses were detected with only very low frequency in Japan, where the use of NAIs is high (Monto et al., 2006; Tamura et al., 2009). NAI-resistant influenza variants showed different degrees of reduced viral fitness in animal models, depending on virus variants studied, resulting in poorer transmission and reduced virulence of NAI-resistant compared to wild type viruses (Ives et al., 2002; Herlocher et al., 2002, 2004; Yen et al., 2005). The need to understand trends in susceptibility of circulating influenza viruses to existing specific antiviral agents is becoming a crucial aim of influenza surveillance. Therefore, we decided to pilot the feasibility of adding antiviral susceptibility in our routine influenza surveillance program. Consequently, the program was running when new oseltamivir-resistant A(H1N1) variants emerged in 2007–2008.

This study reports the extent and characteristics of influenza virus isolates with reduced antiviral susceptibility, detected in physician-based sentinel surveillance of influenza-like illness (ILI) and acute respiratory infection (ARI) during the seasons 2005–2006, 2006–2007 and 2007–2008. Additionally, we discuss clinical challenges of emerging antiviral-resistant influenza strains.

2. Materials and methods

2.1. Clinical specimens

Respiratory secretions (nose and/or throat swabs) collected from patients with ILI or ARI were obtained through the Netherlands Institute for Health Services Research (NIVEL) sentinel surveillance network of general practitioners (GPs), covering 1% of the Dutch population (Donker, 2007). Respiratory secretions from a total of 382, 403 and 606 patients collected during the influenza season 2005–2006, 2006–2007 and 2007–2008, respectively, were analyzed. A minimal dataset was collected for each patient, consisting of date of birth, sex, first day of illness, symptoms, consultation date, the consulted GP, antiviral use, vaccination status and travel history.

2.2. Influenza antiviral drugs

Oseltamivir carboxylate Ro64-0802 (GS4071) and zanamivir (GG167) were kindly provided by Roche Diagnostics (Germany) and GlaxoSmithKline (Netherlands), respectively. Amantadine hydrochloride was purchased from Sigma–Aldrich (United Kingdom).

2.3. Virus isolates

Influenza virus isolates were derived from respiratory secretions by culturing on tertiary cynomolgus monkey kidney cells [supplied by het Nederlands Vaccin Instituut (NVI), Bilthoven, The Netherlands] and Madin-Darby canine kidney-I cells (kindly provided by Dr. G. van Meer, Utrecht University, The Netherlands) and were (sub)typed using the hemagglutinin inhibition assay with turkey erythrocytes and real-time reverse-transcriptase polymerase chain reaction according to published protocols (van Gageldonk-Lafeber et al., 2007; Fouchier et al., 2000).

2.4. Adamantane susceptibility

The adamantane (amantadine and rimantadine) susceptibility of influenza A virus isolates was determined by sequencing the M2 gene on an automated sequencer. After RNA isolation, RNA was transcribed into complementary DNA by using ThermoScript™ reverse transcriptase (Invitrogen, France) and an influenza A virus specific primer (uni12). Complementary DNA was amplified using Phusion™ high-fidelity TAq polymerase (Finnzymes, Finland) and

Table 1
Influenza viruses isolated during the 2005–2006, 2006–2007 and 2007–2008 epidemic and their antiviral susceptibility profile.

Influenza season	2005–2006				2006–2007				2007–2008				Total			
Respiratory secretions tested	382				403				606				1391			
	Isolates	Adamantane-resistant	Oseltamivir-resistant	Zanamivir-resistant	Isolates	Adamantane-resistant	Oseltamivir-resistant	Zanamivir-resistant	Isolates	Adamantane-resistant	Oseltamivir-resistant	Zanamivir-resistant	Isolates	Adamantane-resistant	Oseltamivir-resistant	Zanamivir-resistant
Influenza A(H3N2)	39	29	1[a]	0	50	37	0	0	10	10	0	0	99	76	1[a]	0
Influenza A(H1N1)	0	–	–	–	5	0	0	0	38	0	9[b]	0	43	0	9[b]	0
Influenza B	48	NA	2[a]	2[a]	3	NA	0	0	80	NA	1[a]	0	131	NA	3[a]	2[a]
Total	87				58				128				273			

NA, not applicable.
[a] Extreme outliers for NAI susceptibility detected by box-and-whisker plot analysis.
[b] Oseltamivir-resistant viruses as a result of amino acid substitution H274Y in NA.

