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Antiviral drug profile of seasonal influenza viruses circulating in Portugal from 2004/2005 to 2008/2009 winter seasons

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ABSTRACT

A research project on antiviral drug resistance of influenza viruses circulating in Portugal has been carried out since 2007. Here, the first results obtained regarding the evaluation of susceptibility to amantadine and oseltamivir are presented. Information about antiviral prescription and exposure was available through the National Influenza Surveillance Programme. Amantadine susceptibility was evaluated by pyrosequencing for known resistance markers on 178 influenza A strains from 2004/2005 to 2006/2007. Susceptibility to oseltamivir was evaluated by 50% inhibitory concentration determination on 340 virus strains from 2004/2005 to 2008/2009, 134 of which were further analyzed by sequencing of the neuraminidase gene. This study revealed that influenza antiviral drugs were rarely prescribed at national level. Resistance to amantadine was observed on only A(H3N2) strain isolated during 2005/2006 and on 38 (74.5%) of the 51 A(H3N2) strains from 2006/2007, all carrying the mutation S31N in their M2 sequence. Oseltamivir resistance was observed in 6 (20.7%) of the 29 A(H1N1) strains from 2007/2008 and in all strains from 2008/2009, which exhibited extremely high IC_{50} values and carrying the mutation H275Y in their neuraminidase sequence. The national data generated and analyzed in this study may contribute to increase the knowledge on influenza antiviral drug resistance which is a problem of global concern.

1. Introduction

Two classes of antiviral drugs are currently licensed for the prevention and treatment of seasonal, zoonotic and pandemic influenza: (1) M2 inhibitors or adamantanes, including amantadine and rimantadine, and; (2) neuraminidase inhibitors (NAIs), such as oseltamivir and zanamivir (Crusat and de Jong, 2007). In Portugal, only rimantadine is not licensed for clinical use. Amantadine was introduced into clinical practice in 1973, followed by zanamivir in 1999 and oseltamivir in 2002, all 3 requiring medical prescription (information available at www.infarmed.pt).

The introduction of these antiviral drugs into clinical practice raised public health concerns regarding the potential emergence of resistance and its impact on the clinical effectiveness of the drugs. These concerns led to national and global surveillance activities as well as to specific and detailed research studies on influenza antiviral drug resistance, particularly after the introduction of NAIs.

Resistance to M2 inhibitors has been observed with a high frequency not only in the clinic but also in the community settings, limiting their use on influenza prevention and control. More specifically, resistance was identified on 30–80% of hospitalized patients, within 48–72 h after onset of antiviral therapy (Democratis et al., 2006). In addition, a high increase in the frequency of influenza A(H3N2) virus resistant to M2 inhibitors started to be identified at global level during the 2002/2003 winter season, with the most recent data from 2008/2009 revealing a 100% worldwide frequency of resistance (Bright et al., 2005; WHO, 2009a). High frequencies of resistance to these inhibitors have also been identified for A(H5N1) avian influenza viruses, particularly those from clade 1 and approximately 80% from subclade 2.1. A 100% frequency of resistance was also observed for the recently emerged A(H1N1) swine 2009 pandemic virus (Crusat and de Jong, 2007; Hayden, 2007; WHO, 2009b). Resistance to M2 inhibitors has also been identified for seasonal A(H1N1) influenza virus but on a minor extent and at variable levels, not only between seasons but also between countries. This variability is associated with the co-circulation of 2 hemagglutinin (HA) A(H1N1) lineages, 1 resistant to M2 inhibitors (HA subclade 2C)—represented by A/Hong Kong/2652/2006, and 1 susceptible to this antiviral drug class (HA subclade 2B)—represented by A/Brisbane/59/2007 (Barr et al., 2008; Cheng et al., 2009). However, A/Brisbane/59/2007-like virus have been spreading at global level since they emerged, dur-

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ing 2007/2008, replacing A/Hong Kong/2652/2006-like virus (see interim reports from WHO Influenza Centre, London, available at http://www.nimr.mrc.ac.uk/wic/report/). The most recent data from 2008/2009 revealed that only 2% of the seasonal A(H1N1) virus analysed worldwide belong to the M2 resistant A/Hong Kong lineage (WHO, 2009a).

Regarding the emergence of resistance to NAIs, different situations have been observed for the 2 antiviral drugs available. Resistance to zanamivir has been rarely detected, with the 2 cases identified following treatment of immunocompromised patients being the most notable (Lackenby et al., 2008a). This rare detection can be associated with the high similarity that zanamivir exhibits, at structural level, to the natural substrate of neuraminidase (NA) (Moscona, 2009). However, it is important to consider that zanamivir has been rarely used in the management of influenza, which could also explain the situation observed. Resistance to oseltamivir was, until 2007, detected at a low frequency and mainly following treatment (in 0.32% of the adults and 4.1% of the children under therapy) (Aoki et al., 2007). Two high frequencies of 18% and 16.3% were, however, observed in 2 Japanese clinical trials carried out in children infected with A(H3N2) and A(H1N1) influenza viruses, respectively (Kiso et al., 2004; Ward et al., 2005). During the 2007/2008 winter season, the emergence and widespread circulation of seasonal A(H1N1) influenza virus resistant to oseltamivir, in the absence of drug selective pressure, were identified at global level (ECDC, 2008; Lackenby et al., 2008a). This resistant virus persisted and increased in frequency during 2008/2009, representing 96% of the seasonal A(H1N1) virus that circulated worldwide during that winter season (WHO, 2009a). Sporadic cases of resistance to oseltamivir have been identified for A(H5N1) avian influenza virus, with a total of 5 cases reported (3 in Vietnam and 2 in Egypt), and on the recently emerged A(H1N1) swine 2009 pandemic virus, with a total of 39 cases reported as of 23th October 2009. The majority of these sporadic cases were associated with antiviral drug use (de Jong et al., 2005; Le et al., 2005; Saad et al., 2007; WHO, 2009c,d).

