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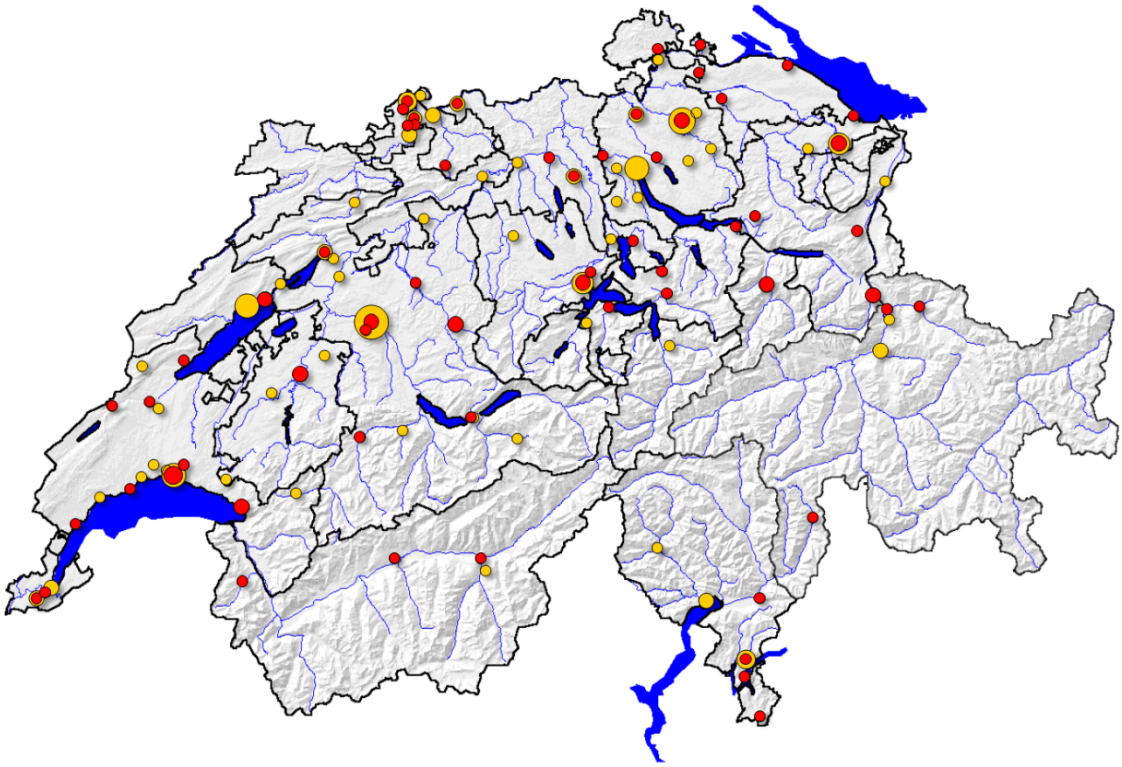
Influenza virus surveillance in Switzerland

Season 2011 – 2012



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Cover : Geographic distribution of Sentinel participating practitioners in Switzerland. See figure 1 of the present report for a detailed legend.

Contents

Abbreviations

FOPH: Swiss Federal Office of Public Health
HA: Hemagglutinin
HI: Hemagglutination inhibition test
HUG: University of Geneva Hospitals
MC-ILI: Medical consultations for influenza-like illness
MN: Microneutralization
MP: Matrix protein
NA: Neuraminidase
NI: Neuraminidase inhibitor
NRCI: National Reference Centre of Influenza
NSABB: National Science Advisory Board for Biosecurity (USA)
RBC: Red blood cells
WHO: World Health Organization

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2. RESUME-SUMMARY- ZUSAMMENFASSUNG

2.1. Résumé

La surveillance Sentinelle de la grippe a débuté le 1er Octobre 2011, puis s'est terminée le 20 avril 2012. Le virus influenza A (H3N2) a prédominé cette année avec 78% de l'ensemble des virus influenza détectés cette saison. Des virus influenza B et A (H1N1)pdm09 ont également été détectés mais dans des proportions plus faibles. Les virus influenza ont commencé à être détecté dès la mi-décembre pour ensuite culminer au cours des semaines 8 et 9/2012. Pendant cette période-là, le taux de prélèvements positifs s'élevait à 71% et les consultations médicales pour symptômes de grippe étaient d'environ 26‰. L'épidémie s'est poursuivie jusqu'à la mi-mars avec un taux de détection de virus influenza qui a chuté sous les 10%, semaine 13 exceptée. En fin d'épidémie, le taux de virus influenza B a légèrement augmenté par rapport aux virus influenza A (H3N2), tout en restant à un taux de détection faible. L'épidémie aura duré 9 semaine, période plus courte que la durée observée habituellement. Les virus influenza B appartenaient aux deux lignées, Victoria et Yamagata. Une moitié de ces virus étaient antigéniquement proches de la souche vaccinale B/Brisbane/60/2008 et l'autre moitié plus proches de la souche B/Bangladesh/3333/2007 et B/Wisconsin/01/2010. Les cas sporadiques de virus influenza A (H1N1)pdm09 étaient proches de la souche influenza A/Hong Kong/2212/2010 (H1N1), elle-même très proche de la souche A/California/7/2009 (H1N1). La majorité des souches influenza A (H3N2) qui ont circulé pendant la saison ont montré une diminution de la reconnaissance par l'antiserum vaccinal A/Perth/10/2009 (H3N2). L'analyse phylogénétique a confirmé cette observation et a montré une parenté plus proche de souche récentes comme les souches influenza A/Victoria/361/2011, A/Stockholm/18/2011 ou A/Brisbane/299/2011.

2.2. Summary

The Sentinel surveillance of influenza began on 1 October 2011 and ended on 20 April 2012. Influenza A (H3N2) viruses predominated during the 2011-2012 season and represented 78% of all influenza viruses detected. Influenza A (H1N1)pdm09 was detected sporadically and influenza B viruses were also detected at a low rate. Influenza viruses started to be detected from mid-December 2011 and then peaked during weeks 8 and 9/2012. During this peak period, the positive detection rate increased to 71% and the medical consultations for influenza-like illness were 26‰ on average. The epidemic continued until mid-March with a positive rate that fell below 10% during the last weeks. At the end of the epidemic, the influenza B virus rate increased compared to influenza A (H3N2) viruses, but the positivity rate remained low. The epidemic threshold lasted 9 weeks, which is at the lower end of an average season. Influenza B viruses belonged to Victoria-like and Yamagata-like lineages. Half of all influenza B viruses were antigenically close to the vaccine strain influenza B/Brisbane/60/2008 while others were closely related to B/Bangladesh/3333/2007 and B/Wisconsin/1/2010 strains. The sporadic cases of influenza A (H1N1)pdm09 were related to influenza A/Hong Kong/2212/2010 (H1N1), which is also related to the vaccine strain influenza A/California/7/2009 (H1N1). Most influenza A (H3N2) circulating during this season displayed a decreased hemagglutination inhibition titer compared to the vaccine antiserum A/Perth/16/2009 (H3N2). Phylogenetic analysis confirmed this observation and showed a relationship of these strains with recent influenza strains, A/Victoria/361/2011, A/Stockholm/18/2011, and A/Brisbane/299/2011.

2.3. Zusammenfassung

Die Grippeüberwachung mittels Sentinella System begann am 1. Oktober 2011 und endete am 20. April 2012. In dieser Saison dominierten die Influenza A (H3N2) Viren mit 78% der nachgewiesenen Viren. Influenza A (H1N1)pdm09 und Influenza B Viren wurden auch nachgewiesen aber mit einer geringeren Häufigkeit. Influenza Viren konnten ab Mitte Dezember nachgewiesen werden und deren grösste Häufigkeit lag in den Wochen 8 und 9 (2012). In diesen Wochen stieg die Nachweishäufigkeit auf 71% an und die Konsultationsrate für grippeartige Erkrankungen lag bei 25.2 respektive 26.7‰. Die Epidemie setzt sich bis Mitte März fort mit einer Nachweisrate welche in den meisten Wochen unter 10% lag. Am Ende der Epidemie nahm die Häufigkeit der Influenza B Viren im Vergleich zu derjenigen von Influenza A (H3N2) zu, blieb aber auf einem tiefen Niveau stehen. Die Epidemie dauerte 9 Wochen was kürzer ist als normalerweise üblich. Die nachgewiesenen Influenza B Viren gehörten zwei verschiedenen Gruppen an und zwar zur Victoria- oder Yamagata-Linie. Die Hälfte dieser Viren war nahe verwandt mit dem im Impfstoff enthaltenen Stamm Influenza B/Brisbane/60/2008 und der Rest war näher verwandt mit dem Stämmen B/Bangladesch/3333/2007 und B/Wisconsin/1/2010. Die vereinzelt gefundenen Influenza A (H1N1)pdm09 waren verwandt mit Influenza A/Hong Kong/2212/2010 (H1N1) ein Stamm welcher auch verwandt ist mit dem im Impfstoff enthaltenen Stamm Influenza A/California/7/2009 (H1N1). Influenza A (H3N2) Viren welche in dieser Saison nachgewiesen wurden, zeigten einen reduzierten HI Titer mit dem Antiserum A/Perth/16/2009 (H3N2). Eine phylogenetische Analyse bestätigte diese Beobachtung und zeigte eine Verwandtschaft dieser Viren mit neueren Influenza A Stämmen A/Victoria/361/2011, A/Stockholm/18/2011 und A/Brisbane/299/2011.

3. Introduction

After two seasons dominated by the pandemic influenza virus A (H1N1)pdm09 virus, influenza A (H3N2) viruses predominated during the season and other influenza viruses circulated at a low level. An interesting aspect was the diversity of influenza A (H3N2) variants that circulated during the epidemic. The present report summarizes the results of influenza surveillance in the Swiss Sentinel network.