M gene specific primers (primers available on request). Sequences were analyzed for substitutions known to confer resistance to adamantanes (Belshe et al., 1988; Hay et al., 1986). From a subset of viruses the sequence-based adamantane susceptibility data were confirmed by determination of the amantadine concentration needed to inhibit virus replication by 50% (IC_{50}). To this end, the inhibitory effect of amantadine on virus replication in cell culture was determined by quantifying the synthesis of influenza nucleoprotein using Enzyme Linked Immuno Sorbent Assay on the ethanol fixed cell monolayer 18 h post-infection, as described previously (Meijer et al., 2004). The hemagglutinin (HA_1) region of the influenza A virus HA gene was sequenced to study possible evolutionary relationships between adamantane-resistant isolates (primers available on request). DNA sequences were assembled, edited, translated and clustered using the Bionumerics V5.10 software package (Applied Maths, Belgium).

2.5. NA inhibitor susceptibility

The NAI (oseltamivir and zanamivir) susceptibility of influenza virus isolates was determined using a fluorescence based NA inhibition assay with 100 μM 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate substrate (Sigma–Aldrich, United Kingdom) and expressed as the concentration of NAI needed to inhibit the NA enzyme activity by 50% (IC_{50}), as described previously (Potier et al., 1979).

2.6. Outlier identification and baseline NAI susceptibility

Box-and-whisker plot analysis was used to identify potential outliers for NAI susceptibility (Massart et al., 2005). IC_{50} values between 1.5 and 3 times the interquartile range (IQR), or more than 3 times the IQR outside the IQR were defined as mild or extreme outliers, respectively. After elimination of outliers, the remaining IC_{50} values were used to calculate the mean and standard deviation (SD) of baseline NAI susceptibility of Dutch influenza viruses. All IC_{50} values were log-transformed prior to analysis and back-transformed afterwards. Outliers for NAI susceptibility were subjected to NA segment gene analysis as described above, and compared to baseline NA sequences to find the origin of the reduced NAI susceptibility. Corresponding amino acid mutations in NA are specified according to N2 numbering (Varghese and Colman, 1991).

2.7. Statistics

Analysis of variance between sets of IC_{50} values was performed by using Single Factor ANOVA. Differences in symptoms of patients infected with antiviral-sensitive or -resistant influenza viruses are expressed by two-tailed Fisher's exact test of significance. Additionally, differences between antiviral-resistant and -sensitive influenza virus infections with respect to the age of the patient and duration of symptoms prior to consultation were analyzed using univariate logistic regression models with data from patients with antiviral-sensitive viruses being the reference category. The latter was performed as it was hypothesized that more virulent influenza virus infections would result in earlier consultation. Age was entered as a continuous as well as a categorical variable based on quartiles and 50th percentile (median) values as detected in the reference category. The categories were scored 1–4 and 1–2, respectively, to conduct a test for trend. Point estimates were considered statistically significant if their 95% confidence interval (95%CI) did not include 1.0. Analyses were performed for each influenza season separately and when possible for multiple seasons, on ILI-patients, ARI-patients or both patients, using SAS 9.1.3 (SAS Institute Inc., NC, USA).

3. Results

3.1. Influenza virus detection

The sentinel surveillance for ILI and ARI yielded 87 influenza virus isolates in influenza season 2005–2006 (23% of 382 patients), 58 influenza virus isolates in 2006–2007 (14% of 403 patients) and 128 influenza virus isolates 2007–2008 (21% of 606 patients) (Table 1).

3.2. Adamantane susceptibility

Results for adamantane susceptibility based on M2 sequence analysis are summarized in Table 1. The percentage adamantane-resistant A(H3N2) viruses increased from 74% in 2005–2006 and 2006–2007 to 100% in 2007–2008, while all isolated A(H1N1) viruses were sensitive. Adamantane-resistant A(H3N2) viruses ($n = 75$) had the single S31N mutation in M2, except one 2005–2006 A(H3N2) virus that had A29T in addition to S31N. Two additional mutations (E16G and M65T) in M2 were found in all adamantane-resistant strains, compared to 2005–2006 adamantane-sensitive strains. Genotypic adamantane resistance was confirmed by and corresponded with phenotypic resistance measurements. Moreover, it indicated that presence or absence of S31N was the major determinant for amantadine resistance, and showed that amino acid substitutions E16G and M65T did not influence the IC_{50} values. Genotypic adamantane-susceptible influenza A(H3N2) viruses ($n = 3$) had an IC_{50} for amantadine of 0.52 ± 0.10 μM (mean ± SD), in contrast to that of S31N variants for which the IC_{50} averaged ($n = 4$) 119 ± 44 μM. The 2005–2006 A(H3N2) virus with substitution A29T in addition to S31N, had an IC_{50} >250 μM for amantadine.

