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Evaluation of the antiviral drug susceptibility of influenza viruses in Italy from 2004/05 to 2009/10 epidemics and from the recent 2009 pandemic

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ABSTRACT

Antiviral monitoring of influenza viruses circulating in Italy has been carried out since 2007 by the National Influenza Centre (NIC), using both phenotypic and sequence-based assays. Here, we report results of the susceptibility evaluation to neuraminidase (NA) inhibitors (NAIs, zanamivir and oseltamivir) and adamantanes of nearly 300 influenza type A and B seasonal viruses isolated in Italy during six recent seasons, together with over 30 pandemic (H1N1) 2009 virus strains. The present work is the first such study conducted in Italy, aimed to develop national data on antiviral drug profile and to establish a nationwide surveillance programme on antiviral susceptibility. Sequencing of the NA gene was undertaken either to confirm the phenotypic findings or to identify any NA change, in potentially resistant viruses (outliers), which might be associated with reduced susceptibility to NAIs. The 50% inhibitory concentration values (IC_{50}s) showed slightly different sensitivities of the seasonal Italian isolates to the two NAI drugs, depending on the specific NA subtype. We found mean zanamivir IC_{50}s of 0.74, 1.33 and 7 nM, and oseltamivir IC_{50}s of 0.67, 2.34 and 30.1 nM for the N2, N1 and B NAs, respectively. The pandemic (H1N1) 2009 viruses showed IC_{50}values overall comparable to the seasonal N1 viruses from previous years, showing mean zanamivir IC_{50}s of 1.02 nM and mean oseltamivir IC_{50}s of 2.82 nM. Oseltamivir resistance was found in a total of 19 seasonal N1viruses of 2007/2008 and 2008/2009, and in three pandemic (H1N1) 2009 strains. A gradual increase of resistance to adamantanes was observed among the N2 viruses isolated in recent seasons; no resistant viruses were found among the seasonal N1 strains, whereas all the pandemic (H1N1) 2009 isolates analysed were resistant to the M2 blockers.

1. Introduction

Although vaccination represents the most effective tool against influenza, antiviral drugs are useful during epidemics and pandemics and can provide a valuable alternative prior to vaccine availability or in those for whom vaccination is unsuitable. Two classes of antiviral agents are available for the prophylaxis and treatment of influenza virus infection in humans: the adamantanes (amantadine and rimantadine) (Mould et al., 2000; Mast et al., 1991; Hayden et al., 1989) and the most common neuraminidase (NA) inhibitors (NAIs: oseltamivir and zanamivir) (McKimm-Breschkin et al., 2003).

The M2 ion channel blockers (adamantanes), unlike NAIs, are able to limit replication of the influenza A viruses only (McKimm-Breschkin et al., 2003) and they are associated with high level of resistance among A(H3N2) subtype, some seasonal A(H1N1) viruses (CDC, 2008) and among all the recently emerged pandemic (H1N1) 2009 viruses (Garten et al., 2009). The NAIs were introduced into clinical practice in 1999 and, although rarely used to treat seasonal influenza, they had been stockpiled by most European countries, including Italy, as part of pandemic preparedness plans (Meijer et al., 2007). During the recent emergence of the pandemic (H1N1) 2009 virus, the sudden increase in usage of these drugs for chemoprophylaxis and treatment of human cases (Dawood et al., 2009) reinforced the need for extensive antiviral susceptibility vigilance among circulating influenza isolates. Moreover, the unexpected emergence and spread of H1N1 oseltamivir-resistant viruses (ORV) among seasonal strains isolated worldwide in 2007/2008 (Lackenby et al., 2008; Hauge et al., 2009), in the absence of drug pressure, demonstrated that the replication and virulence of these mutant viruses may be surprisingly comparable to that of wild type strains (Baz et al., 2010).

The major aim of this study was to investigate the antiviral susceptibility profile of the Italian influenza viruses and to increase

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our knowledge of the origin and spread over time of antiviral resistance in Italy. The National Influenza Centre (NIC), located at the National Institute of Health (Istituto Superiore di Sanita'-ISS) in Rome, has been monitoring antiviral susceptibility since 2007, using methodologies established in collaboration with other European public health laboratories, within the framework of the European Surveillance Network for Vigilance against Viral Resistance (VIRGIL). We examined antiviral susceptibility, to both NAIs and adamantanes, of type A and B seasonal influenza viruses, collected over an extended period of time (from 2004 to 2010) throughout the country, together with pandemic (H1N1) virus isolates collected in 2009. Drug-susceptibility testing was performed for both oseltamivir and zanamivir, using a NA activity inhibition assay in conjunction with NA sequence analyses. A number of Italian influenza A viruses from multiple influenza seasons were also investigated for adamantane resistance, by sequencing the M gene.