Since 2007, a research project has being carried out in Portugal aiming at the evaluation and study of influenza antiviral drug resistance. With the data that has already been obtained, and with data that is expected to be gathered in the course of this project, it may become possible to contribute to the information that is continually being generated at international level, some of which is summarized above, and to advances in the knowledge on this specific area of research.

In this study, the first results of evaluation of susceptibility to oseltamivir and amantadine of seasonal influenza A and B viruses, circulating in Portugal from the 2004/2005 to the 2008/2009 winter seasons, obtained through the national research project mentioned above are reported and discussed.

2. Materials and methods

2.1. Influenza virus strains analysed

The influenza virus strains analysed in this study were isolated from nasopharyngeal swabs collected from influenza-like illness patients who consulted with a Sentinel Medical Practitioner or attended an Emergency Unit participating in the National Influenza Surveillance Programme. The respiratory specimens were accompanied by a notification form containing epidemiological and clinical information related to the patient and, since 2005/2006, information regarding prescription and exposure (either by direct use or contact with a patient on therapy) of patients to influenza antiviral drugs before specimen collection. Every time a notification form indicated drug prescription and the presence of influenza virus was detected on the swab, a second respiratory specimen from the patient was requested, collected at the end of antiviral therapy (4th or 5th day).

Viral isolation was performed in MDCK or MDCK-SIAT1 cells, according to the WHO Manual on Animal Influenza Diagnosis and Surveillance (2002). Antigenic characterization of viral isolates was performed by (1) hemagglutination inhibition assays for determination of HA type or subtype, following the protocol included in the WHO Influenza Reagent Kit for Identification of Influenza Isolates, and; (2) TaqMan real-time PCR for determination of neuraminidase (NA) influenza A subtype, using a modified version of the protocol described by Schweiger et al. (2000).

Susceptibility to amantadine was evaluated for a total of 178 influenza A virus strains, 127 of A(H3N2) and 51 of A(H1N1) subtype, isolated from the 2004/2005 to the 2006/2007 winter seasons (Table 1). This evaluation was carried out at the Health Protection Agency (London, United Kingdom) during 2007, using all influenza A virus strains (from the winter seasons referred) for which a sufficient volume for performing the analysis was available at that moment. The majority of these strains were tested using a 2nd or 3rd cell passage isolate. A total of 340 influenza virus strains isolated from the 2004/2005 to the 2008/2009 winter seasons, 117 of A(H3N2) subtype, 93 of A(H1N1) subtype and 130 of B type, were tested for oseltamivir susceptibility by fluorescence assay (Table 1). The majority of these strains were tested using a 2nd to 4th cell passage isolate. From all the strains isolated during the winter seasons considered for this study, only those that did not exhibited a NA activity equal or higher than 25,000 relative fluorescence units (RFUs), after all the possible re-isolations procedures for increasing activity, were not tested. Wild-type reference influenza virus strains susceptible to NAIs were used as assay controls on fluorescence assay. These were: A/Wisconsin/67/2005 (A(H3N2) subtype); A/Texas/36/1991 (A(H1N1) subtype) and; B/Memphis/20/1991 (B type). These reference strains were kindly provided by Dr. Alan Hay (National Institute for Medical Research, Mill Hill, London, United Kingdom). From the 340 virus strains tested with the fluorescence assay, 134 were further analyzed by NA gene sequencing, including all strains identified as statistical outliers and approximately 25% of non-outliers (randomly selected) (Table 1).

2.2. Oseltamivir

Oseltamivir carboxylate, the active compound of the ethyl ester prodrug oseltamivir phosphate, was provided in the form of a D-tartrate salt by F. Hoffmann-La Roche Ltd. (Basel, Switzerland), through a material transfer agreement.

2.3. Susceptibility to amantadine

Susceptibility to amantadine was evaluated by pyrosequencing using the standard operating procedure (SOP) provided by the European Surveillance Network for Vigilance against Viral Resistance (VIRGIL), which included (1) the amplification of the influenza A M2 gene for amantadine sensitivity pyrosequencing and (2) the preparation of DNA for pyrosequencing using the PyroMark Vacuum Prep Workstation. The 2 steps of the VIRGIL SOP were based on the pyrosequencing manufacturer's protocol (Biotage, Uppsala, Sweden) previously described by Bright et al. (2005). Two relevant modifications were introduced in each step. For the amplification of the M2 gene, 5 μl of viral RNA was used in a 50 μl reaction mixture and the number of amplification cycles was increased to 35. For the preparation of DNA for pyrosequencing, the M2 sequencing primer was used at a final concentration of 0.44 μM and the duration of the annealing step was halved (2 min). Pyrograms were obtained through a SNP (AQ mode) analysis specific for the 5 point mutations associated with the development of resistance to amantadine: L26F/I; V27A/D; A30T; S31N; and G34E (Hay et al., 1985,

Table 1
Number of influenza virus strains analyzed for amantadine and oseltamivir susceptibility/number of influenza virus strains isolated at national level (percentage), by influenza (sub)type or B lineage and winter season.

