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Original article

Virological Surveillance of Influenza in the eight epidemic seasons after the 2009 pandemic in Emilia-Romagna (Northern Italy)

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Summary. *Background and aim of the work*: Influenza virological surveillance is essential for monitoring the evolution of influenza viruses (IVs) as well as for annual updating of the vaccine composition. The aim of this study is to analyse IVs circulation in Emilia-Romagna during the eight epidemic seasons after the 2009 pandemic and to evaluate their match with seasonal vaccine strains. *Methods:* A total of 7882 respiratory specimens from patients with influenza-like illness (ILI), were collected by regional sentinel practitioners and hospital physicians. Viral investigations were conducted by rRT-PCR assay. Genetic characterization was performed for a spatial-temporal representative number of influenza laboratory-confirmed specimens. *Results:* Influenza-positive samples per season ranged between 28.9% (2013-2014) and 66.8% (2012-2013). Co-circulation of IVs type A and type B was observed in all seasons, although with a different intensity. In all seasons, the highest number of positive samples was recorded in younger patients aged 5-14 years with relative frequencies ranging from 40% in the 2013-2014 season and 78% in the 2012-2013 season. Since the 2009 pandemic, A/H1N1pdm09 IVs circulating were closely related to the vaccine strain A/California/7/2009. Antigenic mismatch between vaccine strain and A/H3N2 IVs was observed in the 2011-2012 and 2014-2015 seasons. During 2015-2016, 2016-2017 and 2017-2018 seasons a complete or nearly complete mismatch between the predominant influenza B lineage of IVs type B circulating and vaccine B lineage occurred. *Conclusions:* This analysis confirms the importance of the virological surveillance and highlights the need of a continuous monitoring of IVs circulation, to improve the most appropriate vaccination strategies. (www.actabiomedica.it)

Key words: influenza virus, virological surveillance, antigenic characterisation, B lineage, vaccine virus strain, mismatch

Introduction

Seasonal influenza is an acute, highly contagious viral respiratory infection of great importance from clinical and epidemiological point of view. Worldwide, the annual attack rate is estimated at 5-10% in adults and 20-30% in children with about 3 to 5 million case of severe illness and 290.000-650.000 deaths (1).

Influenza epidemiology mainly depends on the particular characteristics of influenza viruses (IVs), able to spread all over the world and rapidly evolve. Furthermore, a gradual and relatively continuous change in the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), allows them to escape the immunity that comes from prior infections or vaccination (2). Because of this, seasonal epidemics recur every year with different intensity and trend.

The impact of influenza also varies according to different age groups, in terms of morbidity, severe case illness and mortality among high-risk groups; the most

affected patients are the elderly, young children, pregnant women and individuals with comorbidity (1,3-9).

Periodically, in the range of 10-40 years, IVs type A caused pandemic events, due to the emergence of a new variant against which there is no pre-existing immunity in the population. The pandemic in 2009 was caused by a unique quadruple reassortant A/H1N1 IV, including a complex combination of swine, avian and human IV genes (10). This new variant, A/H1N1pdm09, has completely substituted the previous seasonal IVs of the same type and continues to circulate worldwide as seasonal IV, together with A/H3N2 subtype IVs and B type IVs. Moreover, a progressive diversification of B type IVs into two lineages, genetically and antigenically distinct, occurred starting from 1983 (11).

Within this context, influenza epidemiological and virological surveillance plays an essential role and it is carried out, at global level, by World Health Organization Global Influenza Surveillance and Response System (GISRS) (12) and at European level, by the European Centre for Disease Prevention and Control (ECDC) (13). The national influenza surveillance systems, together with the analysis of epidemiological features, have the specific goal to monitor the circulation of IVs, analyse antigenic, genetic and biological characteristics, also including the susceptibility to available antiviral drugs. Moreover, they work to recognize any new viral variants in order to implement the appropriate containment and prevention strategies in a timely manner (14). In Italy, these activities are carried out by influenza surveillance system, named InfluNet, coordinated by the National Influenza Centre at the Istituto Superiore di Sanità (NIC/ISS). Influnet is based on the collaboration of sentinel practitioners who, starting from the 46th week of each year until the 17th of the following year, perform respiratory samples from patients with a clinical presentation of influenza-like illness (ILI) (15-17). The regional influenza Laboratories perform isolations and antigenic characterizations of IVs during the epidemic season, to constantly monitor the different types/subtypes circulation and to evaluate the match between epidemic and vaccine strains. This is fundamental to formulate the vaccine composition of the following season, because the degree of match between circulating and vaccine strains is one of the factor, togheter with the characteristics of the person being vaccinated (such as their age and health), that contribute to the vaccine effectiveness (18-24).