2. Materials and methods

2.1. Viral strains tested

Monitoring of antiviral drug susceptibility in the Italian influenza strains is performed in the context of the national sentinel surveillance activities conducted by the NIC/ISS, according to a specific drug testing strategy (Fig. 1). A total of 294 influenza field isolates, of which 110 were A/H1N1, 107 A/H3N2 and 77 B viruses, were isolated from throat swabs collected from patients with influenza-like illness over an extended period of time (from 2004 to 2010). Thirty-one pandemic (H1N1) 2009 strains were obtained from clinical samples (nasal, pharyngeal, or nasopharyngeal swabs and/or tracheal aspirates) of hospitalized patients, collected between May and November 2009.

Information on antiviral use has not been obtained in association with these clinical samples. With regard to pandemic (H1N1) 2009 viruses, 23 (74%) clinical samples came from patients who developed a mild disease and 8 (25%) from subjects with severe illness. Information about the therapeutic regime of the NAIs treated subjects were obtained only in a few cases. Virus isolation was performed in MDCK cells, after no more than two passages.

2.2. Neuraminidase inhibitors

Oseltamivir carboxylate (GS4071) and zanamivir compounds were provided by Roche and by GlaxoSmithKline, respectively.

2.3. Fluorometric NA inhibition assay

The fluorescence-based enzyme inhibition assay, used in the present study to define viral resistance to the NAI drugs and for the calculation of the inhibitory drug concentration (IC_{50}), had been previously developed and standardised at HPA (Health Protection Agency, London, UK) (Lackenby et al., 2008). Viruses were screened for susceptibility to NAIs, using the methyl umbelliferone *N*-acetyl neuraminic acid (MUNANA) as substrate. Each isolate was initially titrated in black 96-well flat bottom plates, in order to standardise virus input and to ensure equivalent NA activities were compared against inhibitors. After titrating NA activities, the inhibition assay was performed pre-incubating 10 μl of drug and 10 μl

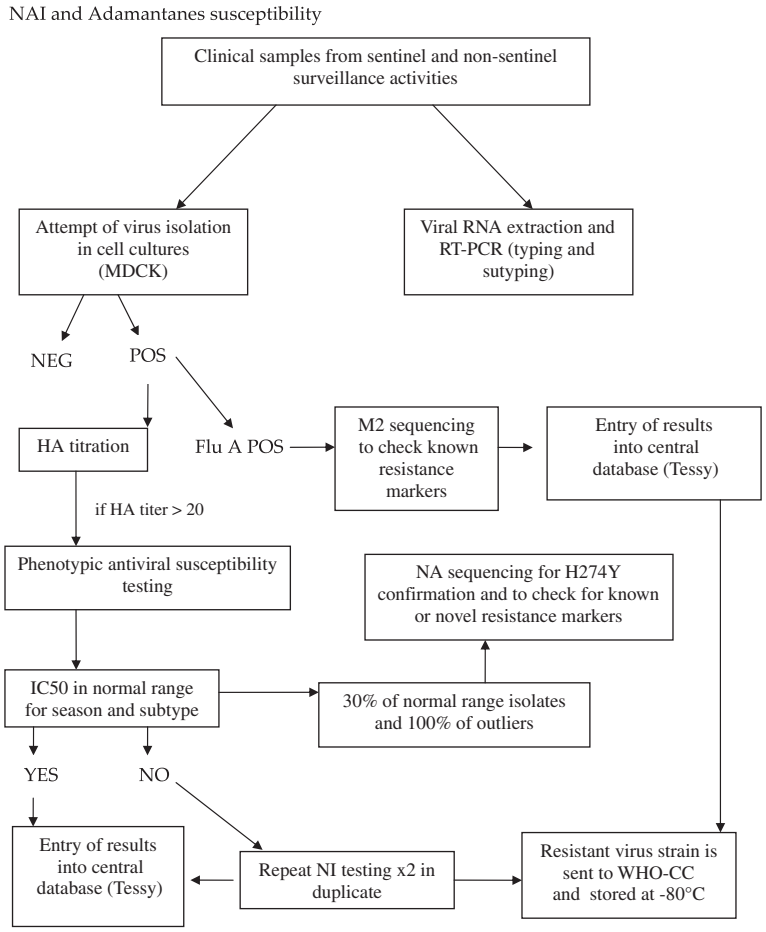

Fig. 1. Drug testing strategy.

of diluted virus for 30 min at 37 °C. The final drug concentration in each test ranged from 0.015 to 4000 nM in serial fourfold dilutions. Then, 100 μM working solution of MUNANA was added to each well and plates were incubated for 1 h at 37 °C. The reaction was stopped and fluorescence was measured in a fluorometer with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Each isolate was tested in duplicate and the 50% IC value was given as a mean of at least two tests.

Before starting analyses, the virus dilution in which enzyme activity yields the equivalent level of fluorescence in 1 h as 10 μM of 4-methyllumelliferone (4-Mu) sodium salt was defined. This dilution was then used in each NAI assay in order to ensure equivalent activities for each virus were compared.