Winter season	A(H3N2)			A(H1N1)			B					Total[a]		
	Amantadine	Oseltamivir		Amantadine	Oseltamivir		Oseltamivir					Amantadine	Oseltamivir	
	Pyro	Fluo	NA seq	Pyro	Fluo	NA seq	Fluo			NA seq		Pyro	Fluo	NA seq
							Yam	Vic	Both	Yam	Vic	Both			
2004/2005	75/89 (84.3)	76/89 (85.4)	20/89 (22.5)	6/6 (100)	4/6 (66.7)	3/6 (50)	31/33 (93.9)	2/2 (100)	33/35 (94.3)	1/33 (3.0)	1/2 (50)	2/35 (5.7)	81/95 (85.3)	113/130 (86.9)	25/130 (19.2)
2005/2006	1/1 (100)	1/1 (100)	1/1 (100)	45/46 (97.8)	39/46 (84.8)	14/46 (30.4)	1/1 (100)	48/48 (100)	49/49 (100)	0/1 (100)	2/48 (4.2)	2/49 (4.1)	46/47 (97.9)	89/96 (92.7)	17/96 (17.7)
2006/2007	51/76 (67.1)	20/76 (26.3)	15/76 (19.7)	0[b]	0[b]	0[b]	1/1 (100)	1/1 (100)	2/2 (100)	1/1 (100)	1/1 (100)	2/2 (100)	51/76 (67.1)	22/78 (28.2)	17/78 (21.8)
2007/2008	0[b]	0[b]	0[b]	0/29 (0)[c]	29/29 (100)	22/29 (75.9)	43/43 (100)	2/2 (100)	45/45 (100)	16/45 (35.6)	1/2 (50)	17/45 (37.8)	0/29 (0)[c]	74/74 (100)	39/74 (52.7)
2008/2009	0/21 (0)[c]	20/21 (95.2)	14/21 (66.7)	0/21 (0)[c]	21/21 (100)	21/21 (100)	0[b]	1/1 (100)	1/1 (100)	0[b]	1/1 (100)	1/1 (100)	0/42 (0)[c]	42/43 (97.7)	36/43 (83.7)
Total	127/187 (67.9)	117/187 (62.6)	50/187 (26.7)	51/102 (50.0)	93/102 (91.2)	60/102 (58.8)	76/78 (97.4)	54/54 (100)	130/132 (98.5)	18/78 (23.1)	6/54 (11.1)	24/132 (18.2)	178/289 (61.6)	340/421 (80.8)	134/421 (31.8)

The abbreviations used in the table correspond to: pyrosequencing (Pyro); fluorescence assay (Fluo); and neuraminidase gene sequencing (NA seq).
[a] Considering both lineages of influenza B virus.
[b] None influenza virus strain from this subtype/B lineage was isolated during this winter season.
[c] Pyrosequencing was not performed on influenza A isolates from this winter season.

1991; Hurt et al., 2007). These procedures were carried out at the Health Protection Agency (London, United Kingdom).

2.4. Susceptibility to oseltamivir

Susceptibility to oseltamivir was first evaluated by a phenotypic fluorescence assay and then through sequencing of NA gene coding region.

2.4.1. Fluorescence assay

Fluorescence assay was performed using the SOP provided by VIRGIL for determination of influenza virus susceptibility to neuraminidase inhibitors, available to the general public from http://www.nisn.org/documents/Zambon_-_VIRGIL_IC50_SOP.pdf. This SOP is based on the method developed by Potier et al. (1979), in which the amount of fluorescent product 4-methylumbelliferone that is cleaved from the substrate 2′,2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate (MUNANA) by the NA of influenza viruses is measured. A NA activity assay was initially performed in order to ensure that equivalent NA activities were compared against oseltamivir. For each influenza virus strain, the dilution in the linear portion of the enzyme activity curve to be used in the subsequent NA inhibition assay was determined. With this second assay, the concentration of oseltamivir required to inhibit 50% of the NA activity of each influenza virus strain (IC_{50} value) was determined.

2.4.2. NA gene sequencing

RNA extraction was performed either using a modified version of the protocol described by Boom et al. (1990) or the QIAamp Viral RNA Mini Kit (QIAGEN, Germany). A conventional two-step RT-PCR was used for the amplification of 3 overlapping segments, with a length varying from 490 to 700 bp, which covered the entire NA gene sequence. RT reaction was performed as described by Ellis et al. (1997) and the PCR reaction consisted of 10 μl of cDNA added to 40 μl of a reaction mixture containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1 mM (N1 subtype and B type) or 1.25 mM (N2 subtype) $MgCl_2$, 12.5 pmol (N1 subtype and B type) or 5 pmol (N2 subtype) of each primer, and 1.5 U of Taq DNA polymerase. PCR cycling conditions included: 2 min at 95 °C; 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °C (N1 and N2 subtypes) or 52 °C (B type) and 2 min extension at 72 °C; and cooling at 8 °C. Purification of the amplified segments was carried out using the QIAquick PCR Purification Kit (QIAGEN, Germany), and the resulting DNA quantified by spectrophotometry. Nucleotide sequences of the purified segments were obtained on an automatic sequencer ABI PRISM Biosystems 3130XL Genetic Analyser after using the Big Dye Terminator Cycle Sequencing Ready Reactions kit v1.1, both according to the manufacturer's instructions (Applied Biosystems). For each of the 3 different amplified segments, 3 sequencing primers were used (2 forward and 1 reverse). The 9 overlapping sequences of NA gene, obtained for each influenza virus strain, were then assembled and edited using the SeqMan II software from the Lasergene package v4.05 (DNASTAR Inc.). The alignment of the NA coding sequences obtained for each NA (sub)type was performed with the Megalign software (from same package) using the neighbour-joining method. NA sequences of reference influenza virus strains, obtained from Influenza Sequence Database at https://isd.eiss.org/, were included in the alignments. The sequences of all primers used are available from the authors upon request.

2.5. Statistical analysis

The temporal distribution of the notification forms indicating (1) prescription or (2) no prescription of influenza antiviral drugs,

Table 2
Frequency (number) of influenza-like illness notification forms received with the indication of prescription and of no prescription of antiviral drugs during the 4 winter seasons for which this information was available (2005/2006 to 2008/2009).