3.3. Phylogenetic analysis

Based on HA_1 clustering, influenza A(H3N2) viruses were assigned to clades as described elsewhere (Saito et al., 2006; Inoue et al., 2007; Simonsen et al., 2007; Hay et al., 2007). Phylogenetic analysis of the HA_1 region of influenza A(H3N2) segregated adamantane-resistant and -sensitive viruses isolated in 2005–2006 into two distinct HA_1 groups, with HA_1 group 1 being adamantane-resistant and HA_1 group 2 being adamantane-sensitive. The adamantane-resistant HA_1 group 1 viruses were characterized by two amino acid substitutions (S193F and D225N) in HA_1, previously classified as clade N viruses (Saito et al., 2006). During the following 2006–2007 and 2007–2008 epidemics, all influenza A(H3N2) virus isolates belonged to group 1 and could be

Table 2
HA_1 segregation and adamantane-sensitive (S) or -resistant (R) genotype of influenza A(H3N2) virus isolates in three following epidemics.

Group assignment[a]	2005–2006	Group and clade assignment[a]	2006–2007	2007–2008
HA_1 group 1 (S193F, D225N)	$n = 29$ (R)	HA_1 group 1, clade A (G50E, K140I)	$n = 36$ (R), $n = 3$ (S)	$n = 10$ (R)
		HA_1 group 1, clade B (N6I, R142G, L157S, K173E)	$n = 10$ (S)	–
		HA_1 group 1, clade C (R142G, N144D)	$n = 1$ (R)	–
HA_1 group 2 (V112I, K173E)	$n = 10$ (S)			

[a] Relative to influenza A/California/7/2004 (H3N2) virus.

further divided in clades 1A, 1B and 1C (Table 2). In 2006–2007, ten adamantane-sensitive A(H3N2) viruses clustered with resistant HA_1 group 1 variants and could be assigned to clade 1B based on mutations in HA_1 (Table 2). One adamantane-resistant A(H3N2) virus was assigned to clade 1C, but most 2006–2007 influenza A(H3N2) viruses (78%) belonged to clade 1A. Clade 1A viruses were predominantly resistant to adamantanes. However, three clade 1 viruses (A/NL/039/07, A/NL/203/07 and A/NL/204/07) were adamantane-sensitive as a result of M protein reassortment with 2005–2006 HA_1 group 2 viruses. Their HA_1 regions clustered with HA_1 group 1 viruses, while their M2 protein clustered with 2005–2006 adamantane-sensitive M2 proteins (bootstrap values of 100%) (Fig. 1). All 2007–2008 influenza A(H3N2) viruses could be assigned to HA_1 clade 1A.

3.4. NAI susceptibility

The results for oseltamivir and zanamivir susceptibilities are summarized in Table 1. While only a few influenza A(H3N2) and influenza B viruses had unusual high IC_{50} values for oseltamivir