4. Methods of detection for influenza viruses

4.1. Clinical identification of influenza cases

During the 2011-2012 season, a network of 157 practitioners participated actively to the clinical surveillance of influenza cases. Surveillance is based on a weekly count of medical consultations for influenza-like illness (MC-ILI). The case definition used is the presence of fever >38°C with or without a feeling of sickness, myalgia, or an alteration of general status. In addition to fever, acute respiratory symptoms such as cough and/or sore throat must be present. The geographic distribution of the participating general practitioners is shown in figure 1.

A subgroup of 76 Sentinel practitioners (49%) provided clinical specimens from selected participants in addition to clinical surveillance. Combined nasopharyngeal and pharyngeal specimens are sent in transport medium by regular mail to the National Reference Centre for Influenza (NRCI) in Geneva for subsequent viral detection and characterization. The sampling selection procedure of specimens is adapted to the epidemic phases as follows:

1) Pre- and post-epidemic phase: the number of MC-ILI by Sentinel practitioners remains below the threshold level of 67 cases per 100,000 inhabitants. During this phase, respiratory screening is performed in all cases that fulfill the case definition.
2) Epidemic phase: the number of MC-ILI cases is over the threshold of 67 cases of MC-ILI per 100,000 inhabitants. During this phase, respiratory screening is performed in a subgroup of cases according to predefined rules and only 1:4 ILI cases are systematically screened.

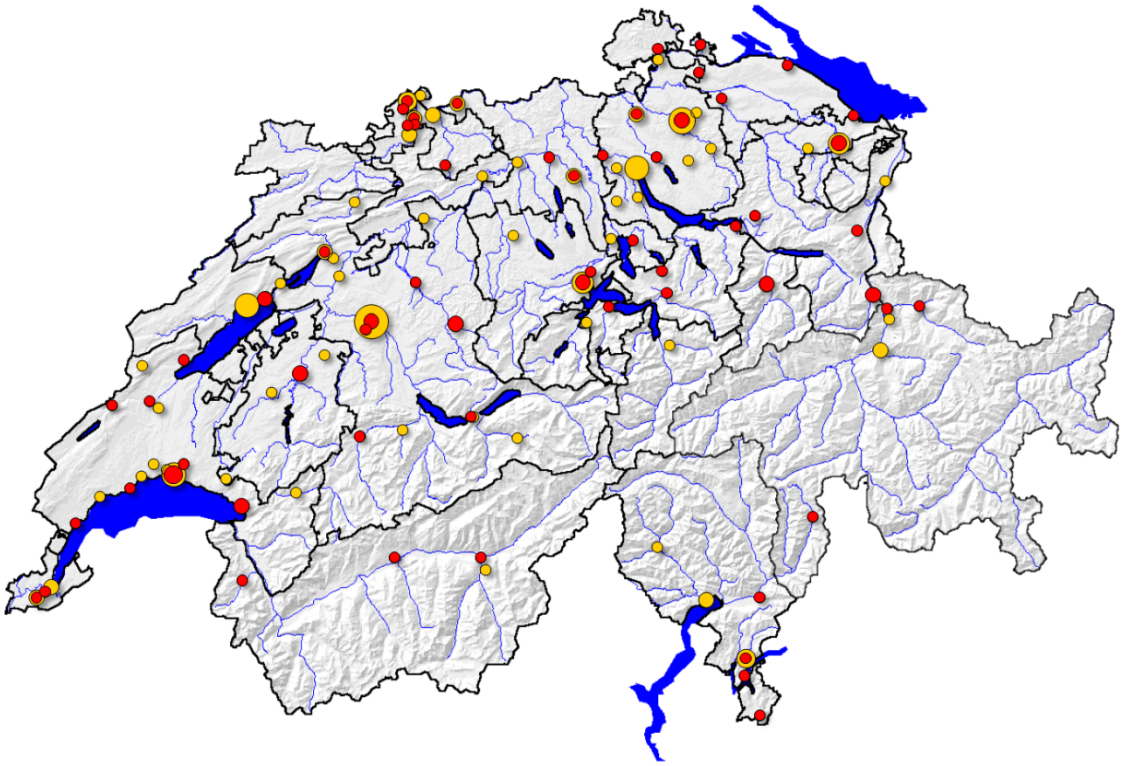


Figure 1: Geographic distribution of the 234 participating practitioners of the Swiss Sentinel network

Yellow circle: location of participants (157) conducting clinical surveillance; red circle: participants conducting both clinical surveillance and specimen collection (76). Participants per community (range: 1-10) = circle size

4.2. Virological detection of influenza viruses

Screening of positive samples containing influenza viruses was organized as follows. Nasopharyngeal swab specimens are submitted to a one-step, real-time reverse transcription polymerase chain reaction (rRT-PCR) for influenza virus detection (figure 2). The method is based on a United States Centers for Disease Control and Prevention (CDC) protocol provided during the pandemic 2009.[2] The primers' composition is complementary to a well-conserved genome fragment, the matrix protein (MP), and allows the detection of both human and animal influenza viruses. Subtyping of positive screening samples is performed with a second rRT-PCR that reveals the subtype of influenza A viruses (H1pdm09, seasonal H1, and seasonal H3) and the lineage of influenza B viruses (Yamagata or Victoria lineage; figure 2). A selection of the first five influenza viruses detected each week with high titers are then submitted to genotyping analysis and culture. Genotyping analysis consisted in the sequencing of the hemagglutinin (HA) gene for phenotyping analysis and the

neuraminidase (NA) gene to evaluate oseltamivir resistance. Positive culture with sufficient hemagglutination titer were then submitted to phenotypic analysis by hemagglutination inhibition (HI). Microneutralization tests (MN) were conducted when required (protocol kindly provided by Katja Hoschler, HPA, London, UK).

During the pre- and post-epidemic phases, a random selection of rRT-PCR negative specimens are regularly inoculated on cells for virus culture (figure 2). The goal of this strategy is to detect influenza strains that could escape RT-PCR detection. This could be the case in the presence of a drifted mutant in regions of the viral genome targeted by RT-PCR primers and probes used for viral screening.

5. Characterization of influenza viruses

Several methods exist for virus characterization. They can be separated into two main groups: phenotypic and genetic analysis. The first method studies the nature of surface proteins and their ability to be recognized by reference ferret antisera. HI and MN assays are part of these type of assays. These assays are used as surrogates of vaccine response. Genetic analysis consists in determining the nucleotide composition of viral genes by sequencing analysis. Gene sequences are then compared and provide information on the viral origin and evolution.

The gold standard of phenotypic analysis is the HI test that has been used for decades at the NRCI in Geneva. This year, this technique has been successfully used for influenza A (H1N1)pdm09 and influenza B viruses. Although influenza A (H3N2) viruses grew on cells under good conditions, no hemagglutination titer could be observed with red blood cells (RBC) of different origins (guinea pig, turkey, or human group O). To overcome this problem and obtain antigenic information on influenza A (H3N2) strains, a MN assay was introduced. The principle of the technique is to evaluate the ability of reference antisera to inhibit cell growth of isolated viruses from nasopharyngeal swabs. As an HI assay, MN cannot be used on clinical specimens without viral amplification by cell culture.

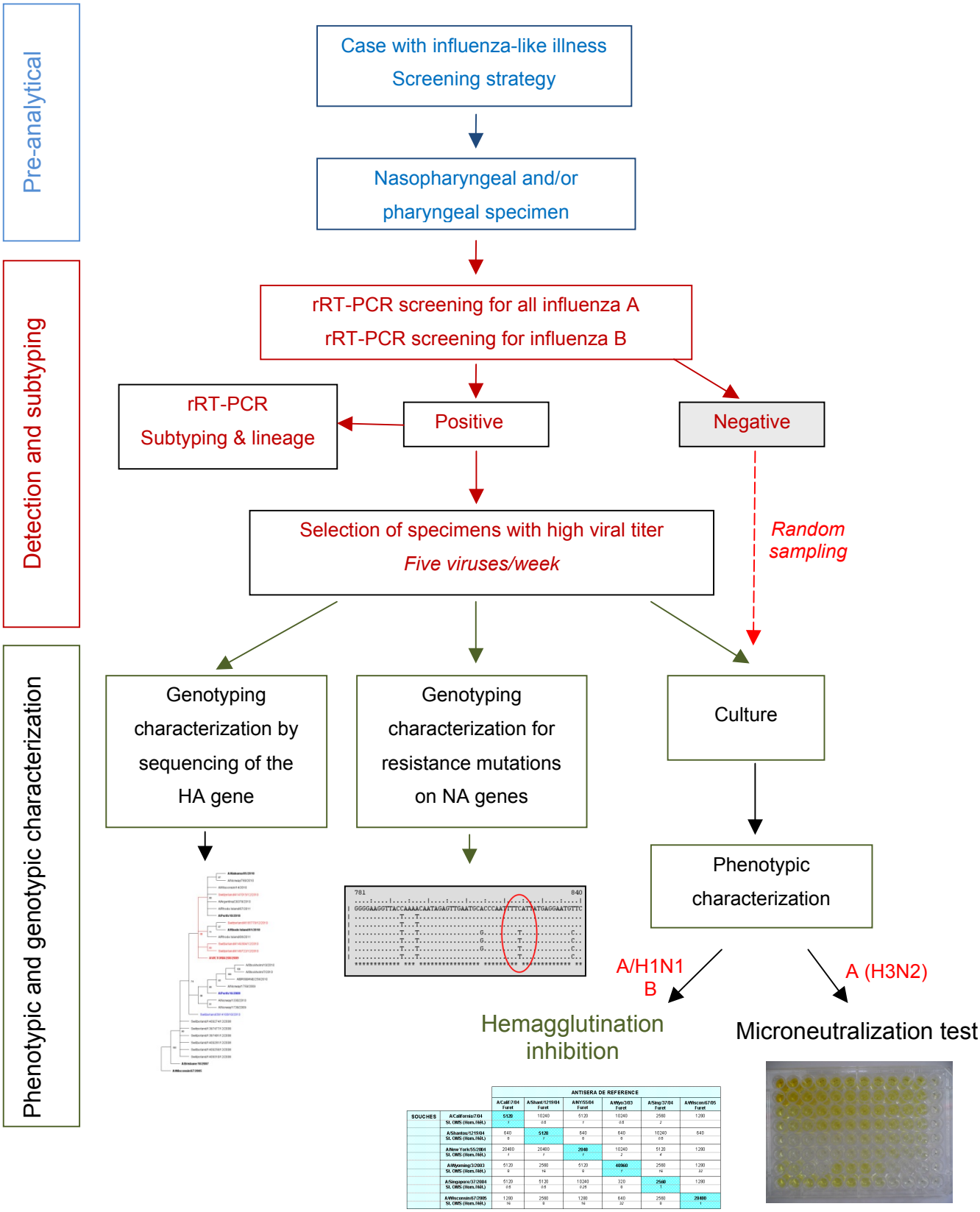


Figure 2: Procedure used for the detection and characterization of influenza viruses by Sentinel surveillance