Type		Winter season				p^a
		2005/2006 (N = 298)	2006/2007 (N = 493)	2007/2008 (N = 226)	2008/2009 (N = 600)	
Prescription	% (n)	2.0 (6)	2.2 (11)	3.1 (7)	1.0 (6)	0.191
	IC95%	0.74–4.33	1.12–3.96	1.25–6.28	0.37–2.16	
No prescription	% (n)	98.0 (292)	97.8 (482)	96.9 (219)	99.0 (594)	
	IC95%	95.67–99.26	96.04–98.88	93.72–98.75	97.84–99.63	
No information	n	240	237	67	129	-

[a] Pearson's Chi-Square Test.

was analyzed by a Pearson Chi-Square Test. Additionally, 95% confidence intervals were determined according to Bliss (1967).

Regarding the fluorescence assay, NA activity and IC_{50} values were calculated through point-to-point curve fitting using the Microsoft Office Excel 2003 (Microsoft Office Professional Edition 2003). The identification of outliers was performed through the determination of lower and upper IC_{50} cut off values for each influenza (sub)type and winter season. The lower cut off value corresponded to 1.65 standard deviations (SD) and the upper cut off value to 3SD above the median IC_{50} value, using a robust estimation of the SD (AMC, 2001). Any influenza virus strain with an IC_{50} value higher than the lower cut off was classified as a minor outlier, and as a major outlier when the IC_{50} value was higher than the upper cut off. All statistical outliers identified were retested twice and the mean IC_{50} value was considered for analysis. An IC_{50} baseline level was also determined for each influenza (sub)type and winter season, corresponding to the median value $\pm$ 1 robust SD (outliers not included). This same determination (median value $\pm$ 1 robust SD) was performed for each of the 2 influenza B lineages in co-circulation (B/Yamagata and B/Victoria) but, in this case, outliers were included. The variation of IC_{50} values between winter seasons and influenza (sub)types/B lineages was analyzed performing an ANOVA one-way. If significant differences were identified, these were analyzed, if possible, by Bonferroni's correction post hoc test. All data obtained with the fluorescence assay was log-transformed before being used for statistical analysis in order to be normally distributed.

All statistical tests were performed using the SPS Statistics software v17.0 and, in all of them, a p value <0.05 was considered to be statistically significant.

3. Results

3.1. Prescription and exposure to influenza antiviral drugs

The information on antiviral drugs indicated on the notification forms received revealed that, from 2005/2006 to 2008/2009, antiviral drugs were rarely prescribed for the treatment of influenza-like illness symptoms. More specifically, indication of antiviral drug prescription was only found on 30 (0.02%) of the 1617 notification forms received during the winter seasons analysed. No significant differences were observed in the prescription frequency throughout the seasons (Pearson Chi-Square Test, p = 0.191) (Table 2).

Of the 30 cases of antiviral drug prescription notified, 27 (90.0%) were received through the Emergency Units Network of the National Influenza Surveillance Programme (Table 3). No particular pattern was observed when analysing the distribution of prescription cases during each winter season and by geographic origin (cases were from 5 regions of mainland Portugal). The same was not observed when considering the patient age group, with the majority of antiviral prescriptions occurring in patients belonging to the 15–44 years age group (22; 73.3%), which includes adolescents and younger adults. Of the 30 notifications of antiviral drug prescription, 4 (13.3%) were for patients who had previously been vaccinated for influenza. Two of these notifications, the 1 from 2005/2006 and the first of 2006/2007, occurred at the beginning of the season and originated on 2 young adults, while the other 2 occurred during the epidemic period of the season and were from an adult and from an older person (Table 3). Oseltamivir was the drug most prescribed, having been indicated in 19 (90.5%) of the 21 known antiviral prescriptions. Laboratory analysis indicated that influenza viruses were detected in only approximately half of the nasopharyngeal swabs associated with antiviral prescription (14; 46.7%). However, it is important to notice that in the last season (2008/2009) there was an absolute association between antiviral prescription and positive influenza infection (Table 3). The second respiratory specimen was obtained only from the 2 influenza positive cases detected in 2007/2008 and from 3 of the 6 ones detected in 2008/2009. Isolation, however, was unsuccessful.

Regarding the situation of exposure of patients to antiviral drugs before specimen collection, only 3 cases were notified between 2005/2006 and 2008/2009 (1 in 2006/2007 and 2 in 2008/2009). However, the antiviral to which the patients were exposed was not referred and the respiratory specimens that came with these notifications were negative for influenza virus infection. Because of this and of the lack of success on viral isolations performed on the second respiratory specimens, it was not possible to analyse the impact of antiviral drug use on influenza virus susceptibility to oseltamivir and amantadine.

3.2. Susceptibility to amantadine

Amantadine resistant marker S31N was identified on the M2 protein sequence of 39 (30.7%) of the 127 influenza A(H3N2) virus strains analyzed, specifically on the single virus strain from 2005/2006 and on 38 (74.5%) of the 51 virus strains from 2006/2007. In the origin of this resistance is a G to A substitution in the second nucleotide of the codon that codes for the amino acid at position 31 of the M2 protein sequence. Using the epidemiological information available, it was possible to observe that A(H3N2) amantadine resistant strains from 2006/2007 were isolated from respiratory specimens collected during the whole winter season and from patients of all age groups and from almost all geographic regions of mainland Portugal.

Regarding the A(H1N1) subtype, all 51 influenza virus strains analysed were found to be susceptible to amantadine, since none of the 5 molecular markers of resistance to this antiviral drug was identified in their M2 protein sequence.

3.3. Susceptibility to oseltamivir

3.3.1. Fluorescence assay

3.3.1.1. IC_{50} baseline level. No significant differences were observed on the IC_{50} baseline levels obtained for the influenza A(H3N2) subtype (ANOVA one-way, p = 0.303) (Table 4). This situation was not observed, however, for both influenza A(H1N1)

Table 3
Epidemiological and laboratory information regarding each of the 30 cases of antiviral drug prescription notified during the 4 winter seasons for which this information was available (2005/2006 to 2008/2009).