Furthermore one of the main Public Health implication of the low vaccine effectiveness, togheter with the decrease and the poor vaccination uptake, even in the target population, is the real reduction of effectiveness of influenza vaccine on field. (19,25-28).

The aim of this paper is to describe the circulation of IVs in Emilia-Romagna and their matching with seasonal vaccine strains, during eight consecutive seasons (from 2010-2011 to 2017-2018) by analysis of virological surveillance data, performed by the regional influenza reference Laboratory of Parma (29-31).

Methods

Respiratory specimens from children and adults with a clinical presentation of ILI, were collected by regional sentinel physicians (general practitioners and pediatricians) and by hospital physicians of the care Units of Parma, Piacenza and Reggio Emilia hospital. Each clinical specimen was accompanied by a case report form filled in with epidemiological data. The "Virocult" diagnostic kit (MWE, England) and the commercial "UTM Viral Transport Media" kit (Copan, Brescia, Italy) were used to collect the clinical samples (32).

Viral isolation was performed in Madin Darby Canine Kidney cells (MDCK), and the presence of the virus was detected by a conventional haemagglutination assay using a 0,8% suspension in PBS of guinea pig red blood cells.

Viral nucleic acid was extracted from respiratory specimens and from viral cell culture supernatant, using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. A one-step Real Time retro-transcription PCR assay (rRT-PCR) was performed with Quantifast Pathogen+IC Kit, (Qiagen, Hilden, Germany**)** with specific primer/probe sets targeting the matrix region of A type IV and the nucleoprotein region of B type IV. For A type IV, samples were further subtyped using specific primer/probe sets for the HA gene to dis-

criminate between A/H1N1pdm09 IVs and A/H3N2 IVs. The genetic lineage of confirmed B type IVs was determined by rRT-PCR. All assays were performed in compliance with institutional guidelines (33-34).

A representative number of influenza laboratory-confirmed specimens and viral isolates, were sent to the NIC/ISS for antigenic characterisations and phylogenetic analysis.

Results

During the study period, the reference Laboratory for influenza virological surveillance of Emilia-Romagna, analysed 7882 nasal or throat swabs, performed by regional sentinel practitioners and doctors working in hospital care Units of Parma, Piacenza and Reggio Emilia. The distribution of samples by season, provenience, age group, vaccination status, virological data, is reported in Table 1. Overall, the percentage of the samples range between 6.2% in 2013-2014 season and 18.6% in 2017-2018 season; 50.3% were outpatients and 49.7% were inpatients. In the first six seasons, the highest numbers of samples were collected in children ≤4 years of age and in school-aged children (5-14 years); in the two latest years, in young-adults (15-64 years) and in elderly ≥65 years of age. The percentage of influenza-positive samples per season ranged between 28.9% (2013-2014) and 66.8% (2012-2013). In the first three seasons after the 2009 pandemic, epidemics were particularly intense due to the highest number of specimens and influenza-positive samples, while in the following five seasons, the proportion of positive samples was lower (less than 50%) compared to the high number of samples (Table 1). In the surveyed area, epidemiological trends observed in the first six influenza

Table 1. Characteristics of patients during virological surveillance in Emilia-Romagna from 2010-2011 to 2017-2018 season