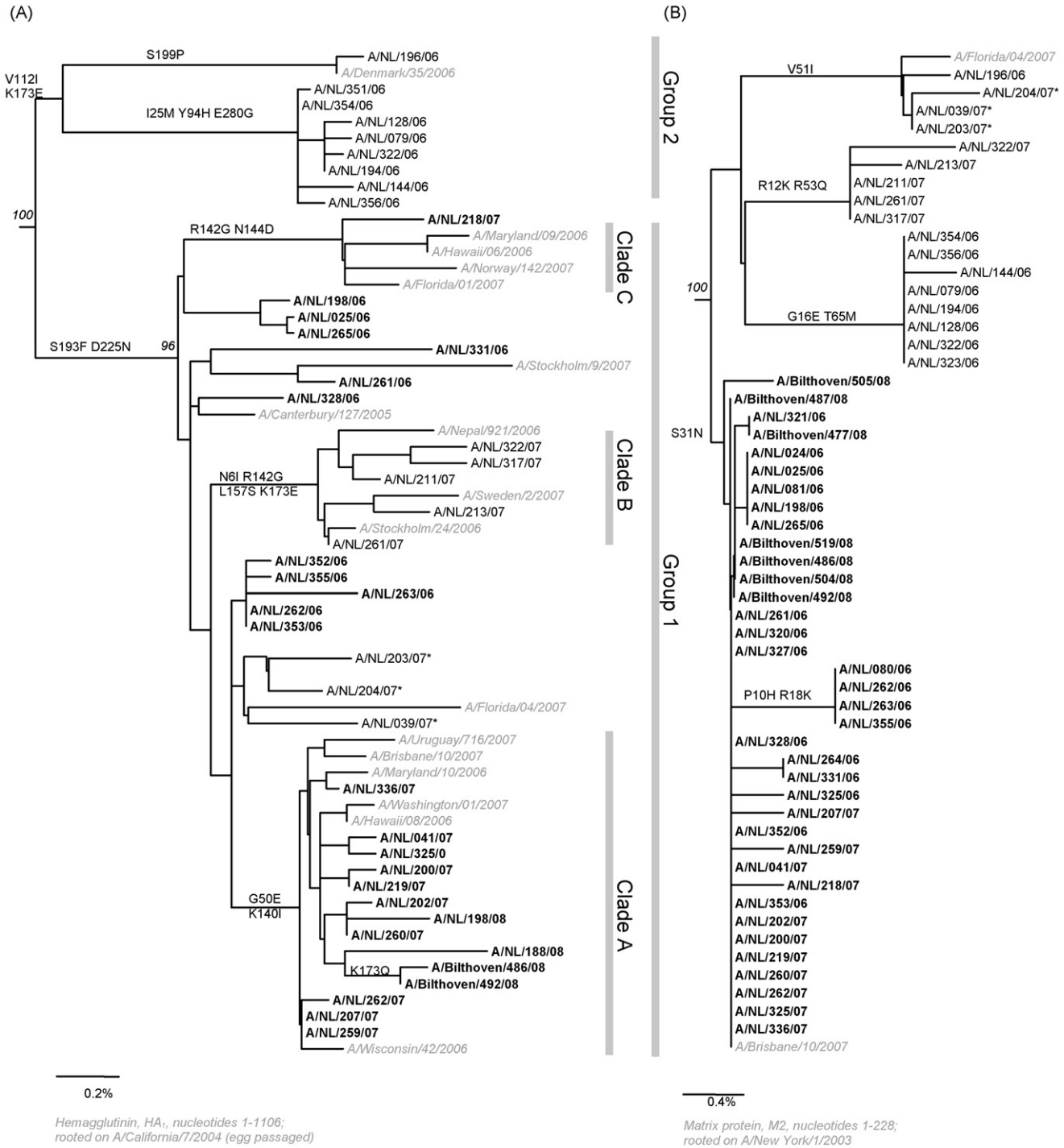

Fig. 1. Phylogenetic HA_1 (A) and M2 (B) clustering of Dutch influenza A(H3N2) viruses. Adamantane-resistant (bold) and adamantane-sensitive viruses isolated in 2005–2006 segregated significant (100%; using 1000 bootstraps) into two distinct HA_1 groups. Similar segregation was observed between 2005 and 2006 adamantane-resistant and -sensitive M2 genes. (*) In 2006–2007, three viruses clustered with HA_1 group 1 viruses, while their M2 ion-channel clustered with 2005–2006 adamantane-sensitive M2 ion-channels.