Winter season	Week	Notification form						lst nasopharyngeal swab		2nd nasopharyngeal swab	
		Source	Geographic origin	Patient			Antiviral	Infection with influenza virus	Isolation in cell culture	Infection with influenza virus	Isolation in cell culture
				Gender	Age (years)[a]	Vaccination					
2005/2006	41	EU	Alentejo	F	54	No	NR	N	–	–	
	44	EU	North	F	69	No	NR	N	–	–	–
	45	EU	Lisboa	F	43	Yes	NR	P (B type)	–	–	–
	48	EU	Alentejo	F	22	No	NR	N	–	–	
	2	EU	North	F	NR	No	NR	N	–	–	
	3	EU	North	M	34	NR	NR	N	–	–	–
2006/2007	44	EU	Center	M	23	No	Oseltamivir	N	–	–	–
	50	EU	Center	F	31	Yes	NR	N	–	–	–
	2	EU	Alentejo	F	29	No	Oseltamivir	N	–	–	
		EU	Alentejo	F	44	No	Oseltamivir	P (subtype AH3)	–	–	
		EU	Alentejo	M	27	No	Oseltamivir	P (subtype AH3)	–	–	–
	6	SMP	Center	M	48	Yes	Oseltamivir	N	–	–	–
		SMP	Alentejo	F	NR	No	NR	N	–	–	–
		EU	Algarve	M	50	No	Oseltamivir	P (subtype AH3)	–	–	
	7	EU	Lisboa	M	28	No	Oseltamivir	P (subtype AH3)	–	–	
	8	EU	Alentejo	M	23	No	Oseltamivir	P (subtype AH3)	–	–	–
		EU	Alentejo	M	39	No	Oseltamivir	N	–	–	–
2007/2008	47	–	Lisboa	M	41	No	NR	N	–	–	–
	51	EU	Alentejo	M	22	No	Oseltamivir	N	–	–	–
		EU	Center	M	29	No	Oseltamivir	P (B type)	P (B/Lisboa/12/2008)	P (B type)	N
		EU	Alentejo	M	42	No	Oseltamivir	N	–	–	–
	8	EU	Alentejo	M	29	No	Zanamivir	N	–	–	
	10	EU	Center	M	38	No	Oseltamivir	P (B type)	P (B/Lisboa/45/2008)	N	
	13	EU	Center	M	17	No	Oseltamivir	N	N	–	–
2008/2009	51	EU	North	F	34	No	Zanamivir	P (subtype AH3)	P (A/Lisboa/44/2008)	N	–
	1	SMP	Algarve	F	73	Yes	Oseltamivir	P (subtype AH3)	P (A/Lisboa/45/2008)	P (A type)	N
	3	EU	North	F	26	No	Oseltamivir	P (subtype AH 1)	P (A/Lisboa/19/2009)	–	
	6	EU	Center	F	29	No	Oseltamivir	P (subtype AH1)	–	N	
	7	EU	Center	M	20	No	Oseltamivir	P (subtype AH1)	P (A/Lisboa/23/2009)	–	–
	9	EU	Center	F	14	No	Oseltamivir	P (subtype AH 1)	P (A/Lisboa/21/2008)	–	–

The abbreviations used in the table correspond to: Emergency Units (EU); Sentinel Medical Practitioners (SMP); male (M); female (F); not referred (NR); negative (N); positive (P).

[a] The following age group distribution was considered in the analysis: 0–4 younger children; 5–14 children and younger adolescents; 15–44 adolescents and younger adults; 45–64 adults; 65 or plus elderly.

Table 4
Oseltamivir IC_{50} baseline levels for each influenza (sub)type and winter season.

Influenza (sub)type	Median ± robust SD					p[a]	
	2004/2005	2005/2006	2006/2007	2007/2008	2008/2009	Between seasons	Between (sub)types
A(H3N2)	0.38 ± 0.13	0.24[b]	0.41 ± 0.04	-[c]	0.38 ± 0.10	0.303	
A(H1N1)	1.71 ± 0.11	1.47 ± 0.49	-[c]	1.88 ± 0.39	526.35 ± 201.75	0.000 (0.044 without 08/09)	0.000
B	13.08 ± 3.47	21.04 ± 3.01	18.82 ± 6.86[d]	19.59 ± 7.09	38.41[b,e]	0.000	

[a] ANOVA one-way.
[b] Only 1 influenza virus strain from this subtype was isolated during this winter season.
[c] None influenza virus strain from this subtype was isolated during this winter season.
[d] Only 2 influenza virus strains from this subtype were isolated during this winter season.
[e] Minor outlier but is the only B IC_{50} value obtained in this winter season.

Table 5
Oseltamivir IC_{50} baseline levels for each influenza B HA lineage and winter season.

Influenza B lineage	Median ± robust SD					p[a]	
	2004/2005	2005/2006	2006/2007	2007/2008	2008/2009	Between seasons	Between (sub)types
Yamagata	13.06 ± 3.13	14.35[b]	14.19[b]	20.12 ± 7.12	-[c]	0.000	
Victoria	21.74 ± 3.97[d]	21.27 ± 3.14	23.45[b]	16.20 ± 0.81[d]	38.41[b]	0.001	0.000
p[a]	0.005	0.011	–	0.254	–	–	

[a] ANOVA one-way.
[b] Only 1 influenza B virus from this lineage was isolated during this winter season.
[c] None influenza B virus from this lineage was isolated during this winter season.
[d] Only 2 influenza B virus from this lineage were isolated during this winter season.

subtype and type B viruses. The IC_{50} baseline levels determined for the A(H1N1) subtype differed markedly between the first 4 winter seasons analysed and the 2008/2009 season (ANOVA one-way, $p < 0.05$, Bonferroni's correction post hoc test). This high difference of approximately 300-fold, indicates a potential shift on the antiviral drug profile of A(H1N1) virus, from susceptible to resistant, between 2007/2008 and 2008/2009. Additionally, when removing the 2008/2009 IC_{50} values from the analysis, A(H1N1) IC_{50} baseline levels of 2005/2006 and 2007/2008 proved to be significantly different (ANOVA one-way, $p < 0.05$, Bonferroni's correction post hoc test). Regarding influenza type B, IC_{50} baseline levels varied significantly throughout winter seasons (ANOVA one-way, $p < 0.05$). However, it was not possible to perform the analysis of these differences through a post hoc test due to the existence of a single value for 2008/2009 (Table 4).