A panel of previously characterized reference viruses were included in each test run as controls for each viral type/subtype. Briefly, the potential intra- and inter-assay variability was firstly evaluated by repeating the test on three reference influenza viruses (A/Parma/8/06 (H3N2), A/Beijing/262/95 (H1N1) and B/RomaISS/1/07, belonging to B/Yamagata-lineage) within the same assay and between assays carried out on 10 different occasions. The A/California/7/09 vaccine strain was used as reference pandemic virus. Validation limits for each reference virus were determined, defined as three standard deviations above and below the median IC_{50}. If the IC_{50} of a reference virus failed to meet the validation criteria for a given drug, the test was invalidated and repeated.

2.4. Statistical analysis

Identification of the outliers was obtained through the determination of the lower (median + 1.6 SD) and upper (median + 3 SD) cut-off values. All viruses with IC_{50}s greater than the lower and upper cut off were considered as minor and major outliers, respectively. All minor and major outliers were tested twice and the median IC_{50} value was included in the analysis.

A virus showing an IC_{50} greater than 10-fold above the mean IC_{50} for oseltamivir and zanamivir were considered as potentially resistant and therefore retested and sequenced.

2.5. Sequencing of the NA and M2 genes

Viral RNAs were extracted from cell culture supernatants by using the Rneasy kit (Qiagen, Germany) and a two-step RT-PCR was carried out as previously described (Puzelli et al., 2004). Briefly, the entire coding region of the NA gene of human influenza A and B viruses and the coding region of the M2 gene of the A type viruses were amplified using specific primers (available from the authors upon request) and the AmpliTaq-Gold enzyme (Applied Biosystems). Amplified products of the expected size were purified with the QIAquick PCR purification kit (Qiagen, Germany), sequenced using the BigDye Terminator Cycle-Sequencing Ready Reaction kit v1.1 (Applied Biosystems) and analysed on ABI Prism 310 DNA sequencer (Applied Biosystems). Sequence assembly was performed with Lasergene package (version 4.0; DNASTAR) and the sequences obtained were then aligned using BioEdit software (version 7.0.0).

3. Results

3.1. Epidemic and pandemic viruses tested

Two hundred and ninety-four epidemic strains, collected between 2004 and 2010, and 31 pandemic (H1N1) 2009 viruses were evaluated for their susceptibility to both NAI drugs by the fluorometric NA inhibition assay and by sequencing. Table 1 summarizes the number of the isolates tested (by type and subtype) and their distribution by season, also reflecting global prevalence during each season in Italy. The number of viruses tested for each of the six seasons ranged from 14 isolates from 2004/05 to 111 from 2007/08 seasons, reflecting the increasing need for testing recent isolates, due to the emergence of ORVs in Europe in 2008.

Overall in Italy, influenza A viruses and, in particular, the H3N2 subtype largely circulated during most of these seasons, with the exception of 2009/2010 characterized by the predominant circulation of the pandemic viruses. The seasonal H1N1 subtype circulated each season, predominating in 2005/2006 and 2007/08, as also observed in the rest of Europe. B viruses also circulated in all six seasons analysed, although at low levels and mainly in the middle or towards the end of each season.

3.2. Viral susceptibility to NA inhibitors by phenotypic assay

The mean IC_{50} values and the outlier cut-off (minor and major) for oseltamivir and zanamivir of all subtypes are also reported in Table 1. Differences in the IC_{50} values, according to subtype and NAIs were noted (Fig. 2). The seasonal H1N1 viruses demonstrated a higher level of sensitivity to zanamivir than to oseltamivir carboxylate (mean IC_{50}: 1.33 and 2.34 nM, respectively); similarly, the pandemic (H1N1) 2009 strains showed higher level of sensitivity to zanamivir, when compared to oseltamivir (mean IC_{50}: 1.02 and 2.82 nM, respectively); B viruses were considerably more sensitive to zanamivir (mean IC_{50}: 7 nM) than to oseltamivir (mean IC_{50}: 30.1 nM), whereas similar IC_{50} values for both drugs were found for the H3N2 isolates (mean IC_{50}: 0.67 nM for oseltamivir versus 0.74 nM for zanamivir).

Overall, mean IC_{50} values for each type/subtype obtained in this study, demonstrate that the Italian influenza A and B viruses were more sensitive to zanamivir than to oseltamivir, as also reported for viruses of other countries (Monto et al., 2006; Stoner et al., 2010).

To study the antiviral susceptibility of our isolates over time, we analysed the data by year of isolation (Figs. 3–5). Although noting a slight seasonal variation for each viral subtype against both drugs, there was not a clear trend towards increasing IC_{50} values with time, particularly for the A/H3N2 and B isolates (Figs. 4 and 5). The same situation was observed for A/H1N1 subtype toward zanamivir but there was a sudden raise of IC_{50} values for oseltamivir from 2007/2008 (range IC_{50}: 1.43–4.68 nM, Fig. 3) to the 2008/2009 season (range IC_{50}: 634–1003 nM, Fig. 3).