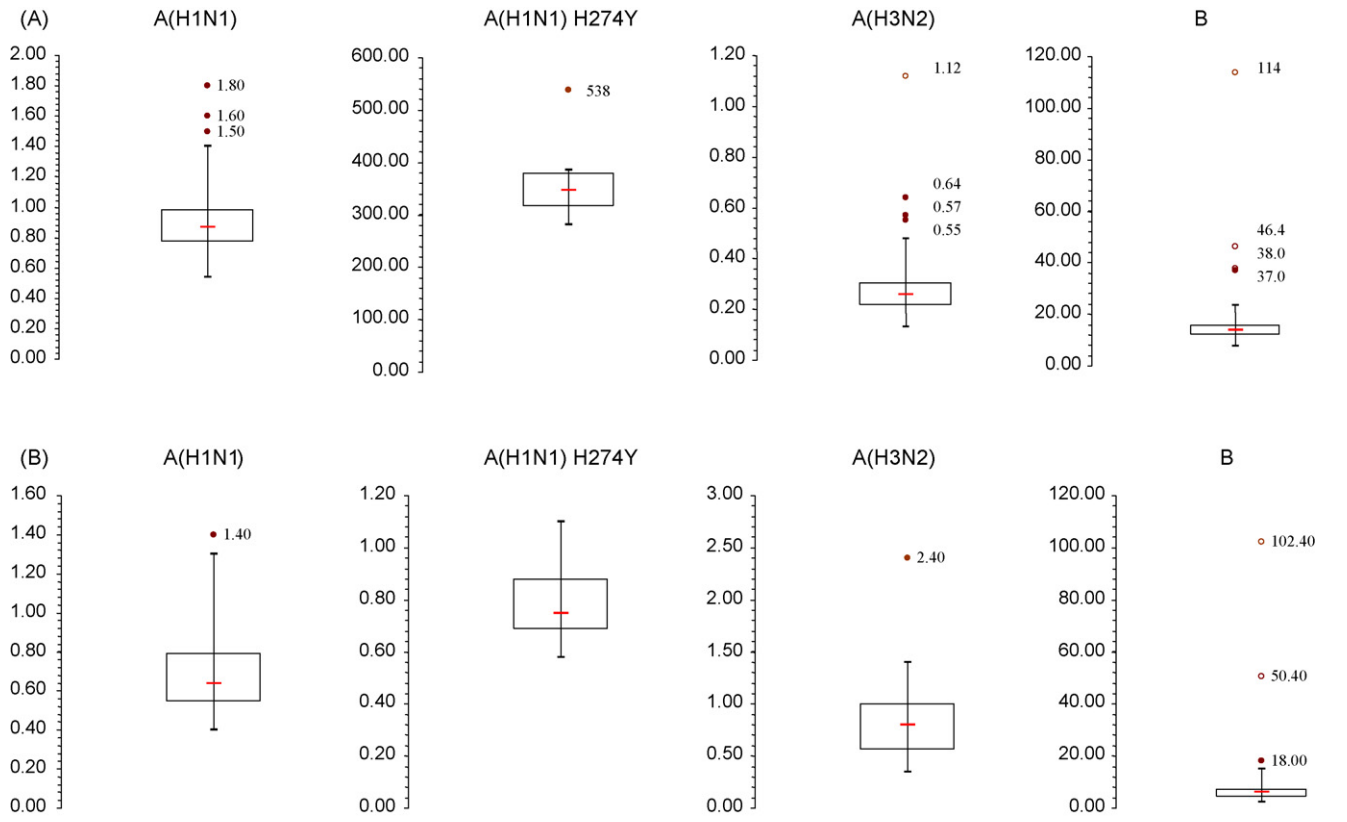

Fig. 2. Box-and-whisker plots showing oseltamivir (A) and zanamivir (B) IC_{50} values (in nM) of influenza viruses obtained during the 2005–2006, 2006–2007 and 2007–2008 influenza season. Closed and open dots represent mild and extreme outliers, respectively.

or zanamivir, the oseltamivir IC_{50} values of the influenza A(H1N1) viruses demonstrated two distinct antiviral profiles (Student's *t*-test, $P < 0.001$) (Fig. 2). Therefore the influenza A(H1N1) data set was analyzed using separate box-and-whisker plots. Baseline oseltamivir and zanamivir susceptibilities are summarized in Table 3. Trend analysis of the baseline IC_{50} values per (sub)type per influenza season did not show significant trends over time (results not shown). However, five (50%) 2007–2008 A(H3N2) viruses characterized by amino acid substitutions D147N and I215V in NA and K173Q in HA had a minor mean 1.8-fold increase in oseltamivir IC_{50} values compared to baseline. Analysis of the influenza A(H1N1) isolates, revealed the presence of oseltamivir resistance in nine (24%) of the influenza A(H1N1) virus isolates obtained during the 2007–2008 influenza epidemic, while in 2006–2007 all five A(H1N1) isolates were oseltamivir- and zanamivir-sensitive. The resistance was characterized by a >300-fold reduction in oseltamivir susceptibility, while remaining sensitive for zanamivir (Table 3). Sequence analysis identified the H274Y amino acid substitution in NA, that is known to be associated with oseltamivir resistance (Gubareva et al., 2001). All oseltamivir-resistant A(H1N1) variants had, in addition to H274Y, amino acid substitution D353G in NA compared to sensitive A(H1N1) virus isolates.