5.1. Phenotypic assay

5.1.1. Cell culture

To apply phenotypic analysis for virus characterization, a high quantity of virus is required. Clinical samples should be enriched in virus by cell cultivation. In order to do so and according to our predefined selection criteria, a subgroup of five specimens per week detected positive by rRT-PCR and with a Ct value lower than 30 were submitted to phenotypic analysis. 0.4ml of transport medium containing nasopharyngeal swab was incubated for 7 days under 5% CO2 at 33°C on Madin Darby Kidney Cells (MDCK) cells and 37°C on sialic acid-enriched MDCK-SIAT1. The presence of virus was confirmed by the presence of a cytopathic effect under visible light (Leica®) and by an immunofluorescence test (figure 3) using monoclonal influenza A and B antibodies combined with mouse FITC-conjugate (Chemicon®, Temecula, CA, USA). Cell supernatants of positive samples were then analyzed by phenotypic assay (HI or MN).

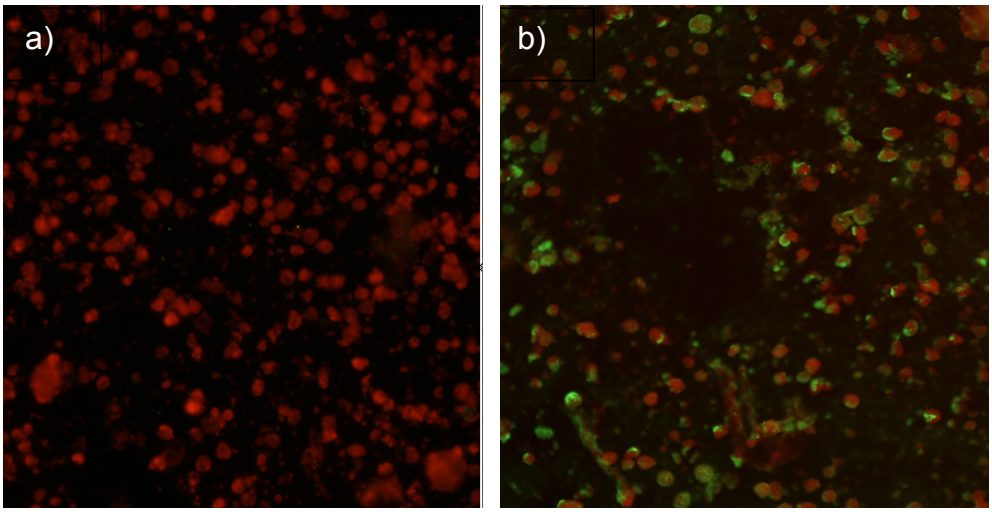


Figure 3: MDCK cells infected by influenza A/Switzerland/01/2009 (H1N1)

a) Negative control; b) influenza viruses detected with monoclonal anti-influenza A primary antibody and monoclonal FITC conjugate (green) revealing the presence of viral antigen in cells (Chemicon®)

5.1.2. Hemagglutination inhibition assay

This assay is based on the ability of HA of influenza virus to bind to sialic acid present at the surface of RBC. The first step consists in evaluating the viral titer by a

hemagglutination test. A two-fold serial dilution is performed using 50µl of viral suspension in 50µl of Veronal buffer (1.5%).

Table 1: Hemagglutination inhibition titers of reference influenza strains tested with the 2011-2012 reference antisera

a) Influenza A (H3N2)

Strains \ Antisera	A/Brisbane/10/07	A/Perth/16/2009	A/Victoria/208/2009	A/Wisconsin/15/09
A/Brisbane/10/07	**256**	<8	<8	<8
A/Perth/16/2009	16	**1024**	1024	128
A/Victoria/210/2009	64	512	**2048**	ND
A/Wisconsin/15/09	<8	256	64	**32**

b) Seasonal influenza A (H1N1) and influenza A (H1N1)pdm09

Strains \ Antisera	A/California 7/09	A/Bayern 69/09	A/HKong 2212/10	A/Brisbane/ 59/2007
A/California/7/09	**1024**	256	256	<8
A/Bayern/69/09	256	**512**	128	<8
A/HKong/2212/10	1024	512	**2048**	<8
A/Brisbane/59/2007	<8	<8	<8	**1024**

c) Influenza B

Strains \ Antisera	B/Brisbane/ 60/2008	B/Hong Kong/ 514/2009	B/Paris/ 1762/2009	B/Florida/ 4/2006	B/Bangladesh/ 3333/2007	B/Wisconsin/ 1/2010
B/Brisbane/60/2008	**512**	256	128	<8	<8	<8
B/Hong Kong/514/2009	128	**512**	128	<8	<8	<8
B/Paris/1762/2009	128	512	**128**	<8	<8	<8
B/Florida/4/2006	32	<8	<8	**4096**	512	256
B/Bangladesh/3333/2007	16	<8	<8	1024	**512**	256
B/Wisconsin/01/10	<8	<8	<8	512	256	**256**

Glutaraldehyde-fixed RBC are added for a 1 h incubation. Hemagglutination titer is determined as the last dilution in which a hemagglutination is still observed. In a second step, HI assays are performed using serial dilutions of reference antisera. 25µl of antiserum is added in the first two wells of a 96-well plate. A two-fold dilution

is prepared adding 25µl of DGV (1.5%) in the second well, 25µl is then collected from the same well, and the procedure repeated to the end of the plate. 25µl of viral suspension containing four hemagglutination units are then added to the antiserum dilution and incubated for 1 h at room temperature. 25µl of RBC are added to each well. The HI titer represents the last antiserum dilution rate where hemagglutination is still inhibited. This titer is compared to homologous titer obtained with reference strains submitted to their corresponding antisera (table 1). A strain is considered as antigenically related to a reference strain when the HI titer obtained is the same or no more than four-fold lower than the one obtained with the reference strain. If the HI titer is lower than four-fold, the strain is considered antigenically different to the reference strain.

Since 2001, two different influenza B virus lineages circulate in Europe. The first is constituted of strains that have been detected in humans for decades worldwide, the B/Yamagata/16/88-lineage. A second lineage was detected in 1988 in the southern hemisphere (Asia and Oceania) before arriving in Europe in the early 2000s, the B/Victoria/2/87-lineage. Since then, both lineages circulate alternatively during different seasons in the community. An influenza virus of one lineage is not (or less) recognized by antisera raised against viruses of the other lineage. In the antigenic table (table 1c), influenza B viruses of the Yamagata lineage (blue section), e.g., B/Florida/4/2006, displayed no, or lower than 16, HI titers with Victoria-lineage antisera, similar to B/Brisbane/60/2008. After RT-PCR detection of influenza B viruses, the lineage is determined mainly by HI analysis.

5.1.3. Microneutralization assay

MN assay represents an alternative to HI assay. The technique allows to evaluate antigenic recognition of influenza viruses by reference immunized ferret antisera. The results provide information on vaccine efficiency against viruses circulating in the community.

MN assay is based on the cell growth property of influenza viruses and its potential inhibition by specific antisera. The technique used at the NRCI was kindly provided by Katja Hoschler (HPA London, UK). Two-fold dilutions of several immunized ferret antisera are prepared (figure 4). A standardized quantity of viruses (10 $TCID_{50}$) are added and incubated during 1 h at 37°C. A standardized number of SIAT cell suspension ($5x10^4$ cells) is added. After 16 h of incubation at 37°C and 5% CO_2,

supernatant is removed and cells are fixed. Viruses are detected by a monoclonal anti-NP antibody and its HRP conjugate. After addition of tetramethylbenzidine substrate (TMB), the optical density is measured by a spectrophotometer. Growth inhibition is determined by comparing viral growth with and without antiserum.