It must be noted that H3N2 viruses collected during the 2007/2008 winter showed higher sensitivity to zanamivir than to oseltamivir (Fig. 4), in contrast to data from the literature which usually report a major susceptibility to oseltamivir for N2 viruses (Sheu et al., 2008). However, these differences may be due to the limited number of H3N2 strains tested from that season.

None of the A and B viruses tested showed phenotypic resistance to zanamivir, as shown by the IC_{50} values, which never significantly exceeded the minor and major outlier cut-off (Table 1).

Among the 294 seasonal influenza viruses under study, we identified 19 seasonal A/H1N1 ORVs; 1 from the 2007/2008 and 18 from the 2008/2009 seasons. No ORV was detected among the A/H3N2 and B isolates. All ORVs exhibited a 350/400-fold reduction in susceptibility to oseltamivir, showing elevated IC_{50}s, ranging from 634 to 1003 nM. No history of antiviral use was obtained in association with these clinical samples.

Two out of 30 pandemic (H1N1) 2009 viruses, analysed by NAI, were found to be oseltamivir-resistant, showing elevated IC_{50} values (968 and 577 nM respectively, data not shown), although remaining sensitive to zanamivir. These two pandemic (H1N1) 2009 resistant strains were isolated in northern Italy from hospitalized children, with severe influenza illness and under prolonged treatment with oseltamivir.

Table 1
Susceptibility of seasonal and pandemic viruses to both NAI drugs, obtained in the fluorometric neuraminidase inhibition test.

Drug	Virus	IC_{50} (nM)[a] per season						Total IC_{50} (nM)[a]
		2004/2005	2005/2006	2006/2007	2007/2008	2008/2009	2009/2010	
		Mean IC_{50} (n)[b] (m.o. and M.o.)[c]	Mean IC_{50} (n)[b] (m.o. and M.o.)[c]	Mean IC_{50} (n)[b] (m.o. and M.o.)[c]	Mean IC_{50} (n)[b] (m.o. and M.o.)[c]	Mean IC_{50} (n)[b] (m.o. and M.o.)[c]	Mean IC_{50} (n)[b] (m.o. and M.o.)[c]	Mean IC_{50} (n)[b] (m.o. and M.o.)[c]
Oseltamivir	A(H1N1)	2.33 (4) (2.84–3.26)	1.88 (11) (2.66–3.29)	1.34 (10) 1.98–2.49)	2.58 (66)[d] (3.77–4.95)	*	n.a.	2.34 (91)[d] (3.62–4.81)
	A(H3N2)	0.64 (5) (1.09–1.53)	0.29 (2) (0.35–0.41)	0.51 (19) (0.68–0.83)	1.24 (3) (1.64–2.14)	0.7 (78) (1.3–1.89)	n.a.	0.67 (107) (1.2–1.7)
	B	21.2 (5) (38.14–50.6)	45.3 (11) (72.3–96)	23 (4) (40.2–57.4)	27.4 (41) (42.7–55.1)	44.5 (5) (64.7–91)	24.7 (11) (35.9–46.8)	30.1 (77) (50.5–70.1)
	Pandemic (H1N1)2009						2.82 (28)[e] (3.53–4.45)	
Zanamivir	A(H1N1)	1.46 (4) (2.23–2.78)	0.94 (11) (1.51–2.04)	1.05 (10) (1.27–1.57)	1.37 (67) (2.22–2.93)	1.6 (18) (2.1–2.6)	n.a	1.33 (110) (2.1–2.8)
	A(H3N2)	1 (5) (1.8–2.8)	0.45 (2) (0.47–0.49)	0.57 (19) (0.87–1.19)	0.56 (3) (0.65–0.76)	0.77(78) (1.05–1.33)	n.a.	0.74 (107) (1.12–1.5)
	B	4.16 (5) (8.36–12.78)	5.2 (11) (9.3–13.5)	3 (4) (5.2–7.2)	7.3 (41) (12.9–18.8)	10.4 (5) (20.7–27.2)	9.1(11) (14–18.2)	7 (77) (11.9–17.8)
	Pandemic (H1N1)2009						1.02 (30) (1.66–2.38)	

n.a not available: H1N1 viruses did not circulate during the 2009/10 season in Italy; no isolate was obtained from the only two H3N2 viruses detected during the same influenza season in Italy.

[a] Original scale (not log transformed).
[b] Number of isolates.
[c] m.o. minor outlier cut-off; M.o. major outlier cut-off.
[d] H1N1 ORVs (1 from 2007/08, 18 from 2008/09) were excluded from statistical analysis.
[e] Pandemic (H1N1)2009 ORVs (2 from 2009/10 season) were excluded from statistical analysis.