	FLU SEASON 2010-2011	FLU SEASON 2011-2012	FLU SEASON 2012-2013	FLU SEASON 2013-2014	FLU SEASON 2014-2015	FLU SEASON 2015-2016	FLU SEASON 2016-2017	FLU SEASON 2017-2018
Overall n.	747	695	922	491	1327	1134	1095	1471
Outpatients n (%)	478 (64%)	617 (88.8%)	511 (55.4%)	251 (51.1%)	645 (48.6%)	618 (54.5%)	415 (37.9%)	428 (29%)
Inpatients n (%)	269 (36%)	78 (11.2%)	411 (44.6%)	240 (48.9%)	682 (51.4%)	516 (45.5%)	680 (62.1%)	1043 (71%)
Age group (years) n (%)								
0-4	212 (28.4%)	298 (43%)	313 (33.9%)	158 (32.2%)	393 (29.6%)	340 (30%)	249 (22.7%)	311 (21.2%)
5-14	208 (27.9%)	192 (27.6%)	215 (23.4%)	111 (22.6%)	301 (22.7%)	324 (28.6%)	191 (17.5%)	168 (11.4%)
15-64	249 (33.3%)	155 (22.3%)	254 (27.5%)	122 (24.9%)	258 (19.4%)	258 (22.7%)	227 (20.7%)	401 (27.3%)
≥65	63 (8.4%)	45 (6.4%)	129 (14%)	99 (20.1%)	366 (27.6%)	209 (18.4%)	425 (38.8%)	591 (40.1%)
Unknown	15 (2%)	15 (0.7%)	11 (1.2%)	1 (0.2%)	9 (0.7%)	3 (0.3%)	3 (0.3%)	-
Vaccination Status n (%)								
Unvaccinated	514 (68.8%)	464 (66.8%)	695 (75.4%)	351 (71.5%)	977 (73.6%)	944 (83.2%)	867 (79.2%)	1104 (75.1%)
Vaccinated	151 (20.2%)	169 (24.3%)	136 (14.7%)	84 (17.1%)	233 (17.6%)	190 (16.8%)	228 (20.8%)	278 (18.9%)
Missing Information	82 (11%)	62 (8.9%)	91 (9.9%)	56 (11.4%)	117 (8.8%)	-	-	89 (6%)
Outcome n (%)								
Positive	379 (50.7%)	449 (64.6%)	616 (66.8%)	142 (28.9%)	581 (43.8%)	394 (34.7%)	392 (35.8%)	597 (40.6%)
Negative	368 (49.3%)	246 (35.4%)	306 (33.2%)	349 (71.1%)	746 (56.2%)	740 (65.3%)	703 (64.2%)	874 (59.4%)
Influenza Virus type/subtype n (%)								
A/H3N2	14 (3.7%)	447 (99.5%)	16 (2.6%)	103 (72.5%)	268 (46.2%)	34 (8.6%)	380 (97.2%)	15 (2.5%)
A/H1N1pdm09	177 (46.7%)	-	152 (24.8%)	37 (26.1%)	239 (41.1%)	49 (12.4%)	1 (0.2%)	233 (39.2%)
Influenza B	188 (49.6%)	2 (0.5%)	446 (72.6%)	2 (1.4%)	74 (12.7%)	311 (79%)	10 (2.6%)	347 (58.3%)

seasons were quite similar and analogous to that of the period prior to the 2009 pandemic. Concerning the official virological surveillance period, all six seasons after the 2009 pandemic started at the beginning of December (weeks 50-51) and peaked in February (weeks 5-6). The first IVs were detected between the end of the year and the beginning of the new one. All six seasons were characterized by temporally long epidemics, that declined on March and April. During 2016-2017 and 2017-2018 seasons, a clear shift was observed in epidemiological trend: influenza activity started about four weeks in advance, with a rapid increase of ILI and influenza-positive samples, and peaked between late December and early January (weeks 51-52).