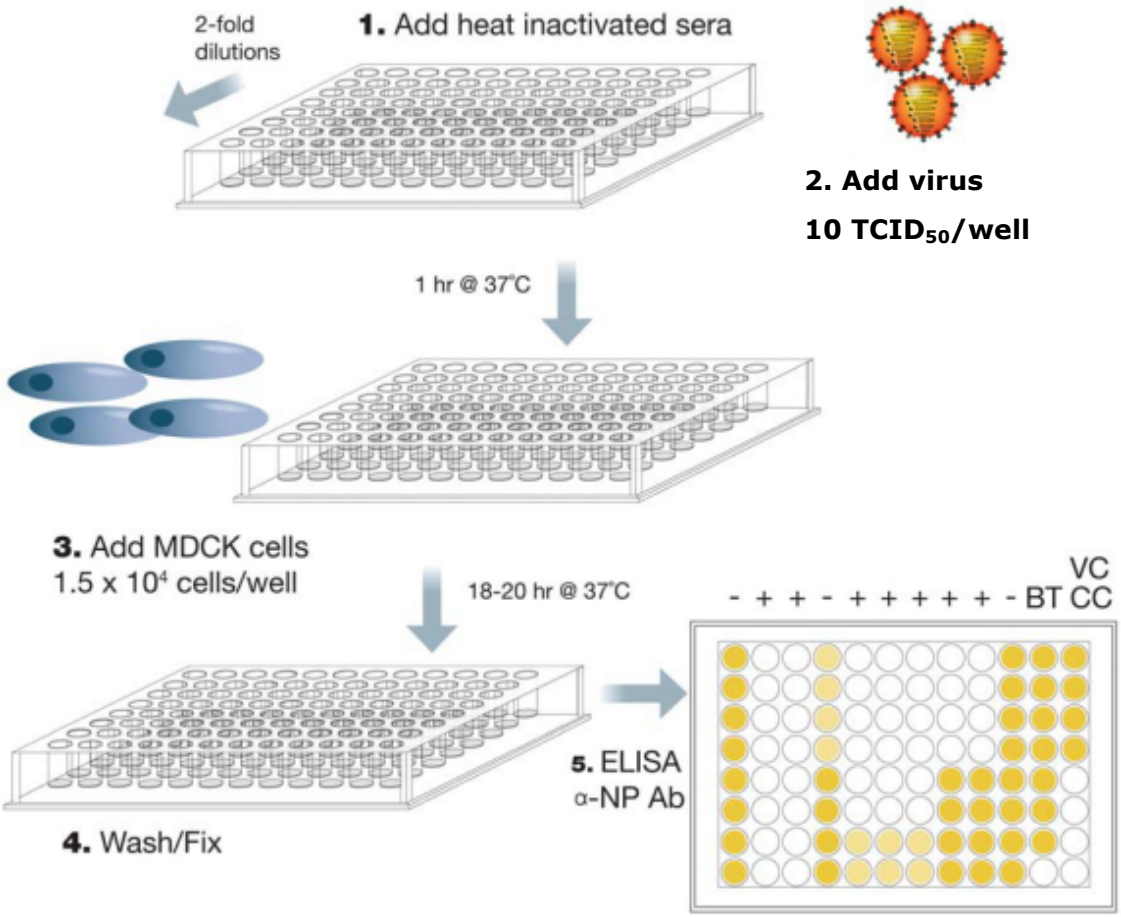


Figure 4: Schematic representation of a microneutralization assay.

Schema reproduced from :
http://www.who.int/influenza/gisrs_laboratory/2010_12_06_serological_diagnosis_of_influenza_by_microneutralization_assay.pdf

5.2. Genetic characterization

Genetic analysis has been widely used over the last few years to characterize better the genetic relationship between strains. The most variable sequence of influenza virus, the HA1 part of the HA gene (1000 nucleotides in the 5'-end), is the target of choice for sequencing analysis. Phylogenetic analysis then consists in comparing these sequences with HA sequences of reference strains. The different nucleotide compositions help to evaluate the drift or identify specific mutations. Genotyping could provide also additional information, such as for influenza A (H1N1) 2009

viruses; for example, the presence of the D222G mutation in the receptor-binding region of the HA sequence might be potentially associated with more severe cases.[5] Thus, determining the HA sequence of the circulating H1N1 viruses could help also to understand better their pathogenicity.

5.3. Antiviral resistance

A key issue for any antiviral use is the monitoring of antiviral resistance. This mechanism can be a complex association of known or unknown mutations and only precisely characterized mutations are easily targeted. Sequencing of NA and MP genes provide information on resistance against neuraminidase inhibitors (NI: oseltamivir and zanamivir) and the matrix inhibitor (amantadine). Specific mutations in the NA gene induce change in enzymatic activity that remains active in the presence of NI (table 2). Such resistance mutations can be type- or subtype-specific. In addition, each drug has its own resistance pattern. During the 2009-2010 and 2010-11 seasons, H275Y mutation in the NA gene of influenza A (H1N1) 2009 has been observed sporadically in several countries, but at a limited rate and mainly in immuno-compromised patients under antiviral treatment.[7,8,11]

Table 2: Key mutations conferring antiviral resistance to influenza viruses

LR: low resistance

	Mutations conferring antiviral resistance - 06.05.2011 -		
	Oseltamivir (NA)	Zanamivir (NA)	Amantadine (M2)
H3N2	E119V N146K, S219T, A272V D151V,A,N,G I222V (LR) N249S N294S R292K	Q136K D151V,A,N,G R292K (LR)	
H1N1	E119V Y155H G248R+I266V H275Y	E119V Q136K Y155H G248R+I266V	L26F V27A A30T S31N G34E
H1N1p	I117M E119V D199E I223R (LR) I223V H275Y N295S Q313R & I427T	E119V I223R (LR) Q313R & I427T	
B	D197N/E/Y (LR) I221T (LR) H273Y (LR) R374K	D197N/Y D197N/E (LR) I221T (LR)	

LR: low reactor

During the 2010-2011 season in the United Kingdom, a limited number of resistant viruses in individuals with no known exposure to oseltamivir were observed.[7] A similar observation was made during the 2008-2009 season with seasonal influenza A (H1N1) viruses that became predominant in the European community in the absence of systematic treatment of influenza infection.[6] Likewise, a S31M mutation in the M gene conferring amantadine resistance has been observed in almost all influenza A (H3N2) viruses[1] and influenza A (H1N1) 2009 viruses[3] circulating in the community over the last years. For some years now, the detection of antiviral resistance either in the community or in patients treated in healthcare facilities has become an important aspect of influenza surveillance.

6. Results of the 2011-2012 season

6.1. Detection of influenza viruses in nasopharyngeal samples in the Sentinel network

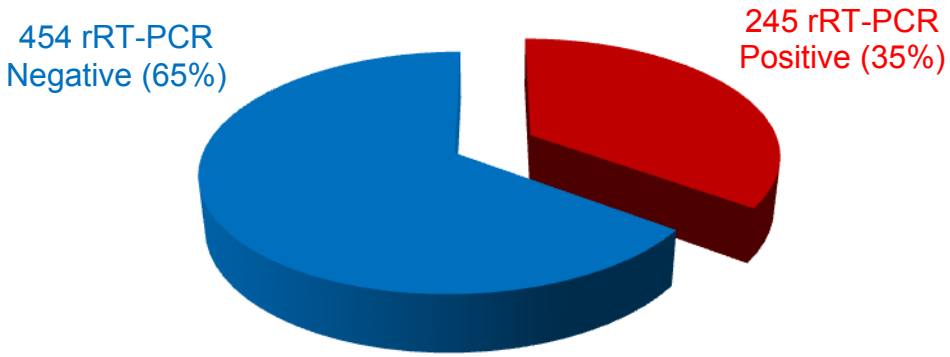
The influenza surveillance period lasted 29 weeks, from 1 October 2011 (week 40) to 20 April 2012 (week 16). Of 97 practitioners, 77 sent at least one nasopharyngeal swab for virus detection, representing a total of 699 samples received. Of these, 245/699 (35%) influenza viruses have been detected by rRT-PCR (figure 5a). Detailed data on influenza virus quantities detected are provided in annex 1.

212/245 were of type A (87%) and 33/245 (13%) of type B. 190/245 (77%) were influenza A (H3N2) viruses and 11/245 (5%) were influenza A (H1N1)pdm09. 17/245 (7%) were influenza B virus of the Victoria lineage and 14/245 (5%) were of Yamagata lineage (figure 5b). Finally, 11 influenza A (5%) and two influenza B viruses (1%) could not be further subtyped due to low viral titer.

The first influenza virus detected was an influenza B during week 47 in mid-November 2011. From then onwards, influenza A (H3N2) viruses started to be detected regularly during week 51 (mid-December). The number of influenza viruses detected increased and reached a maximum positivity rate of 71% during weeks 8 and 9 (between 18 February to 2 March 2012). The rate then decreased slowly until week 16. The epidemic phase lasted for approximately nine weeks (figure 6). Influenza A (H3N2) virus predominated during the whole season from weeks 51 to 15 with a ratio varying between 50% to 100% of all influenza viruses detected. Influenza B viruses started to circulate in a sustained manner during the second part of the

epidemic (figure 6). The ratio of influenza B virus varied from 0% to 33% of influenza viruses detected up to week 10, increased up to 50% during week 14, and was finally the only virus detected at the end of the epidemic.

a)



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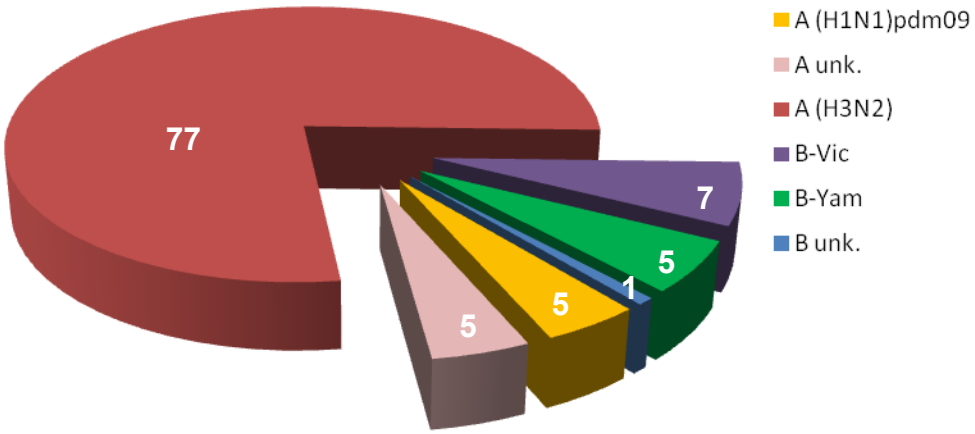


Figure 5: Distribution of influenza viruses recovered in nasopharyngeal specimens collected during the 2011-2012 season (n=699)

a) Number and % of RT-PCR-positive versus -negative specimens
b) Distribution of the different types and subtypes of influenza viruses (%)

MC-ILI values bypassed the epidemic threshold during week 5 (end January 2012) and remained above this threshold up to week 13. Based on the number of MC-ILI cases, the epidemic phase lasted for 9 weeks with a maximum value reached during week 9. This represents a relatively short season compared to former years.