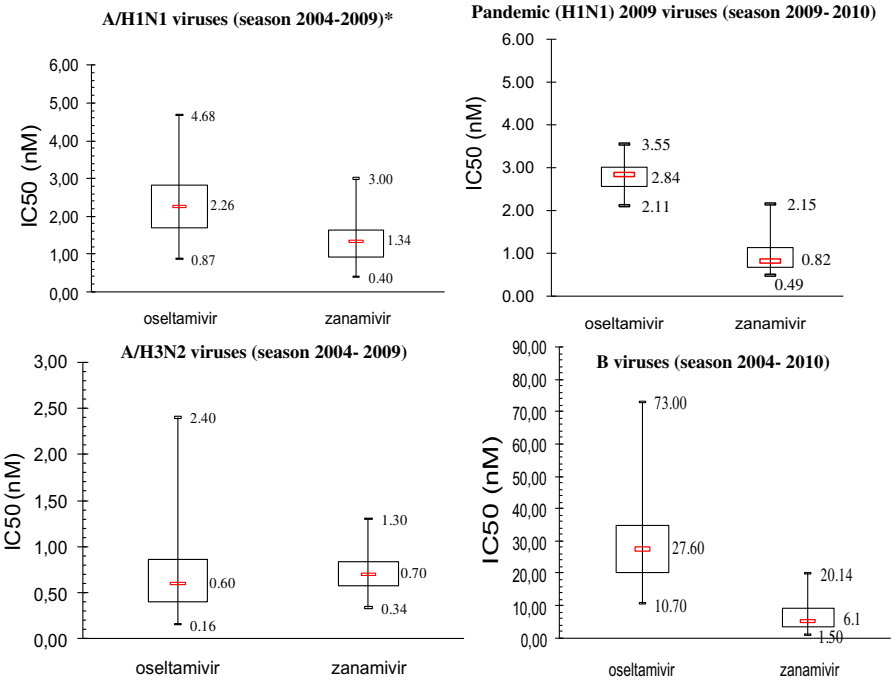

Fig. 2. Box plots of Oseltamivir and Zanamivir IC50 values (nM) for A/H1N1, A/H1N1pdm, A/H3N2 and B Italian isolates, obtained in the fluorometric neuraminidase inhibition test. Results obtained from six consecutive influenza seasons are grouped together for each virus type and subtype. The box stretches from the lower hinge (25th percentile) to the upper hinge (75th percentile). The median is shown as a line across the box. *A/H1N1 ORVs were excluded from statistical analysis.

3.2.1. Characterization of outliers

Among the seasonal A/H1N1 viruses isolated between 2006 and 2008, we found seven minor outliers for oseltamivir and four for zanamivir. With regard to the A/H3N2 viruses, we detected only four minor outliers for oseltamivir and six for zanamivir, collected between 2004 and 2009; moreover, one major outlier for zanamivir was also found in 2008/09 season. Among the B isolates, respectively one major outlier for oseltamivir and two for zanamivir have been found between 2005 and 2008, whereas two minor outliers for oseltamivir and seven for zanamivir were identified between 2004 and 2009. All the viruses reported as ORV in this study showed IC_{50}s > 100 nM.

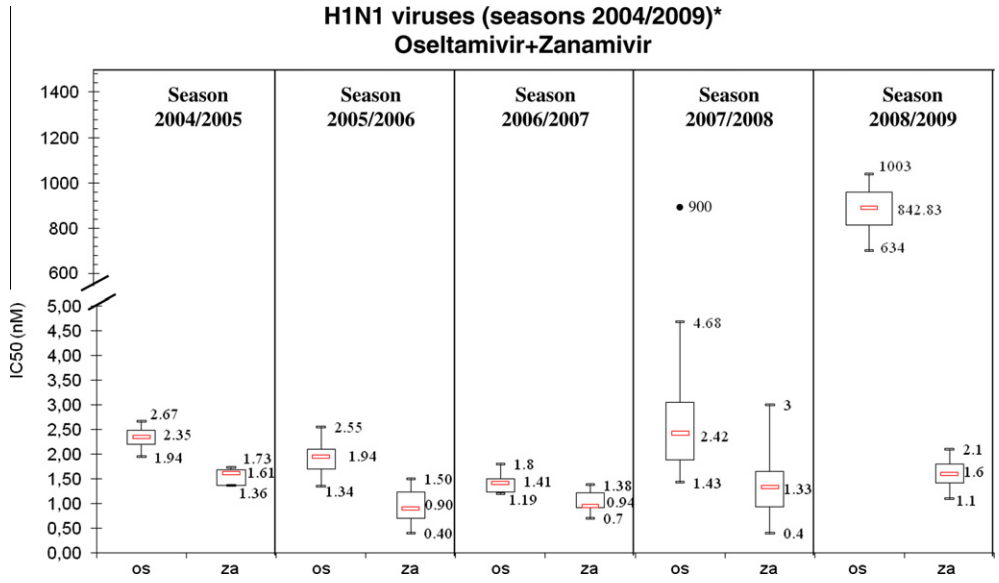

Fig. 3. Box plots of Oseltamivir and Zanamivir IC50 values (nM) for A/H1N1 virus isolates, by season (2004–2009), obtained in the fluorometric neuraminidase inhibition test. The box stretches from the lower hinge (25th percentile) to the upper hinge (75th percentile). The median is shown as a line across the box. *H1N1 viruses did not circulate during the 2009/2010 season in Italy.