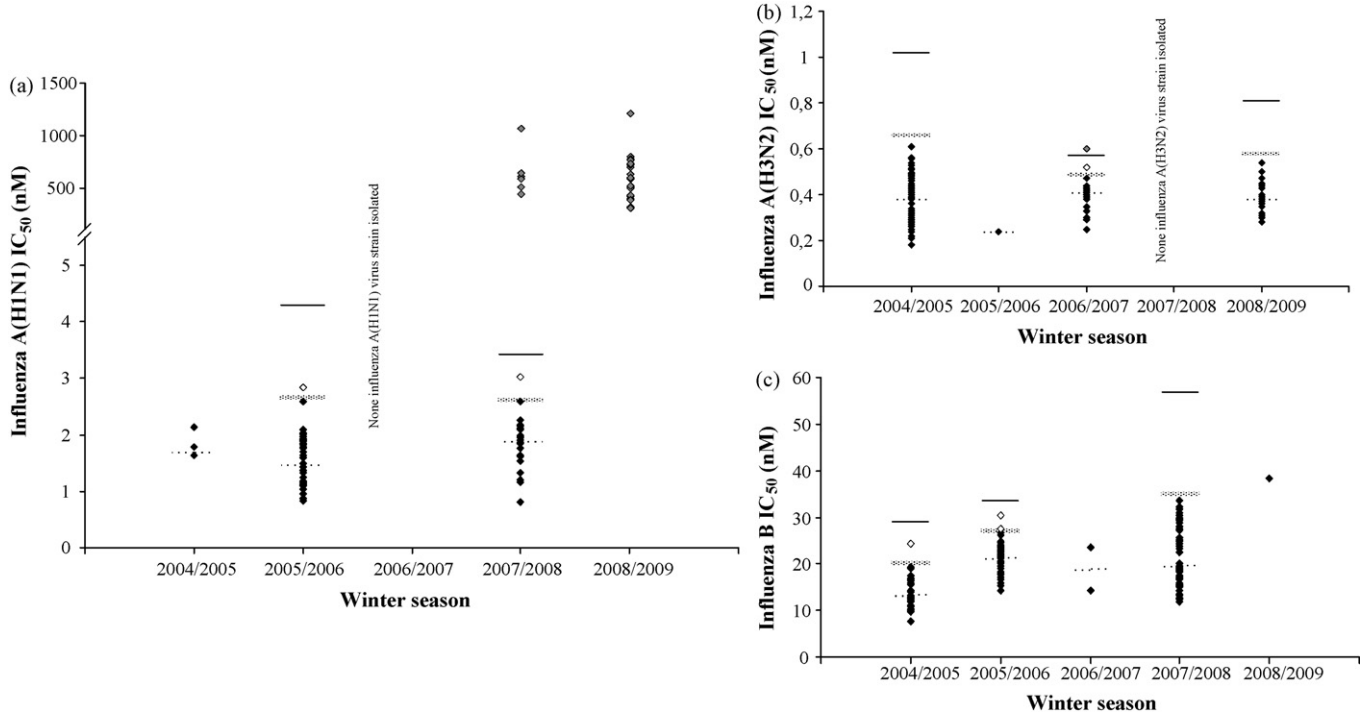

Fig. 1. IC_{50} values obtained by fluorescence assay for the influenza A(H1N1) (a), the influenza A(H3N2) (b) and the influenza B virus strains analyzed (c), in each of the winter seasons studied. Each (⧫), (◊) and (❖) represents an IC_{50} value obtained, being that the last 2 symbols represent also the minor and major outliers identified, respectively. The (·····) represents the IC_{50} median value, the (,,,,,,,,) the lower IC_{50} cut off value and the (_____) the upper IC_{50} cut off value. *Note*: Lower and upper cut off values were only determined when the number of virus strains tested were equal or higher than 10. When this did not occur, we considered for analysis the cut off values of the previous and/or the following winter season.

Oseltamivir IC_{50} baseline levels proved to differ significantly according to the NA (sub)type of influenza viruses, with NA type B virus exhibiting the higher levels, followed by the NA subtype A(N1) and by the NA subtype A(N2) virus (ANOVA one-way, $p < 0.05$) (Table 4).

Additionally, oseltamivir IC_{50} baseline levels differed significantly between the 2 co-circulating influenza B HA lineages, B/Yamagata and B/Victoria (ANOVA one-way, $p < 0.05$) (Table 5). B/Yamagata-like virus from 2004/2005 and 2005/2006 showed significant lower IC_{50} baseline levels when compared to B/Victoria-like virus from the same winter seasons (ANOVA one-way, $p = 0.005/p = 0.011$). For the remaining 3 seasons, the difference observed was not statistically significant, or it was not possible to perform the analysis. Significant differences were also observed on the IC_{50} baseline levels obtained for each influenza B lineage throughout the winter seasons (ANOVA one-way, $p < 0.05$). These differences appeared to occur between the 2007/2008 and the previous 3 winter seasons for the B/Yamagata lineage and between the 2008/2009 and the previous 4 winter seasons for the B/Victoria lineage (Table 5). However, the impossibility of performing a post hoc test does not allow confirmation at statistical level.