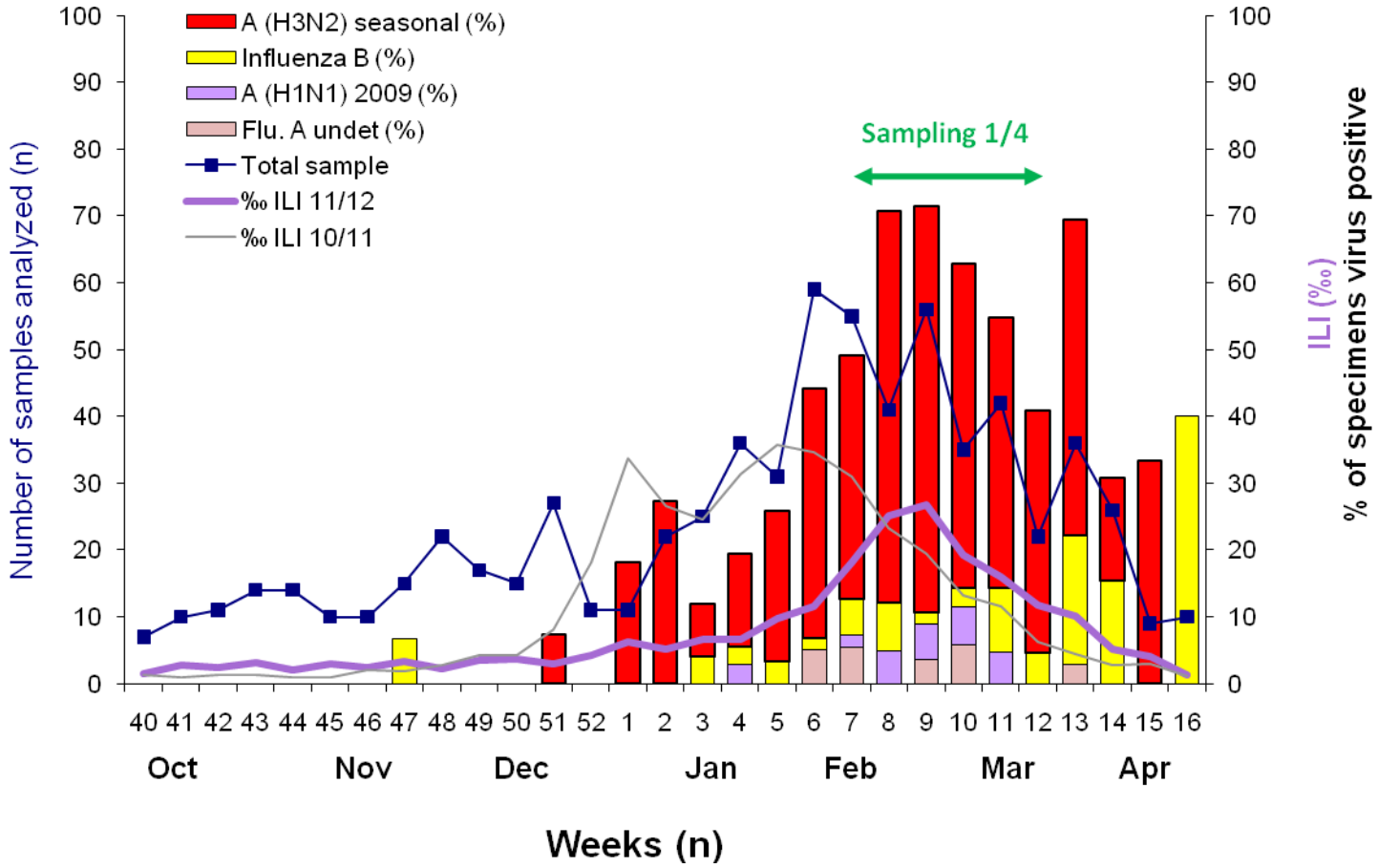


Figure 6: MC-ILI, positivity rate, and distribution of rRT-PCR-positive cases according to influenza types

Proportion of influenza A (H3N2), A (H1N1) 2009 and B viruses (%), total number of samples tested, and MC-ILI case (%) distribution per week during the 2011-2012 season. MC-ILI (%) 2010-2011 is shown also for comparative purposes. Sampling: green arrow indicates the weeks when the 1:4 sampling strategy was introduced by Sentinel practitioners.

6.2. Characteristics of Sentinel-screened individuals

Many studies have confirmed the observation that influenza A (H1N1)pdm09 viruses affected preferentially individuals < 20 years old.[14] Recently, an epidemiological study revealed that during the 2011-2012 season, an excess mortality was observed in individuals > 65 years old in several European countries, including in Switzerland.[9] To evaluate the impact of influenza viruses that circulated in the community during this season in Switzerland, the viral positivity rate has been distributed according to each age group (figure 7).

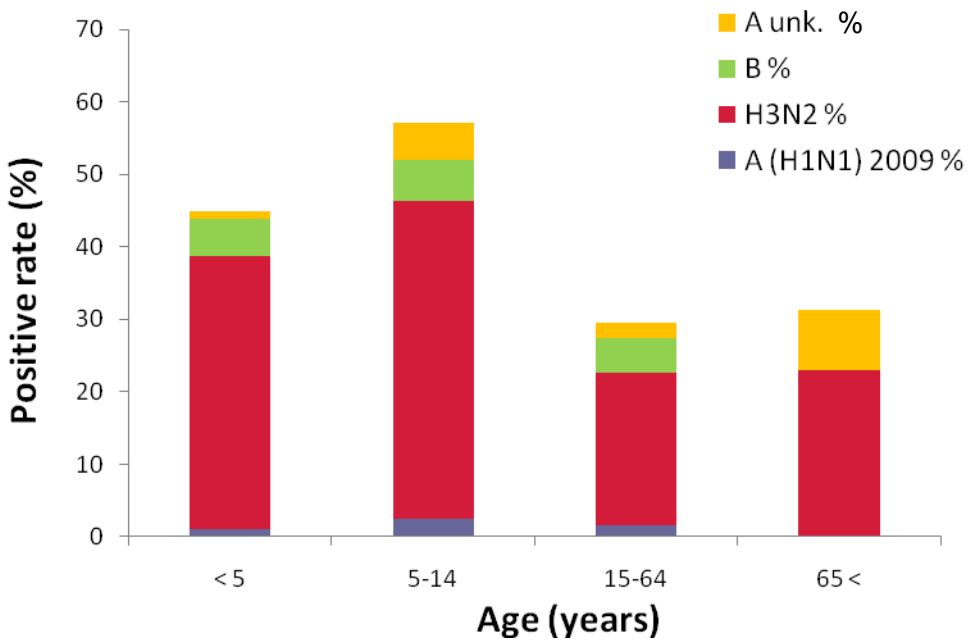


Figure 7: Distribution of viruses (%) detected according to age groups

A unk.: influenza A viruses with an unknown subtype; B: influenza B viruses; H3N2: influenza A (H3N2) viruses; A (H1N1) 2009: influenza A (H1N1)pdm09 viruses

Influenza A (H3N2) viruses affected all age groups, with approximately a two-fold higher rate observed in the younger age range (<14 years) compared with the older groups. Of note, this virus was the only one detected in individuals > 65 years and no influenza A (H1N1)pdm09 or influenza B viruses were identified in this age group. However, a higher rate of unknown influenza A viruses can be observed in this age group (27%) than in others (2-9%). This viral population could be composed also of a non-negligible influenza A (H1N1)pdm09 virus rate not detected by subtyping reaction. A possible explanation is that samples collected in the elderly have usually a lower viral titer than in younger individuals, thus resulting in a lower rate of

subtyping of influenza A viruses in this age group. It should be taken into account also that as the Sentinel surveillance network is based on individuals selected on the basis of the presence of ILI symptoms, this could be more in favor of young children and healthy adults who consequently would be more represented.

6.3. Antigenic and genetic characterization of influenza viruses

Eighty-five selected specimens, positive by rRT-PCR with a Ct value lower than 30, were inoculated on cells in order to perform phenotypic assays, such as HI and MN. 61/85 (72%) revealed to be culture-positive: 10 influenza B, three influenza A (H1N1)pdm09, and 48 influenza A (H3N2) viruses. In addition, 67 influenza viruses equally distributed during the epidemic and with Ct values lower than 30 were analyzed by gene sequencing for phylogenetic comparison: 57 HA genes were analyzed in 36 influenza A (H3N2), two influenza A (H1N1)pdm09, and 19 influenza B viruses.

6.3.1. Influenza A (H1N1)pdm09 virus

The three influenza A (H1N1)pdm09 analyzed by HI assay showed HI titers similar to those obtained with the influenza A/Hong Kong/2212/2010 (H1N1) strain (annex 4) and remained well recognized by the 2011-2012 vaccine antiserum against A/California/7/2009 (H1N1) virus. As reported previously, all influenza A (H1N1)pdm09 sent by European countries and analyzed by the WHO collaborating centre in London, UK, were well recognized by the influenza A/California/7/2009 (H1N1) vaccine antiserum.[10]

HA gene sequencing revealed that the two influenza A (H1N1)pdm09 viruses analyzed had mutations (S143G, S185T and A197T) of the genetic group 7 represented by the influenza A/St-Petersburg/100/2011 (H1N1) strain. Similar strains were also detected in Europe and the USA during this season (Grenoble, France; Stockholm, Sweden; Texas, USA).[10] These mutations were not present in Swiss strains detected during the last two seasons. However, no major genetic differences could be observed between strains that circulated during both seasons. The D222G mutation[5] was checked for in the two HA sequences and was absent in all.