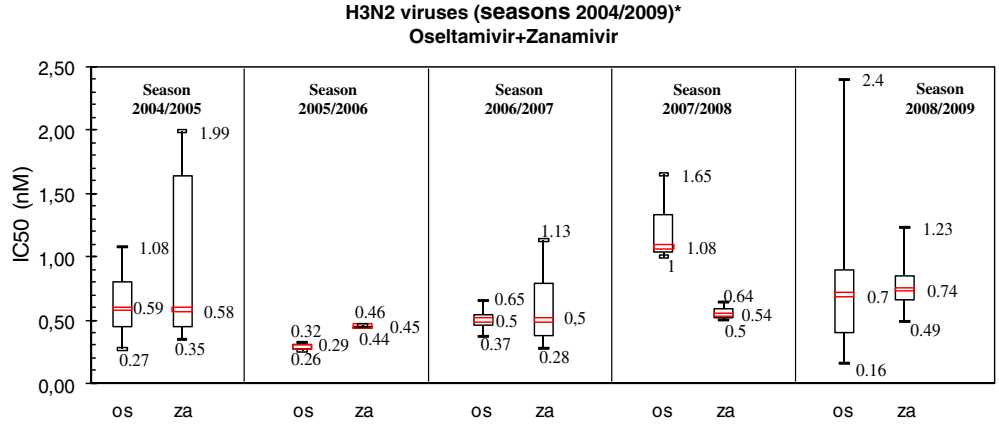

Fig. 4. Box plots of Oseltamivir and Zanamivir IC50 values (nM) for A/H3N2 virus isolates, by season (2004–2009), obtained in the fluorometric neuraminidase inhibition test. The The box stretches from the lower hinge (25th percentile) to the upper hinge (75th percentile). The median is shown as a line across the box. *No isolate was obtained from the only 2 H3N2 viruses detected during the 2009/2010 season in Italy.

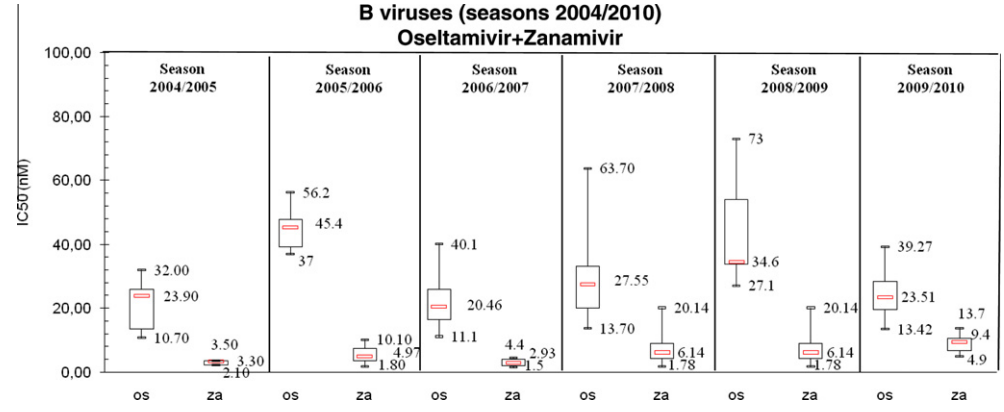

Fig. 5. Box plots of Oseltamivir and Zanamivir IC50 values (nM) for B virus isolates, by season (2004–2010), obtained in the fluorometric neuraminidase inhibition test. The box stretches from the lower hinge (25th percentile) to the upper hinge (75th percentile). The median is shown as a line across the box.

Table 2
Number of human influenza isolates analysed for M2 protein mutations.

Subtype	Season	No. of isolates tested	No. of resistant isolates	Percentage of resistance	Sequence changes	Total No. of isolates tested by subtype
H1N1	2004/2005	5	0	0%		
	2005/2006	12	0	0%		
	2006/2007	14	0	0%		**64**
	2007/2008	17	0	0%		
	2008/2009	16	0	0%		
H3N2	2004/2005	9	3	**33.3%**	**S31N**	
	2005/2006	3	0	0%		
	2006/2007	16	9	**56%**	**S31N**	**52**
	2007/2008	3	2	**67%**	**S31N**	
	2008/2009	21	21	**100%**	**S31N**	
H1N1v	2009/2010	17	17	**100%**	**S31N**	**17**

3.3. Viral susceptibility to NA inhibitors by sequence analyses

NA sequencing of all the ORVs, major and minor outliers detected in this study has been carried out, together with a representative number of wild type strains. All 19 A/H1N1 and the two pandemic (H1N1) 2009 ORVs, detected by phenotypic assay, were shown to possess the H274Y substitution (N2 numbering), commonly associated with oseltamivir resistance [The nucleotide sequences obtained in this study are available from GenBank under accession numbers JF513094 to JF513114]. One further pandemic (H1N1) 2009 oseltamivir-resistant strain with the typical H274Y mutation, not previously analysed by phenotypic assay because of the unavailability of the isolated virus, was found by sequencing analysis of the NA gene. This latter resistant virus, A/Pavia/21/2009, representing the first pandemic (H1N1) 2009 ORV case reported in Italy, was detected in nasal secretion of a 2 year-old child affected by acute lymphoid leukaemia with influenza-like illness, after 18 days of treatment with oseltamivir (Campanini et al., 2010). In two of the above three pandemic ORVs, we also found a T332K (N1 numbering) amino acid substitution, located outside of the active site, the significance of which is not clear.