The distribution of detected IVs is presented in Figure 1. During the study period, co-circulation of A type IVs and B type IVs was observed, although with a different intensity; in five seasons A type IVs predominated over B type IVs. An overview of the eight seasons shows a mixed IVs circulation: co-circulation of A/H1N1pdm09 IVs and B type IVs in 2010-2011 season (46.7% vs 49.61%); co-circulation of A/H1N1pdm09 IVs and A/H3N2 IVs in 2014-2015 season (41.1% vs 46.2%). During 2011-2012, 2013-2014, 2014-2015 and 2016-2017 seasons, A/H3N2 IVs were predominant (99.5%, 72.5%, 46.2% and 97.2% respectively); during 2012-2013, 2015-2016, 2017-2108 seasons, B type IVs were predominantly detected (72.6%, 79.0%, 58.3% respectively). In all seasons, the highest number of positive samples were recorded in younger patients 5-14 aged with relative frequencies ranging from 40% in the 2013-2014 season and 78% in the 2012-2013 season (Table 2, Figure 2). During every season, a spatial-temporal representative number of influenza-positive samples was genetically characterized and the phylogenetic analysis was performed by the NIC/ISS (16).

The antigenic and molecular characteristics of IVs circulating were analysed, with particular attention to the match with seasonal vaccine strains (Table 3).

In Emilia-Romagna most of IVs detected in the 2010-2011, and 2017-2018 seasons were A/H1N1pdm09 IVs. Since 2010-2011 season, A/H1N1pdm09 IVs were closely related to the vaccine strain A/California/7/2009. In particular, A/H1N1pdm09 IVs isolated in Emilia-Romagna during 2015-2016 season fell into genetic groups 6 and 8 (A/St.PetersburG/27/2011-like, A/Norway/2552/2010-like and A/South Africa/3626/2013-like). During 2016-2017 and 2017-2018, A/H1N1pdm09 IVs fell into genetic subgroup 6B.1, characterized by the amino acid substitutions S84N, S162N, I216T in HA1, antigenetically correlate to vaccine strains A/California/7/2009 and A/Michigan/45/2015 that was recommended vaccine strain for the 2018-2019 season (Table 3) (35).

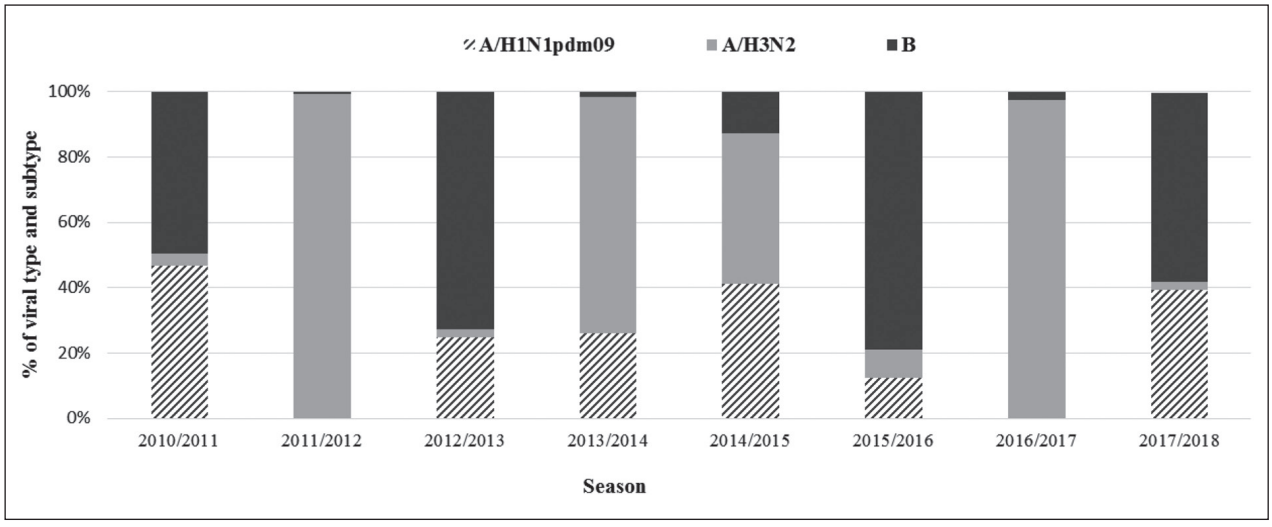

Figure 1. Influenza virus types/subtypes distribution in Emilia-Romagna from 2010-2011 to 2017-2018 season

Table 2. Number (%) of influenza virus positive by age groups and influenza season