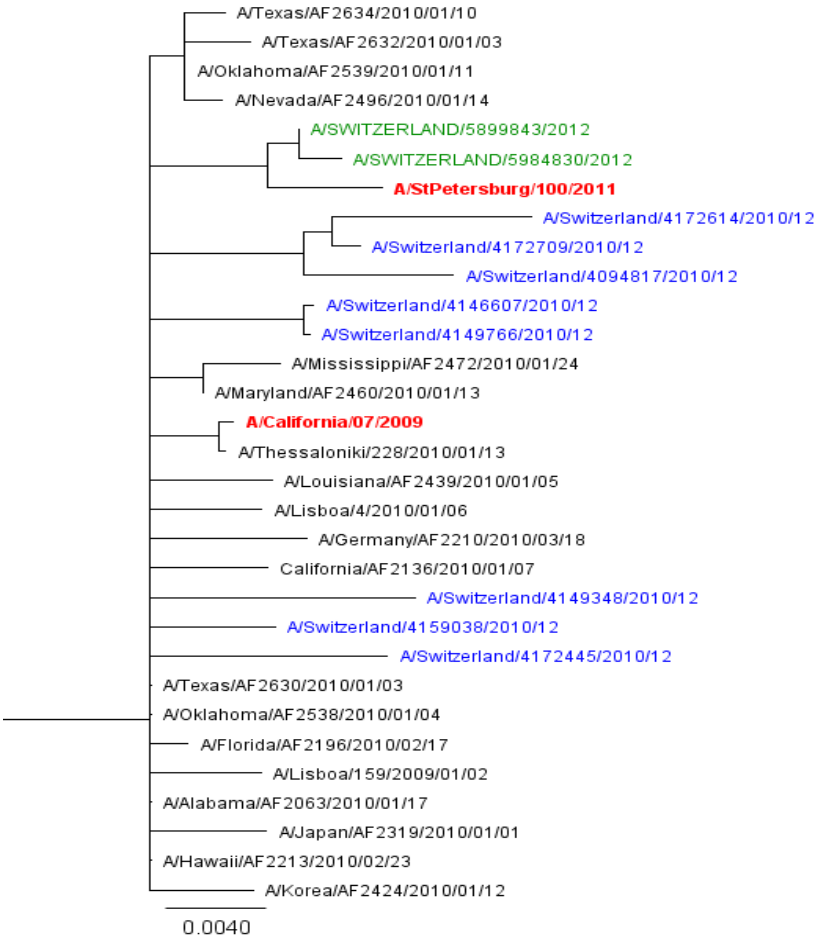


Figure 8: Phylogenetic analysis of the influenza A (H1N1) 2009 hemagglutinin

Red: vaccine and reference strains. Green: Swiss influenza viruses detected during the 2011-2012 season. Blue: Swiss influenza viruses detected during the 2010-2011 season.

6.3.2. Influenza A (H3N2) viruses

6.3.2.1. Phenotypic assays

Influenza A (H3N2) viruses grew quite normally on SIAT cells with a clear cytopathic effect visible in a few days, as usually observed. However, cell supernatant did not hemagglutinate glutaraldehyde-fixed RBC, thus indicating an apparent absence of this basic property of influenza viruses. Immunofluorescence testing confirmed the presence of influenza nucleoprotein in fixed cells and rRT-PCR confirmed a high dose of influenza genome in cell supernatant. Similarly, an evaluation of guinea pig, turkey, and group O human RBC revealed to be also without significant hemagglutinin titers.

The lack of RBC sensitivity of H3N2 observed in our laboratory during this season was also discussed during the WHO annual consultation meeting on the composition of influenza vaccine for the northern hemisphere that took place on 22 February 2012 in Geneva.[10] For this reason, MN tests were introduced at the NRCI. The goal of this analysis was to evaluate antigen recognition of influenza A (H3N2) viruses circulating in the population by the vaccine A/Perth/16/2009 antiserum and the capacity of the vaccine antiserum to inhibit infection. The principle of this technique is detailed in chapter 5.1.3.

Table 3: Microneutralization titer of influenza A (H3N2) viruses detected in samples from the Sentinel network

Blue: influenza viruses detected in Sentinel samples that showed a high MN titer with A/Perth/16/2009 antiserum

	Samples	**A/Perth/16/2009 AS MN titer**
	Perth 10 TCID	**64**
1	5920857	<32
2	5952619	<32
3	5952760	<32
4	5952968	<32
5	6015981	<32
6	6048351	<32
7	6114397	1024
8	6124837	128
9	6125030	<32
10	6146939	<32
11	6157769	<32
12	6215618	<32
13	6246188	<32

10 $TCID_{50}$ of influenza A/Perth/16/2009 (H3N2) gave a MN titer of 64 (table 3; annex 2a). By contrast, 2/13 viral suspensions obtained from Sentinel samples containing 10 $TCID_{50}$ of A (H3N2) viruses only showed a MN titer with A/Perth/16/2009 ferret antiserum (table 3, blue label; annex 2b). No viral growth inhibition was observed with the 11 other influenza A (H3N2) viruses. To confirm this observation, an additional A/Perth/16/2009 (H3N2) specific immune serum was used for the MN assay. With human serum obtained from a vaccinated individual immunized against influenza A/Perth/16/2009 (H3N2) virus, a higher MN titer was obtained with the A/Perth/16/2009 strain than with the ferret immunized antiserum: 512 and 64, respectively (table 4; annex 2c). 5/6 influenza A (H3N2) viruses obtained from

Sentinel samples yielded an eight- or 16-fold decrease with the human antiserum. Only one Sentinel sample was inhibited by the vaccinated human serum, the 5952968.

These results strongly suggest a decreased affinity of the A/Perth/16/2009 (H3N2) antiserum for the majority of influenza A (H3N2) strains circulating this season in Switzerland.

Table 4: Microneutralization analysis of influenza A (H3N2) viruses from Sentinel subjects with ferret and human antiserum against influenza A/Perth/16/2009 (H3N2) virus

	Antisera	
Viruses 10 $TCID_{50}$	**Ferret A/Perth/16/2009**	**Vaccinated individual**
A/Perth/16/2009	**64**	**512**
5920857	<32	64
5952619	<32	64
5952760	<32	32
5952968	<32	256
6048351	<32	64
6157769	<32	64

A more adapted antiserum for currently circulating strains that could help us to improve the phenotypic characterization of these strains is not available yet. A selection of eight of these strains were sent to MRC, London, UK, for HI and MN testing with more recent antisera. The results confirmed an eight- to 16-fold decrease of HI and MN titers with A/Perth/16/2009 (H3N2) ferret antiserum for all eight influenza A (H3N2) strains (data not shown). As expected, these A (H3N2) viruses were well recognized with the more recent antisera influenza A/Alabama/5/2010, A/Hong Kong/3969/2011, A/Stockholm/18/2011, A/Finland/190/2011, A/England/259/2011 and A/Norway/1789/2011.

6.3.2.2. Genetic analysis

For further characterization, the HA gene of 36 influenza A(H3N2) viruses were sequenced and a phylogenetic comparison was performed (figure 9). A relatively high diversity of sequences has been observed in the HA gene of influenza A (H3N2)

viruses. These viruses were distributed within three main genetic groups with clades 3 (3b and 3c) and 6 being the most frequent, while clade 7 was less frequently observed.[10] The clade 7 strain was close to influenza viruses detected in Switzerland during the 2010-2011 season. Interestingly, these strains were related to influenza A/Perth/16/2009. No travel information was reported for the 2011-2012 case previously mentioned. Genetic analysis carried out on additional strains at MRC London confirmed that Swiss influenza A (H3N2) strains were related to clades 3a, 3b, 3c, and 6. Hence, all influenza viruses genetically characterized at the NRCI were closely related to the A/Victoria/208/2009 clade and no virus belonging to the A/Perth/16/2009 clade (vaccine strain) was detected. The same observation was made in Europe where most influenza A (H3N2) viruses characterized genetically fell also into the Victoria/208 clade, more specifically in subclades 3 and 6.[10,16] Genetic analysis allowed us to detect the genetic diversity of influenza A (H3N2) viruses that circulated in Switzerland and our results were in accordance with those for European countries as communicated by WHO.[16] These viruses showed a decreased recognition by vaccine A/Perth/16/2009 antiserum in approximately 60% of cases, which explains why the influenza A (H3N2) strain was replaced in the 2012-2013 vaccine composition by an influenza A/Victoria/361/2011 strain.

Some amino acids are specific determinants of antigenic recognition of HA protein by specific antibodies. They constitute five distinct regions called epitopes A to E with some separated regions, i.e., B1, B2, C1, and C2. To evaluate the impact of genetic change in influenza A (H3N2) viruses detected in Switzerland this season on immune recognition, an alignment of the epitope region of 29 amino acid sequences was performed together with the A/Perth/16/2009 virus. The comparison revealed four differences in three epitope regions between the vaccine strain and viruses detected in Sentinel samples: K140N/D/T, T212A, S214I/T, and K62E (figure 10, red arrows). Some strains presented additional mutations in three epitope regions: I140M, N145S, D53N, and N278K/D (blue arrows). The impact of such mutations on immune