3.3.1.2. IC_{50} values and outlier determination. Regarding A(H1N1) influenza subtype, IC_{50} values ranged from 1.63 to 2.13 nM in 2004/2005, from 0.83 to 2.6 nM in 2005/2006, from 0.8 to 2.6 nM in 2007/2008 and from 314.04 to 1208.84 nM in 2008/2009 (Fig. 1a). All A(H1N1) virus strains from 2008/2009 were directly considered as potential oseltamivir-resistant strains, as a result of the high IC_{50} values exhibited by all of them. During the previous 4 winter seasons, 2 minor and 6 major A(H1N1) outliers were identified by statistical analysis. The virus strains classified as minor outliers exhibited an approximately 2-fold decrease in the susceptibility to oseltamivir (based on the IC_{50} median value). These were: A/Lisboa/25/2006 (IC_{50} of 2.85 nM), from 2005/2006; and A/Lisboa/8/2008 (IC_{50} of 3.02 nM), from 2007/2008. The 6 major outliers identified in 2007/2008 were: A/Lisboa/2/2008 (IC_{50} of 609.09 nM); A/Lisboa/3/2008 (IC_{50} of 584.82 nM); A/Lisboa/11/2008 (IC_{50} of 441.90 nM); A/Lisboa/20/2008 (IC_{50} of 1064.15 nM); A/Lisboa/27/2008 (IC_{50} of 642.05 nM), and; A/Lisboa/28/2008 (IC_{50} of 511.44 nM). These virus strains exhibited a 230–560-fold decrease in susceptibility to oseltamivir (based on the IC_{50} median value), having been, therefore, considered as potential oseltamivir-resistant strains (Fig. 1a).

Influenza A(H3N2) IC_{50} values ranged from 0.18 to 0.61 nM in 2004/2005, from 0.25 to 0.47 nM in 2006/2007 and from 0.28 to 0.54 nM in 2008/2009 (Fig. 1b). Only 2 A(H3N2) outliers were identified by statistical analysis, 1 minor and 1 major. These 2 outliers were from the 2006/2007 winter season, corresponding to the virus strains A/Lisboa/1/2007 (IC_{50} of 0.52 nM—minor) and A/Lisboa/54/2007 (IC_{50} of 0.6nM—major) (Fig. 1b). However, the difference observed between their IC_{50} values and the seasonal IC_{50} median value was less than 2-fold.

For influenza type B, IC_{50} values ranged from 7.73 to 19.39 nM in 2004/2005, from 14.35 to 26.72 nM in 2005/2006 and from 11.90 to 33.61 nM in 2007/2008 (Fig. 1c). A total of 4 minor outliers were identified, corresponding to the virus strains: B/Lisboa/12/2005 (IC_{50} of 24.41 nM), from 2004/2005; B/Lisboa/5/2006 (IC_{50} of 30.51 nM) and B/Lisboa/11/2006 (IC_{50} of 27.53 nM), from 2005/2006, and; B/Lisboa/1/2009 (IC_{50} of 38.41 nM), from 2008/2009 (Fig. 1c). Both B/Lisboa/12/2005 and B/Lisboa/1/2009 outlier strains exhibited an approximately 2-fold decrease in the susceptibility to oseltamivir (based on the IC_{50} median value), while for the other 2 outlier strains the difference observed was less than 2-fold.

3.3.2. NA gene sequencing

The analysis of the A(H1N1) virus NA sequences revealed the presence of the mutation His275Tyr (H275Y, N1 numbering) in the sequence of the 6 virus strains from 2007/2008 classified as major outliers on the fluorescence assay, and in all virus strains from 2008/2009. This mutation is associated with a high level of resistance to oseltamivir in the NA subtype (N1) virus, resulting from a C to T nucleotide substitution at position 823 of the coding sequence. Its identification in the NA sequence of the A(H1N1) virus strains that exhibited high fluorescence IC_{50} values allowed to confirm that 6 (20.7%) of the 29 A(H1N1) virus strains from 2007/2008 and that all (100%) A(H1N1) virus strains from 2008/2009 were resistant to oseltamivir. The NA sequence of these oseltamivir-resistant viruses is also characterized by the presence of the mutation Asp354Gly (D354G, N1 numbering), in relation to the reference strain A/Brisbane/59/2007. No amino acid changes different from those observed in the other co-circulating A(H1N1) virus strains were observed in the NA sequence of the 2 strains classified as minor outliers on the fluorescence assay.

The lack of different amino acid changes that could explain the reduction observed for oseltamivir susceptibility was also verified in the NA sequence of the 2 A(H3N2) virus strains classified as outliers on the fluorescence assay. However, the presence of a mixed population of virus with either an Asp (D) or an Asn (N) at residue 151 (G and A peak at position 451 of the N2 coding sequence) was found in the NA sequence of the non-outlier A/Lisboa/8/2007 virus strain, from 2006/2007. The mutation D151N has been associated with a reduction in the susceptibility of influenza A and B viruses to oseltamivir and/or zanamivir. However, this virus strain exhibited one of the lowest IC_{50} values (0.29 nM) obtained during 2006/2007.

Regarding influenza type B, the NA sequences of the 4 virus strains classified as minor outliers in the fluorescence assay were analysed in relation to NA sequences of reference B virus strains, and not to NA sequences of co-circulating B virus strains as performed for A(H1N1) and A(H3N2) subtypes. This difference is a consequence of the low number of influenza B strains isolated during 2006/2007 and 2008/2009 and of the few influenza B strains from 2004/2005 that were sequenced for NA. Two mutations were found in the NA sequence of the outlier B/Lisboa/12/2005: Ile240Val (I240V) and Glu404Gly (E404G) (B numbering). In the NA sequence of the outlier B/Lisboa/1/2009, 2 mutations were also identified: Arg65Leu (R65L) and Ala358Glu (A358E). None of these 4 mutations are associated with reduction or with development of resistance to oseltamivir. In the NA sequence of the 2 outliers from 2005/2006, B/Lisboa/5/2006 and B/Lisboa/11/2006, no different amino acid changes were observed that could explain the reduced susceptibility.