Flu season	Age group (years)	Results		
		Flu negative n. (%)	Flu positive n. (%)	All n.
2010-2011	0-4	100 (47.2%)	112 (52.8%)	212
	5-14	66 (31.7%)	142 (68.3%)	208
	15-64	147 (59.0%)	102 (41.0%)	249
	≥65	47 (74.6%)	16 (25.4%)	63
2011-2012	0-4	98 (32.9%)	200 (67.1%)	298
	5-14	54 (28.1)	138 (71.9%)	192
	15-64	68 (43.9%)	87 (56.1%)	155
	≥65	23 (51.1%)	22 (48.9%)	45
2012-2013	0-4	106 (33.9%)	207 (66.1%)	313
	5-14	48 (22.3%)	167 (77.7%)	215
	15-64	95 (37.4%)	159 (62.6%)	254
	≥65	55 (42.6%)	74 (57.4%)	129
2013-2014	0-4	117 (74.1)	41 (25.9%)	158
	5-14	67 (60.4%)	44 (39.6%)	111
	15-64	86 (70.5%)	36 (29.5%)	122
	≥65	78 (78.8%)	21 (21.2%)	99
2014-2015	0-4	223 (56.7%)	170 (43.3%)	393
	5-14	136 (45.1%)	165 (54.8%)	301
	15-64	152 (58.9%)	106 (41.1%)	258
	≥65	232 (63.4%)	134 (36.6%)	366
2015-2016	0-4	237 (69.7%)	103 (30.3%)	340
	5-14	123 (38.0%)	201 (62.0%)	324
	15-64	197 (76.4%)	61 (23.6%)	258
	≥65	182 (87.1%)	27 (12.9%)	209
2016-2017	0-4	163 (65.5%)	86 (34.5%)	249
	5-14	87 (45.5%)	104 (54.5%)	191
	15-64	154 (67.8%)	73 (32.2%)	227
	≥65	296 (69.6%)	129 (30.4%)	425
2017-2018	0-4	164 (52.7%)	147 (47.3%)	311
	5-14	58 (34.5%)	110 (65.5%)	168
	15-64	254 (63.3%)	147 (36.7%)	401
	≥65	398 (67.3%)	193 (32.7%)	591

In Emilia-Romagna A/H3N2 IVs were identified only sporadically during 2010-2011 season, whereas they predominated in the 2011-2012, 2013-2014, 2014-2015 and 2016-2017 seasons.

A/H3N2 IVs circulating in 2010-2011 season were closely related to the vaccine strain A/Perth/16/2009, reconfirmed in the following season. In the first period of 2012-2013 season, A/H3N2

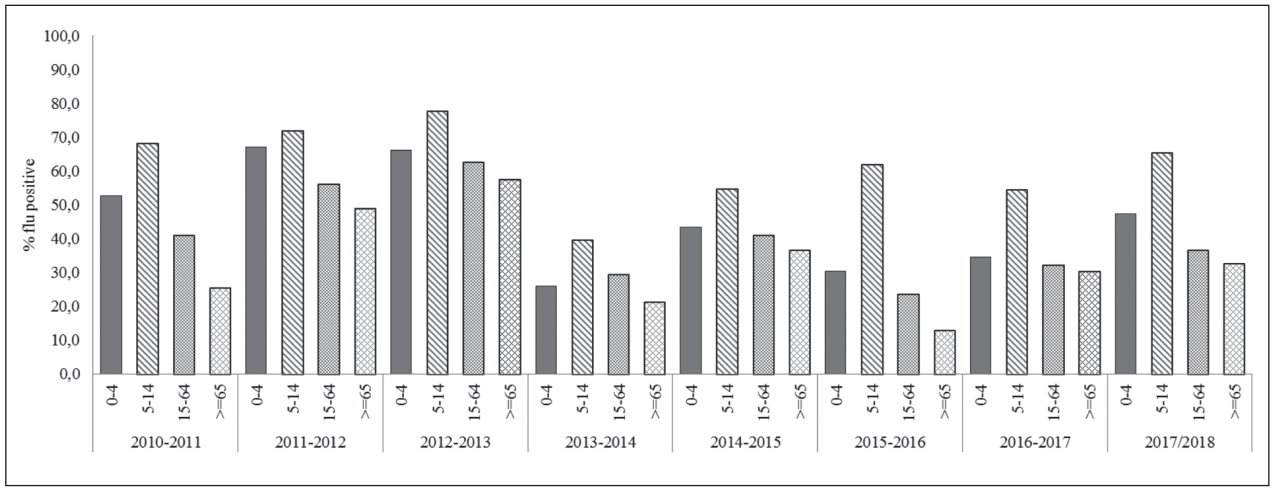

Figure 2. Distribution (%) of influenza positive samples by age group and by season (from 2010-2011 to 2017-2018)