The 273 influenza virus isolates tested for oseltamivir and zanamivir susceptibility, yielded in addition to the nine oseltamivir-resistant (H274Y) A(H1N1) viruses, 13 outlier IC_{50} values according to the box-and-whisker plot analysis. These outliers for NAI susceptibility include four influenza A(H3N2) virus isolates (three minor and one extreme outlier), five influenza A(H1N1) virus isolates (all minor outliers) and four influenza B virus isolates (one minor and three extreme outliers). Two of these influenza B virus isolates were extreme outliers for both NAI (Fig. 2 and Table 4). Sequence comparison of influenza viruses B/NL/034/06 and B/NL/329/06 NA genes revealed the presence of a mixed virus population in both isolates of which one population contained an amino acid substitution (T148K) near catalytic residues D151 and R152 in the 150-loop (N2 numbering). This substitution was not observed in NAI-susceptible influenza B isolates. Sequence analysis of influenza virus B/NL/203/08 identified amino acid substitution D199N in NA as most likely cause of the 2-fold reduced oseltamivir susceptibility of the virus isolate. No sequence analysis was performed on NA of influenza virus A/NL/262/06.

3.5. Clinical effects of antiviral resistance

Qualitative comparison of symptoms between persons infected with adamantane-resistant A(H3N2) and adamantane-sensitive A(H3N2) viruses showed no significant clinical effect as a result

Table 3
Mean (−SD, +SD) baseline oseltamivir and zanamivir susceptibilities per influenza virus (sub)type.

Virus (sub)type	Oseltamivir IC_{50} (nM)	Zanamivir IC_{50} (nM)
Influenza A(H1N1)	0.85 (0.68, 1.05)	0.68 (0.49, 0.92)
Influenza A(H1N1) H274Y	336 (299, 377)	0.78 (0.64, 0.95)
Influenza A(H3N2)	0.25 (0.19, 0.34)	0.74 (0.52, 1.05)
Influenza B	13.6 (11.1, 16.8)	5.62 (3.95, 8.00)

Table 4
Extreme outliers for NAI susceptibility detected by box-and-whisker plot analysis.

Influenza virus isolate	Influenza season isolated	Oseltamivir IC_{50} (nM)	Zanamivir IC_{50} (nM)
A/NL/262/06 (H3N2)	2005–2006	1.12	0.84[a]
B/NL/034/06	2005–2006	114	102.4
B/NL/329/06	2005–2006	46.4	50.4
B/NL/203/08	2007–2008	38	18[b]

[a] No outlier for zanamivir susceptibility.
[b] Minor outlier for zanamivir susceptibility.

Table 5
Symptoms (in %) of adamantane-sensitive (S) and adamantane-resistant (R) influenza A(H3N2) viruses obtained from ILI-patients and ARI-patients during the 2005-2006 and 2006-2007 epidemic.

Symptom	Acute	Fever	Malaise	Myalgia	Coughing	Rhinorrhoea	Sore throat	Headache	Diarrhoea	Other
A(H3N2) $n=23$ (S)	96	96	83	65	83	39	74	48	4	4
A(H3N2) S31N $n=67$ (R)	96	93	75	69	88	43	58	46	4	3
Fisher's P[a]	1.000	1.000	0.572	0.799	0.494	0.809	0.219	1.000	1.000	1.000

[a] Qualitative differences between clinical symptoms as a result of the adamantane susceptibility of the influenza A(H3N2) isolate are expressed by two-tailed Fisher's exact test of significance.

Table 6
Symptoms (in %) of oseltamivir-sensitive (S) and oseltamivir-resistant (R) influenza A(H1N1) viruses obtained from ILI-patients and ARI-patients during the 2007–2008 epidemic.

Symptom	Acute	Fever	Malaise	Myalgia	Coughing	Rhinorrhoea	Sore throat	Headache	Diarrhoea	Other
A(H1N1) $n=29$ (S)	100	89	78	56	89	33	56	44	11	11
A(H1N1) H274Y $n=9$ (R)	83	90	59	62	90	34	38	38	7	0
Fisher's P[a]	1.000	1.000	0.693	0.721	1.000	0.273	1.000	1.000	1.000	0.452

[a] Differences between clinical symptoms as a result of the oseltamivir susceptibility of the influenza A(H1N1) isolate are expressed by two-tailed Fisher's exact test of significance.