4. Discussion

This study revealed that antiviral drugs were rarely prescribed at national level for the treatment of seasonal influenza, from 2005/2006 to 2008/2009. This may be a direct consequence of the perception that these antivirals would be crucial and should only be used during a pandemic. The fact that the information obtained is limited to the population under observation, in the context of the National Influenza Surveillance Programme, should also be taken into account. In fact, the information was collected from patients that use primary and secondary public health care systems where the delay in consultation upon onset of symptoms leads to unsuitability of antiviral drug prescription. Most of the antiviral prescriptions were indicated for patients belonging to the 15–44 age group (adolescents and younger adults), but no information was available about their risk of developing serious complications from influenza. This information could have been useful when analyzing the 4 cases of drug prescription in previously vaccinated

individuals that, most probably due to recent vaccination, did not have a sufficient level of immunity against circulating influenza viruses. Oseltamivir was the antiviral drug most prescribed. The reasons for this could have been its advantage over zanamivir in terms of administration route and pharmacokinetics, and the high level of resistance observed for M2 inhibitors (Democratis et al., 2006; Deyde et al., 2007). Additionally, M2 inhibitors in Portugal are essentially used for treatment of Parkinson's disease. One important finding of this study, which raises concerns on how antiviral drugs are being used, was that approximately half of the prescriptions were made for individuals who were not infected with influenza.

Resistance to amantadine was only observed for the A(H3N2) influenza subtype, with the identification of the mutation S31N on the M2 protein sequence of the only strain isolated in 2005/2006 and of 74.5% of the strains from 2006/2007. This mutation is the most common mechanism of resistance to M2 inhibitors, being already known that is occurrence on the M2 protein constricts the size and increases the polarity of the antiviral drug binding site (Stouffer et al., 2008; Weinstock and Zuccotti, 2006). The situation observed at the national level is in agreement with the global spread of an A(H3N2) virus strain carrying the S31N mutation that has been observed since 2002/2003 (Bright et al., 2005).

In respect to oseltamivir, resistance was only observed for seasonal A(H1N1) influenza virus and at an increasing frequency of 20.7% in 2007/2008 to 100% in 2008/2009. These resistant viruses exhibited extremely high IC_{50} values, between 314.04 and 1208.84 nM, and carried the mutation H275Y (N1 numbering) in their NA protein sequence. The mechanism of resistance associated with the presence of this mutation is already known, consisting of prevention of formation of the pocket in the NA active site of influenza N1 subtype virus, which is essential for the high affinity binding of oseltamivir (Moscona, 2005; Wang et al., 2002). The role of the D354G mutation that was also found in the NA sequence of all A(H1N1) oseltamivir-resistant virus is still unclear. The situation observed at the national level is in agreement with the widespread and sustained transmission of oseltamivir A(H1N1) resistant virus observed worldwide since 2007/2008 (ECDC, 2008). Until now, no clear reasons were found for the emergence and global spread of this A(H1N1) resistant virus.

No different mutations, comparing to those observed in other co-circulating strains from the same subtype, were identified in the NA sequence of the A(H1N1) and A(H3N2) virus strains that exhibited a reduction for oseltamivir susceptibility at the phenotypic level (fluorescence minor outliers). For this reason, a more detailed genotypic analysis of these strains will be attempted, involving the sequence of other segments of the genome, particularly HA. The presence of the mixed population of D151 wild-type and N151 mutant virus on the non-outlier A/Lisboa/8/2007 virus strain is now being further studied, given the described association of D151N mutation to a reduction on NAIs susceptibility (McKimm-Breschkin et al., 2003; Sheu et al., 2008). For influenza type B, due to the limited number of sequences available, it was not possible to verify if the 2 mutations identified exclusively on 2 of the minor outliers (B/Lisboa/12/2005 and B/Lisboa/1/2009) were associated with the reduction on oseltamivir resistance, observed at phenotypic level, or are simply a result of antigenic drift. However, it has to be taken into consideration that (1) B/Lisboa/12/2005 belongs to the B/Victoria lineage and was isolated during a season of B/Yamagata lineage dominance and; (2) that B/Lisboa/1/2009 was the only B virus isolated during 2008/2009, a season in which the occurrence of a HA and NA antigenic drift on the B/Victoria lineage, from B/Malaysia/2506/2004 to B/Brisbane/60/2008-like virus, was described. Further genotypic analysis of influenza B virus, involving NA sequencing and sequencing of other segments of the genome of outlier virus strains would be essential.

Susceptibility of influenza viruses to oseltamivir proved to differ significantly according to the NA (sub)type. This difference was described in previous studies and was expected to occur here, having been associated with the existence of minor structural differences between the NA active sites of each influenza NA sub(type) virus, which would result in different oseltamivir binding affinities (Boivin and Goyette, 2002; Escuret et al., 2008; Ferraris et al., 2005; Hurt et al., 2004; McKimm-Breschkin et al., 2003). The reduction in susceptibility to oseltamivir observed from 2005/2006 to 2007/2008 in the A(H1N1) subtype was most probably a result of NA antigenic drift, from A/New Caledonia/20/1999-like virus to A/Brisbane/59/2007-like virus, that occurred between these winter seasons (results not shown). The co-circulation of 2 HA B lineages (B/Yamagata and B/Victoria) could help to explain the variation observed in the susceptibility of influenza B virus during the period of this study. These 2 lineages differed significantly in their susceptibility to oseltamivir, with B/Yamagata-like virus exhibiting significant lower IC_{50} values for 2 of the 3 winter seasons for which this analysis was possible to be performed. Additionally, this difference between B lineages has already been described by Lackenby et al. (2008b).

For the coming winter season(s), it would be of great importance to continue these studies and to advance to more specific research studies, even more now that the importance and critical role of antiviral drugs, particularly oseltamivir, has been demonstrated and highlighted for the A(H1N1) 2009 influenza pandemic.

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