Table 3. Circulating influenza viruses in Emilia-Romagna vs vaccine strains from 2010-2011 to 2017-2018 season (Predominant strain is indicated in bold; mismatch is highlighted)

Season	Trivalent vaccine composition (vaccine strains)			Circulating influenza viruses		
	A/H1N1pdm09	A/H3N2	B(lineage)	A/H1N1pdm09	A/H3N2	B(lineage)
2010-2011	A/California/7/2009	A/Perth/16/2009	B/Brisbane/60/2008 (VIC)	A/H1N1pdm09		**VIC**
2011-2012	A/California/7/2009	A/Perth/16/2009	B/Brisbane/60/2008 (VIC)		**A/H3N2**	
2012-2013	A/California/7/2009	A/Victoria/361/2011	B/Wisconsin/1/2010 (YAM)	A/H1N1pdm09		**YAM**
2013-2014	A/California/7/2009	A/Texas/50/2012	B/Massachusetts/2/2012(YAM)	A/H1N1pdm09	**A/H3N2**	
2014-2015	A/California/7/2009	A/Texas/50/2012	B/Massachusetts/2/2012(YAM)	A/H1N1pdm09	**A/H3N2**	
2015-2016	A/California/7/2009	A/Switzerland/9715293/2013	B/Phuket/3073/20132(YAM)			**VIC**
2016-2017	A/California/7/2009	A/Hong Kong/4801/2014	B/Brisbane/60/2008(VIC)		**A/H3N2**	**YAM**
2017-2018	A/Michigan/45/2015	A/Hong Kong/4801/2014	B/Brisbane/60/2008(VIC)	A/H1N1pdm09		**YAM**

IVs were correlated to the vaccine strain, while from January they were antigenically similar to variants of recent isolation (A/Alabama/5/2010-like, A/Hong Kong/3969/2011-like and A/Stockholm/18/2011-like). Genetic characterization showed different amino acid substitutions (K62E, K144N, T212A9) and molecular homology with a new variant, A/Victoria/361/2011, that replaced the vaccine strain of previous seasons. During 2012-2013 season, A/H3N2 IVs were antigenically correlated to the A/Texas/50/2012 strain, antigenically indistinguishable from the vaccine strain A/Victoria/361/2011, but more genetically stable for propagation and, for these reasons, recommended vaccine strain in the following season.

In 2013-2014 season, most of the A/H3N2 IVs were correlated to the different variants antigenically related to the vaccine strain A/Texas /50/2012. Phylogenetic analyses showed that most of them fell into genetic group 3C.3, with amino acid substitutions T128A, R142G and N145S in HA1 (reference virus: A/Samara/73/2013).

During 2014-2015 season A/H3N2 IVs presented with a mixed circulation of viral variants antigenically distinct from the vaccine strain. The HA

sequences of these viruses fell into two genetic subgroups: 3C.2, with amino acid substitutions N145S in HA1 and D160N in HA2 (reference virus A/Hong Kong/146/2013) and clade 3C.3a, with amino acid substitutions A138S, F159S, N225D in HA1, similar to A/Switzerland/9715293/2013, reference strain for 2015-2016 vaccine. The heterogeneous circulation of different A/H3N2 IVs strains was highlighted during the following seasons, with the emergence of variants grouped into genetic subgroup 3C.2, clade 3C.2a, with further amino acid substitutions (N144S, F159Y, K160T, N225D, Q311H). The reference strain A/Hong Kong /4801/2014, was the new vaccine strain. Also in the last two seasons, A/H3N2 IVs fell into genetic subgroup 3C.2a. However, in the last phase of 2017-2018 epidemic, viruses fell into sub-clade 3C.2a1, and shared similary with A/Singapore/INFIMH160019/2016, the reference strain for the vaccine of 2018/2019 season.