Among the seasonal A/H1N1 ORVs, we identified the additional change G248K, previously highlighted by others (Monto et al., 2006; Sheu et al., 2008; Ferraris et al., 2005). With regard to major and minor outliers, no significant changes were detected, when compared with the reference strains used in our analysis, for each subtype under study. Only in one A/H3N2 isolate, identified as minor outlier for oseltamivir, we found a D151G substitution in the enzyme catalytic site. However, mutations in this position (D151G or D151E, N2 numbering) were also detected in three additional A/H3N2 isolates found to be drug sensitive. A further interesting change, E277D, was identified in a framework residue of the NA active site of a B isolate collected in the 2007/2008 season, but this isolate remained drug sensitive.

With regard to the pandemic (H1N1) 2009 viruses, the majority (29/31) shared the I106 and D248 (N1 numbering) amino acids in the NA protein, which makes these strains closely related to A/New York/18/09-like viruses (Deyde et al., 2010). In contrast, the other two strains showed the V106 and N248 (N1 numbering) amino acids, such as A/California/4/09-like viruses. No further changes were detected in those residues, located in and around the NA active site, known to alter the susceptibility of these viruses to NAIs (Deyde et al., 2010). Moreover, all the above pandemic (H1N1) 2009 viruses were wild type at positions 15 and 189, in contrast to most viruses from southern hemisphere with the specific changes M15I and N189S (Barr et al., 2010). They were also wild type (isoleucine) at position 223 of the NA gene, a residue which may be involved in a significant increase of oseltamivir resistance, when an I223V change is found in association with H274Y, as recently reported (Pizzorno et al., 2011).

3.4. Viral susceptibility to M2 inhibitors by sequence analyses

Overall, 133 Italian virus isolates (64 A/H1N1, 52 A/H3N2 and 17 pandemic (H1N1) 2009 strains), collected between 2004 and 2009, were screened for those specific M2 protein mutations known to confer resistance to adamantanes (Table 2).

Our results showed that all the pandemic (H1N1) 2009 viruses under study were resistant to M2 ion channel blockers and that there is an increasing trend of adamantane resistance, particularly among the A/H3N2 isolates from the most recent seasons. Respectively, 56%, 67% and 100% of the isolates belonging to H3N2 subtype tested from the 2006/07, 2007/08 and 2008/09 seasons were found resistant. Very few A/H3N2 viruses of 2007/08 season were included and analysed in this study, due to their limited circulation in that winter, characterized by the predominance of seasonal A/H1N1 viruses. All M2 blocker resistant variants harboured an amino acid change at residue 31 (S31N), the most common mutation known to confer resistance to adamantanes (Bright et al., 2005, 2006). No adamantane resistant strains were found among the seasonal A/H1N1viruses analysed here.

4. Discussion

Antiviral agents can play a major role in the control of seasonal influenza outbreaks and are also expected to confer significant prophylactic and therapeutic benefits during an influenza pandemic.

The NA inhibitors, represented by orally bio-available oseltamivir and inhaled zanamivir, have been available for the treatment of influenza infection in many countries of the world since 1999. However, the global spread of oseltamivir-resistant seasonal A/H1N1 viruses (Meijer et al., 2009), since 2007, raised public health concerns as it may significantly compromise the clinical benefits of this agent, which have been stockpiled at national level to fight a pandemic emergency. The recent pandemic (H1N1) 2009 influenza virus, which has been circulating globally since April 2009, is currently susceptible to both NAIs (WHO, 2010), although adamantane-resistant. Moreover, resistance to the M2 inhibitors in seasonal virus isolates has been heavily documented in the past decade (Bright et al., 2005, 2006). For all the above reasons, continued national and global surveillance activities on influenza antiviral drug resistance remains essential in order to determine the level of resistance among all the currently circulating influenza subtypes, particularly to NAIs. The use of both phenotypic and sequence-based assays may help either in rapidly detecting any resistant virus harbouring those recognized molecular changes associated with resistance phenotype or in identifying possible novel mutations which may also confer reduced susceptibility to these drugs.

The present study is the first one conducted in Italy, on a large number of influenza isolates collected over an extended period of

time, to specifically develop data on virus sensitivity to antiviral drugs and aimed at the establishment of a nationwide surveillance programme on influenza antiviral susceptibility.

Nearly 300 seasonal virus isolates, belonging to the different influenza types and subtypes and collected along six recent seasons, together with over 30 pandemic (H1N1) 2009 strains, were assayed for sensitivity to both the NAIs. A set of 133 A type strains, collected in various seasons, was also screened to establish the susceptibility to adamantanes.