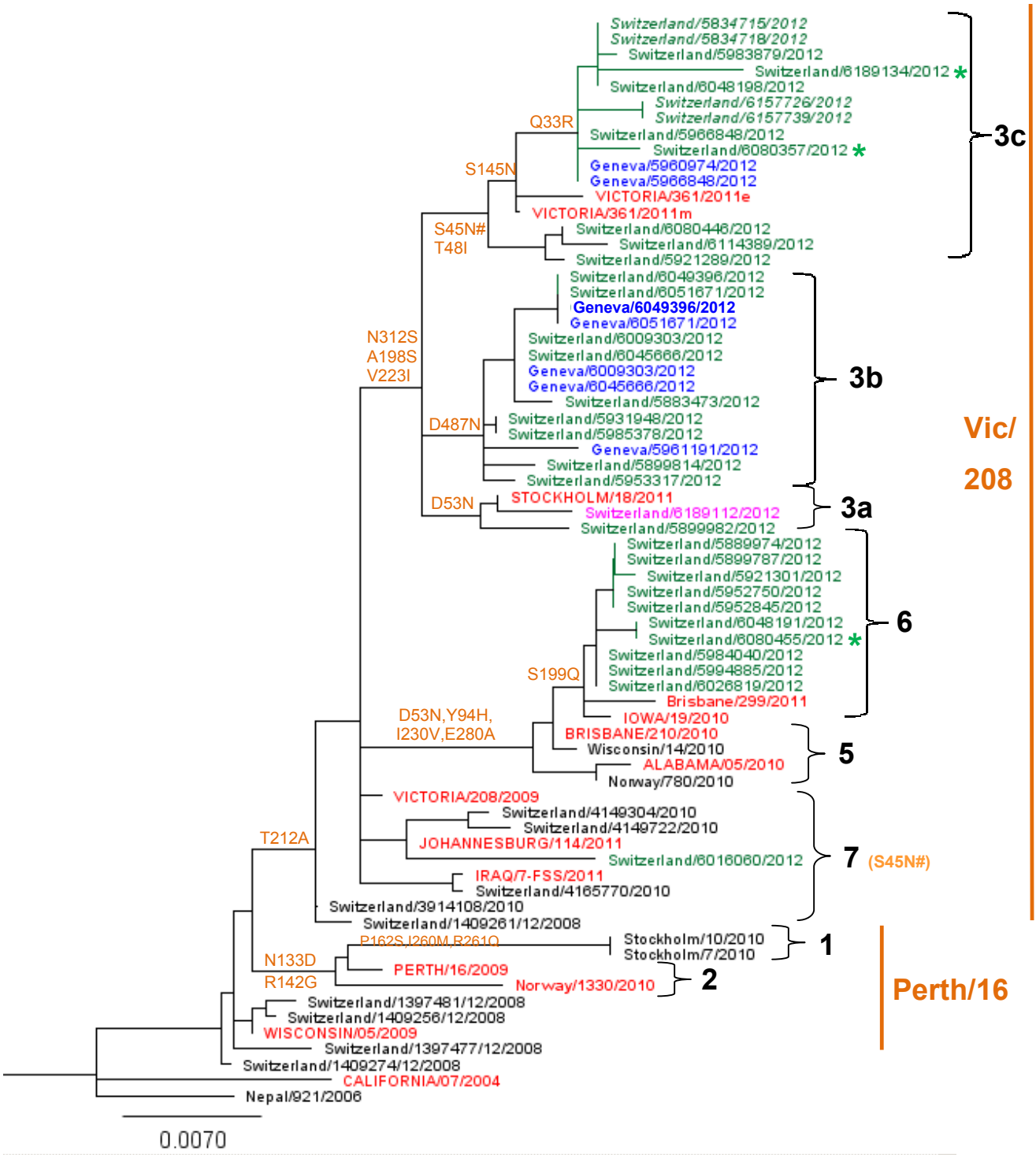


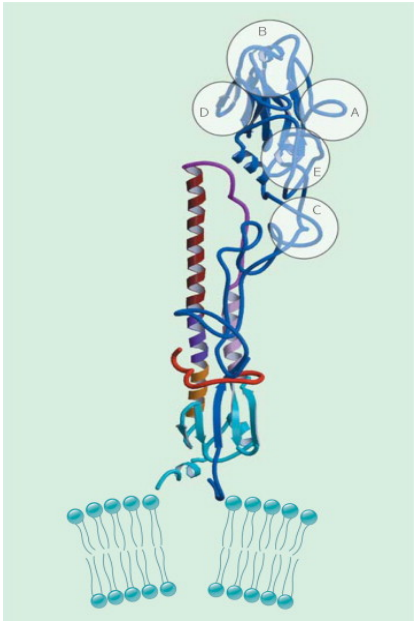
Figure 9: Phylogenetic analysis of the HA gene of A (H3N2) viruses

Green: influenza virus detected in the Sentinel network during 2012. Blue: influenza virus detected during 2012 in hospitalized patients at the University of Geneva Hospitals. Pink: virus detected in a Sentinel patient with pneumonia. *Italic:* influenza viruses detected in household contacts.*: vaccinated patients

recognition by the 2011-2012 vaccine-induced human antibodies is unknown, but it suggests a decreased affinity of H3N2-specific antibodies for these viruses. This

observation is concordant with the decreased MN titer obtained with the vaccine antiserum.

a)



b)

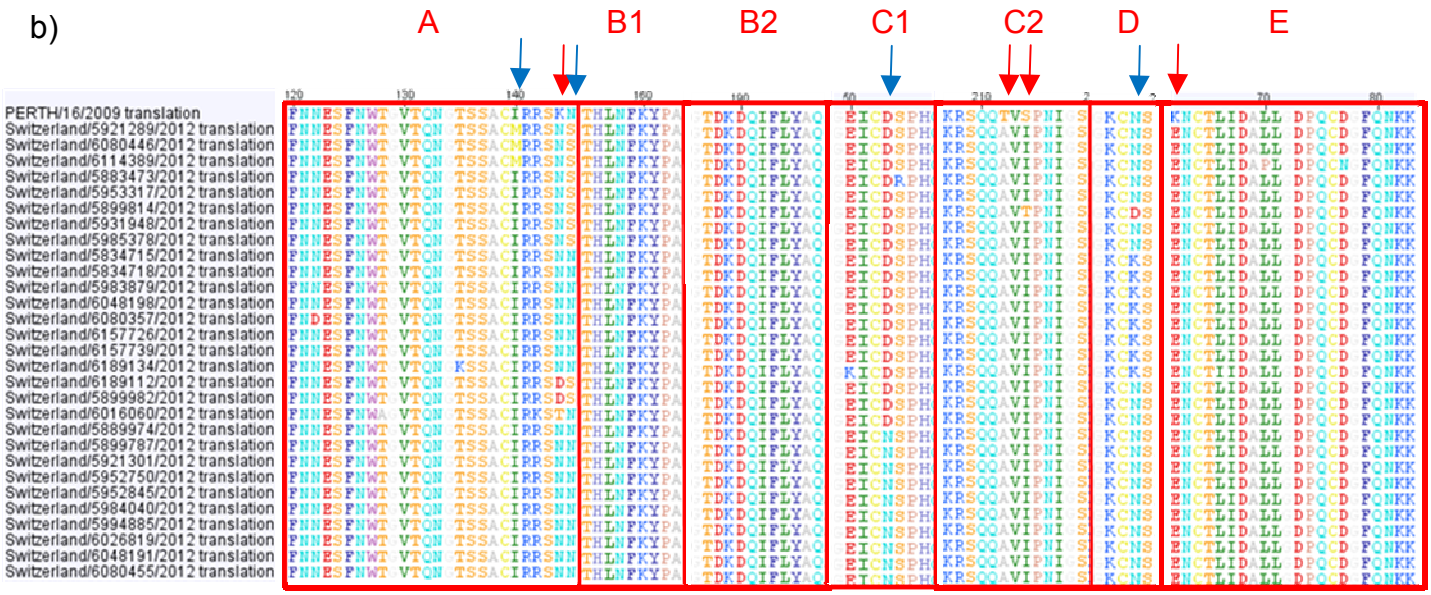


Figure 10: Amino acid sequence of influenza A (H3N2) viruses HA protein detected in Switzerland during the 2011-2012 season

a) Schema of an HA protein with the five epitope regions labeled by letters A-E; reproduced with permission from Wilschut and Elhanet, 2005.[17]

b) Amino acid alignment of epitope regions. Differences between the vaccine strain A/Perth/16/2009 (H3N2) and viruses of the Sentinel network HA amino acid sequences are indicated by arrows. Red arrows indicate a difference in all the Swiss sequences and blue arrows indicate differences in sporadic cases.

6.3.2.3. Influenza strains detected at the University of Geneva Hospitals

An epidemiological study has been conducted in hospitalized patients with a suspicion of ILI at the University of Geneva Hospitals (HUG). Among 176 suspected cases, 49 (27.8%) samples were positive for influenza A, two (1.1%) for influenza B, one (0.5%) for influenza A (H1N1)pdm09, 47 (26.7%) for other respiratory viruses, and 95 (53.9%) were negative. HA sequencing of seven influenza A (H3N2) viruses was determined. All HA genes had the Victoria/208 clade and subclade 3 specific mutations: T212A, N312S, A198S, and V223I (figure 9, blue labeled strains). Four strains presented the 3b specific amino acids S45, T48, S145, and R54.

6.3.3. influenza B viruses

33 influenza B viruses were detected. Of these, 17 were influenza B Victoria-like and 14 Yamagata-like viruses. Seven influenza strains were analyzed by HI assays; 4 were Victoria-like and 3 Yamagata-like viruses. To complete the virological characterization, 17 HA genes of these B viruses have been sequenced (nine Victoria-like and eight Yamagata-like).

6.3.3.1 Influenza B Victoria-like

Four influenza B Victoria-like strains analyzed by HI assay showed that they were antigenically related to the strain influenza B/Hong Kong/514/2009, close to the vaccine strain B/Brisbane/60/2008 (annex 3). HI titers were even higher with the more recent reference antisera B/Paris/1762 and B/Odessa/3886/2010, as confirmed by the WHO collaborating centre in London. One influenza B strain, detected in an 11-year-old girl living in the canton of Vaud, showed HI titer with more than a 32-fold reduction. However, this strain was well recognized by an older antiserum raised against the influenza B/Malaysia/2506/2004. Further sequencing analysis showed that this virus was closely related to clade 5 strains of the B-Victoria lineage. These viruses are mainly detected in Asian countries,[10] but no travel history was reported by the Sentinel practitioner for this subject. Sequencing analysis of eight HA genes of Sentinel samples showed that all influenza B Victoria-like strains were of clade 1,

Brisbane/60, with characteristic mutations N75K, N165K, S172P, L58P, and V146I (figure 11).