In Emilia-Romagna, type B IVs co-circulated with type A IVs in all seasons and predominated over type A during 2012-2013, 2015-2016, 2017-2018 seasons. With the exception of two seasons (2011-2012, 2013-2014), the co-circulation of both B lineages (B/Victoria/2/87 and B/Yamagata/16/88) was always observed, although with different intensity every year. The analysis of the HA gene sequence on a selection of type B IVs circulating in 2010-2011 season, showed that most of them belonged to B/Victoria lineage (B/Vic), antigenically similar to the vaccine strain B/Brisbane/60/2008 (B/Vic) and few to B/Yamagata lineage (B/Yam).

In 2012-2013 season, B/Yam viruses were predominant, related to B/Massachusetts/02/2012, strain recommended for 2013/2014 vaccine. During 2015-2016, 2016-2017 and 2017-2018 seasons a complete or nearly complete mismatch between the predominant influenza B lineage and vaccine B lineage occurred. In 2015-2016 season, type B IVs belonged to B/Vic lineage (clade 1A), related to B/Brisbane/60/2008, recommended in the 2016-2017 trivalent vaccine formulation.

During 2016-2017 season type B IVs circulating in Emilia-Romagna belonged to B/Yam lineage (clade 3), while in Italy, both B lineages co-circulated. The reference strain B/Brisbane/60/2008 (B/Vic) was reconfirmed for the following two seasons, where almost the whole B IVs belonged to B/Yam lineage circulated. Only one virus detected in Emilia-Romagna in 2016-2017 season belonged to B/Vic lineage.

Conclusions

This study provides the results of the virological surveillance in the eight epidemic seasons after the 2009 pandemic in Emilia-Romagna. The aim of this paper is to describe genetic and antigenic changes of IVs, and to evaluate their match with vaccine strains.

The circulation of IVs in Emilia-Romagna was similar to that of the other regions (36-39). Type B IVs co-circulated with type A IVs in all seasons. With the exception of 2011-2012 season during which only A/H3N2 subtype IVs circulated, in the others, both subtypes co-circulated, although with different intensity.

From a general point of view, yearly variations by distribution and frequency of viral types/subtypes were observed, as well as an alternation of the predominant type/subtype. The seasons with a modest circulation of a specific type/subtype, have been followed by seasons with its greater circulation and vice versa.

During all seasons, A/H1N1pdm09 IVs detected were closely related to the vaccine strain A/California/7/2009 and circulated intensely in 2010-2011 season, with the higher morbidity rates in school-aged children (aged 5-14 years) (Figure 2). Different considerations must be made for type A/H3N2 IVs and type B IVs. The continuous and rapid evolution of A/H3N2 IVs and the co-circulation of different A/H3N2 IVs strains during the same season caused an incomplete match between the vaccine strains and seasonal A/H3N2 IVs, so a new vaccine strain was recommended in the vaccine formulation in four seasons (35).

During seasons in which A/H3N2 IVs were predominant, the higher number of ILI occurred in the age group ≥65 years, more vulnerable to severe consequences of A/H3N2 IVs infection (40-47).

Furthermore, because of the presence of two genetically and antigenically distinct type B IVs lineages, co-circulating in the same season with different intensity, it was very difficult to predict the type of virus that will circulate in the following season. During the study

period, a complete mismatch between the type B IVs circulating and the vaccine strain, was observed in three consecutive seasons with a high number of cases and positive samples in children, young adults and elderly.

Overall, from this study we highlight that in none of the epidemic influenza season full match was achieved. Probably the Health Technology Assessment instruments, implemented with new studies on Artificial Intelligence, could help fill the information gap in the setting of the new influenza vaccine (48-55).

This study also confirms the importance of the virological surveillance and the integration of epidemiological and virological data, and highlights the need of a continuous monitoring of types/subtypes of IVs circulation during epidemic season, to acquire useful informations for improve the most appropriate vaccination strategies.

Conflict of interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article

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