of resistant A(H3N2) virus infections obtained from ILI-patients, ARI-patients or both patients. The same was found for comparison of symptoms as a result of oseltamivir-resistant A(H1N1) and oseltamivir-sensitive A(H1N1) virus infections (Tables 5 and 6). To assess the effect of age of the patient on susceptibility for adamantane-resistant or -sensitive influenza A(H3N2) virus infections, age categories were based on quartiles (0–12, 13–40, 41–60, and >60 years) and 50th percentile values ($\leq$40 and >40 years) as found in infections with sensitive viruses. Adamantane-resistant influenza A(H3N2) viruses obtained from ILI-patients, ARI-patients or both categories of patients were not significantly associated with age in years or in categories in 2005–2006, 2006–2007, or both influenza seasons, with the point estimate (95%CI) being 1.0 (0.97–1.01) for age in years, 0.9 (0.54–1.4) for trend of age in quartiles and 0.6 (0.22–1.55) for trend of age in 2 categories, for both influenza seasons. Similarly, the 2007–2008 oseltamivir-resistant A(H1N1) infections were not significantly associated with age in years (1.0; 95%CI 0.92–1.02), age in quartiles (1.1; 95%CI 0.31–3.86), or age in 2 categories (0.6; 95%CI 0.06–6.10). The duration of symptoms of ILI-patients, ARI-patients or both patients prior to consultation did not significantly differ for adamantane-resistant virus infections and oseltamivir-resistant virus infections compared to antiviral-sensitive virus infections. For adamantane-resistant A(H3N2) virus infections the point estimates were (95%CI) 0.9 (0.51–1.71) during the 2005–2006 epidemic, 0.8 (0.49–1.25) during the 2006–2007 epidemic, and 0.8 (0.58–1.21) for both epidemics. For oseltamivir-resistant A(H1N1) virus infections the point estimate was 1.1 (0.84–1.44) during the 2007–2008 epidemic.

4. Discussion

This study shows that already over a short period of time and in a small country acquisition and spread of adamantane and NAI resistance is a dynamic process: resistance may emerge very rapidly, as seen in the case of A(H1N1) oseltamivir resistance, or partially disappear, as seen for A(H3N2) adamantane resistance. While in 2005–2006 and 2006–2007, 2% of the tested influenza virus isolates were found extreme outliers for NAI susceptibility, we detected the emergence of oseltamivir resistance in 9 (24%) influenza A(H1N1) virus isolates obtained during the 2007–2008 influenza epidemic. However, we also observed that adamantane-sensitive A(H3N2) variants re-emerged in the Netherlands in 2006–2007 by seeding and as a result of gene segment reassortment. Our results show that standard oseltamivir therapy for treatment and prophylaxis of recent A(H1N1) infections is hampered but that in individual cases adamantane treatment could be considered on the basis of susceptibility testing. For the successful control of influenza in future, rapid antiviral characterization of each specific virus causing infection will become a clinical necessity.

The observed high frequency of adamantane resistance is in line with data from other studies. The origin of adamantane resistance, however, remains unclear (Sweet et al., 1991; Bright et al., 2005). Our study shows a relation between adamantane resistance and evolution of influenza A(H3N2) viruses during the 2005–2006 epidemic. During this epidemic, genotypic adamantane-resistance (S31N) in the ion-channel was accompanied by two amino acid substitutions (S193F and D225N) in HA_1. Similar substitutions were detected in adamantane-resistant influenza A(H3N2) viruses in Japan and Vietnam in 2005, and it was suggested that these adamantane-resistant variants belonged to the same genetic (N) lineage that possibly emerged in China at the beginning of 2005. It was postulated that amino acid substitutions in HA_1 may play a role together with other advantageous mutations in the viral genome that facilitate S31N fixation by beneficial M2 interactions, and thereby inducing adamantane resistance by a non-drug induced mechanism (Saito et al., 2006; Inoue et al., 2007; Simonsen et al., 2007). In our study, thirteen influenza A(H3N2) virus isolates obtained in 2006–2007 were classified as N lineage based on HA_1, but were adamantane-sensitive. Three of those adamantane-sensitive A(H3N2) isolates, re-emerged as adamantane-sensitive as a result of gene reassortment. A similar gene reassortment pattern was observed for influenza A/Florida/04/2007 (H3N2) virus, obtained from GenBank. Similarly, reversion of adamantane-resistant to -sensitive A(H3N2) viruses by gene reassortment was detected by whole genome sequencing in Japan in 2006–2007, suggesting that this was not an isolated event (Furuse et al., 2009). Although it is not clear where and when the reassortment took place, our results suggest absence of a major beneficial advantage of HA_1 containing amino acid substitutions S193F and D225N together with S31N in M2.