With regard to the NAI results our data demonstrated that, in accordance with the specific NA type, the Italian influenza isolates showed slightly different sensitivities to the two drugs. Based on the mean IC_{50} values obtained on NAI susceptible strains, type A viruses resulted more drug sensitive than B viruses. Moreover, A/H3N2 viruses were more sensitive to oseltamivir than seasonal A/H1N1 isolates, as also reported by other colleagues (McKimm-Breschkin et al., 2003; Monto et al., 2006; Sheu et al., 2008). The pandemic (H1N1) 2009 viruses showed IC_{50}values overall comparable to the seasonal H1N1 viruses from previous years, showing in both cases a higher sensitivity to zanamivir than to oseltamivir. Of note, 19 seasonal H1N1viruses (one isolated in 2007/2008 and 18 in 2008/2009) and three pandemic (H1N1) 2009 strains were found to be resistant to oseltamivir by the fluorometric NA inhibition assay. These viruses showed a 350/400-fold reduction in susceptibility to oseltamivir in accordance with previous literature data (Ferraris and Lina, 2008; Wetherall et al., 2003; Gubareva et al. 2001; Mishin et al., 2005).

The resistance profile of all the above 22 viruses have been also confirmed by NA sequencing which showed the H274Y (N2 numbering) mutation. None of the above isolates exhibited phenotypic or genotypic resistance to zanamivir.

It must be highlighted that, in contrast to the seasonal A/H1N1 ORVs for which H274Y change emerged naturally (Baz et al., 2010), the three oseltamivir resistant pandemic (H1N1) 2009 viruses were isolated from immuno-compromised patients, characterized by a long phase of viral shedding and under prolonged oseltamivir treatment, thus suggesting that, in these cases, resistance emerged through selective pressure driven by drug usage, as also reported by others (ECDC, 2010).

The 107 A/H3N2 viruses included in this study generally showed lower IC_{50} values compared to the N1 viruses and comparable level of sensitivity against both the NAIs drugs. The NA sequencing of A/H3N2 viruses found a D151G change in one isolate, a minor outlier to oseltamivir, the significance of which is still unclear. Recent studies confirm that isolates harbouring a change at position 151 of NA cannot be considered as resistant viruses, although this residue directly interacts with NAIs (Sheu et al. 2008; Aoki et al. 2007; Deyde et al. 2009).

The 77 influenza B viruses analysed showed a season to season variation, particularly for oseltamivir, the significance of which is difficult to determine in view of the limited number of isolates tested in some seasons. In general, the mean IC_{50} values for B viruses towards zanamivir were four-fold lower than those against oseltamivir, similar to other studies (Sheu et al., 2008; McKimm-Breschkin et al., 2003; Okomo-Adhiamobo et al., 2010), although showing some variations if single seasons were considered.

The results reported confirm that no gradual shift in the susceptibility of clinical isolates to the NAIs was observed in the N2 and B isolates, after more than a decade from the licensing of these drugs in Italy. Notably, there were no major seasonal or geographical variations in the IC_{50} values and no naturally occurring N2 or B resistant variants were isolated.

Starting from 2004/05 winter season, an increase of resistance to adamantanes among the Italian influenza A/H3N2 virus isolates has been observed, harbouring the typical mutation (S31N) in the M2 protein. No adamantane-resistant viruses were found among the seasonal A/H1N1 strains, whereas all the pandemic (H1N1) 2009 isolates analysed were resistant to these drugs. It is still unclear if drug selection pressure could have played a key role in the decreasing trend in susceptibility of A/H3N2 strains to adamantanes, as this information was not available until 2007/08 season. The lack of data on patient's exposure to antiviral drugs (NAIs and adamantanes) did not allow thus far a full understanding of the real impact of drug usage in the community. For this reason, we are now conducting further studies particularly focusing on those samples for which clinical information on drug usage are also provided. To date, preliminary findings from the antiviral susceptibility testing carried out on the current 2010/2011 pandemic (H1N1) 2009 Italian isolates showed equivalent trends, when compared to the previous winter. In particular, among the 43 pandemic (H1N1) isolates so far analysed at NIC/ISS, 2 cases of oseltamivir-resistance (4.6%) were detected in hospitalized at-risk children, sampled post-treatment. No oseltamivir-resistance was found among the 4 A/H3N2 and the 28 B strains thus far tested.

Future work will be: (i) maintaining a constant and active surveillance for antiviral resistance in both seasonal and pandemic virus strains, in order to provide continuous and timely information about the possible emergence of drug-resistant human virus variants and to determine their clinical importance and epidemiological consequences; (ii) transferring the resistance testing methodologies, employed in the present work, to various peripheral laboratories, preferably from hospitals, selected according to their geographical location; (iii) including N1 animal Italian influenza isolates in the antiviral resistance analyses, in order to gather more information about the variability in IC_{50} amongst N1 containing influenza viruses from different animal species.

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