For NAIs, the majority (98%) of the tested influenza virus isolates in 2005–2006 and 2006–2007 was sensitive. However, the 2007–2008 epidemic demonstrated a significant (24%) emergence of oseltamivir-resistant A(H1N1) viruses. By qualitative analysis of clinical data we showed that both adamantane- and oseltamivir-resistant seasonal influenza virus variants did not result in significantly altered symptoms during infection, compared to sensitive variants. Additionally, no significant deviation was observed between the duration of symptoms prior to consultation of (adamantane and oseltamivir)-resistant and -sensitive influenza

virus infected patients. Combined with the frequent occurrence of adamantane-resistant (up to 100%) and oseltamivir-resistant (24%) influenza A(H3N2) and A(H1N1) viruses, respectively, our results suggest that both types of resistance did not result in reduced viral fitness, virulence and transmissibility, in agreement with findings of Hauge et al. (2009) and Dharan et al. (2009). However, our results are limited to virus isolates retrieved from sentinel specimens only and clinical symptoms at presentation of the patient to the GP. Therefore, we are carrying out a more in depth study of patients with oseltamivir-resistant A(H1N1) including patients with genotypic resistance data only, progress of disease data and data from non-sentinel surveillance (viruses isolated in peripheral hospital laboratories) of which the results will be published elsewhere. Although Herlocher et al. (2004) demonstrated with A/New Calendonia-like (H1N1) virus that NA mutant H274Y viruses did transmit, but required a higher dose compared to wild type virus in animal models, the apparently more fit 2007–2008 H274Y influenza A(H1N1) virus variant co-circulated with oseltamivir-sensitive A(H1N1) viruses and became dominant in Europe (Meijer et al., 2009). Subsequently, this oseltamivir-resistant A(H1N1) variant emerged in other parts of the Northern Hemisphere and the Southern Hemisphere and spread into the 2008–2009 season with almost 100% oseltamivir-resistant A(H1N1) viruses (Besselaar et al., 2008; Dharan et al., 2009; Goddard et al., 2009). Because of low oseltamivir use in Europe, we expect that the emergence of oseltamivir resistance, in A(H1N1) virus isolates since 2007–2008, is due to a similar non-drug induced mechanism as for the emergence of adamantane-resistant A(H3N2) viruses, facilitated by advantageous mutations in other viral genes (Rameix-Welti et al., 2008; Kramarz et al., 2009).

The increasing prevalence of antiviral-resistant influenza viruses calls for rapid high throughput antiviral susceptibility tests to monitor the emergence of antiviral resistance and re-emergence of antiviral-sensitive seasonal and pandemic influenza strains. Moreover, treatment or prophylaxis of influenza viruses should be based upon the antiviral profile of each specific infection, as an important component of vigilance, in addition to antiviral resistance monitoring as part of national influenza virus surveillance. Since phenotypic NAI susceptibility assays as the fluorescence based assay we used, are conducted on virus isolates, this obstructs rapid antiviral susceptibility profiling of an influenza virus infection. Recently, pyrosequencing protocols have been proposed for rapid and sensitive detection of NAI resistance (Lackenby et al., 2008; Duwe and Schweiger, 2008). However, due to insufficient understanding of molecular markers for NAI resistance, phenotypic NAI susceptibility assays remain necessary. Indeed, this study identified amino acid substitution T148K in NA of influenza B virus as a potential antiviral resistance marker for both oseltamivir and zanamivir. In addition, amino acid substitution A29T in M2 was identified, that could contribute to adamantane resistance by changing hydrophobicity, size and charge of amino acid residue 29 within the transmembrane region of the molecule (Hay et al., 1985; Cady and Hong, 2008). Nonetheless, more structural and anecdotic studies on acquisition and spread of antiviral resistance are needed to allow implementation of rapid molecular based high throughput antiviral susceptibility profiling.

Acknowledgments

We are grateful to all patients and their GPs for providing information and specimens for investigation. We also thank Gé Donker for management of the sentinel surveillance network and Marianne Heshusius-van Valen for administrative support (both from NIVEL). From the RIVM we thank Wichard Tilstra, Mariam Bagheri, Astrid Glas, Shireen Jenny and Piet Overduin for virological technical assistance.

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