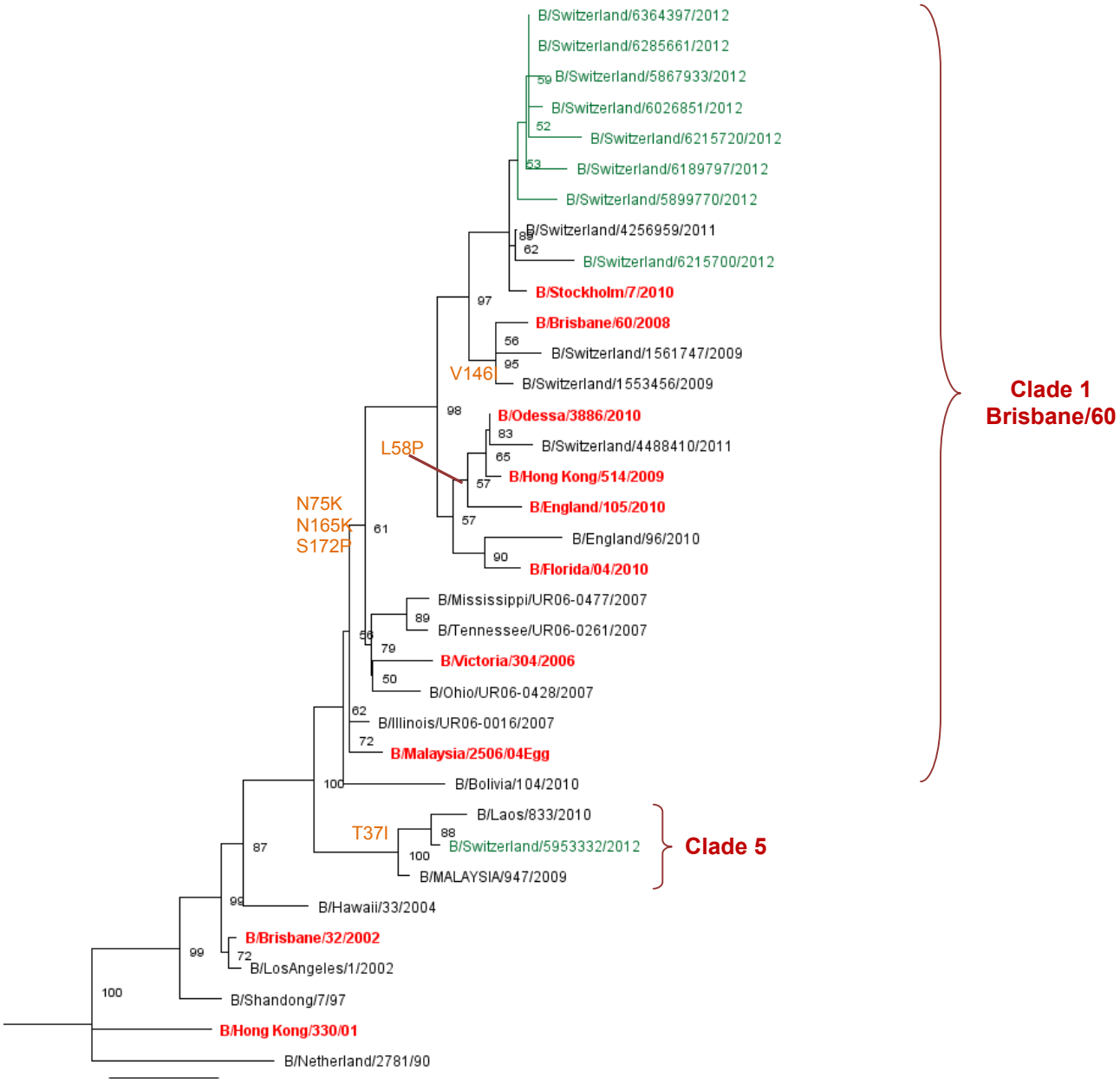


Figure 11: Phylogenetic analysis of the HA gene of influenza B Victoria-like viruses

Red: vaccine strain; green: influenza virus detected in Switzerland during the 2011-2012 season

6.3.3.2. Influenza B Yamagata-like

Three influenza B/Yamagata-like viruses have been characterized by HI analysis. All these strains showed a decreased HI titer with B/Bangladesh/3333/2010 and

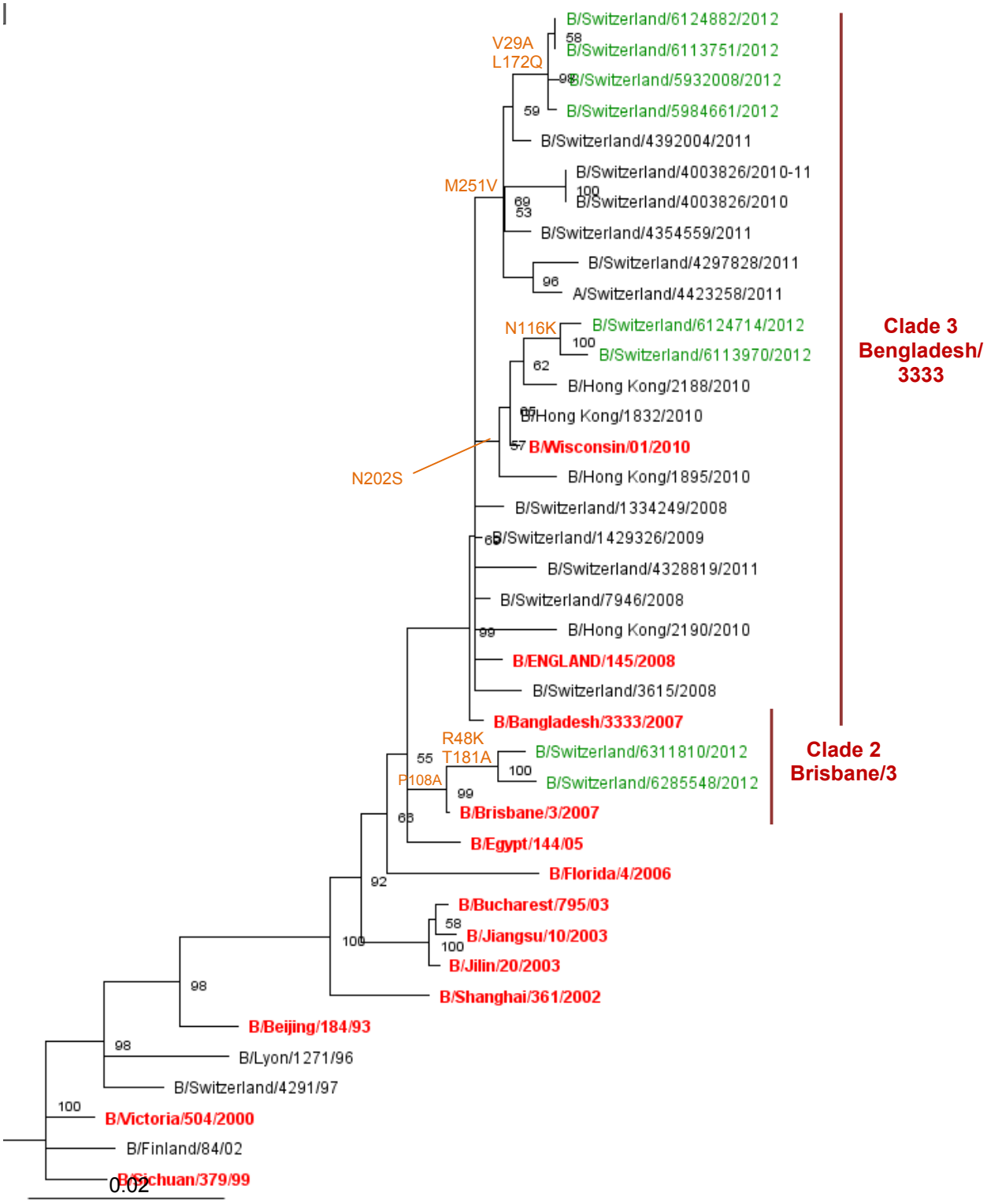


Figure 12: Phylogenetic analysis of the HA gene of influenza B Yamagata-like viruses

Red: vaccine strain; green: influenza virus detected in Switzerland during the 2011-2012 season

decreased affinity of antisera for cell grown viruses as later ones have been produced with egg-adapted influenza viruses. The significance of this low HI reaction remains to be confirmed.

Eight HA genes of influenza B/Yamagata-like strains have been sequenced. 6/8 of these sequences were distributed in clade 3 and clustered with the B/Bangladesh/3333/2007 and B/Wisconsin/1/2010 reference strains (figure 12). In a more detailed analysis, these strains appeared to be distributed in two different genetic groups previously described.[10] The first group is constituted by two strains harbouring N202S and N116K mutations that appeared close to influenza B/Wisconsin/1/2010. The second group had the M251V together with V29A & L172Q mutations. These strains previously analyzed by HI showed a decreased titer with B/Wisconsin/1/2010 and B/Bangladesh/3333/2007 antisera. 2/8 Yamagata-like influenza B viruses were closer to the Brisbane/3 clade 2, which is associated with more ancient strains.

7. Antiviral resistance

To detect any antiviral-resistant variant, 10 NA genes of six influenza A (H3N2) and four influenza A (H1N1)pdm09 viruses were analyzed. No opportunistic mutation of NA conferring resistance to NI was detected during the 2011-2012 influenza season in Switzerland. The same observation was made for European countries. A total of 12 countries have screened 962 influenza A(H3N2), A(H1N1)pdm09 and B viruses for susceptibility to the NI, oseltamivir and zanamivir. None of these viruses showed resistance or reduced susceptibility. All A(H1N1)pdm09 and A(H3N2) viruses screened for susceptibility to adamantanes were found to be resistant.

8. WHO recommendation for the composition of influenza virus vaccines for use in the 2012-2013 northern hemisphere influenza season

The WHO annual consultation for the recommendation of the composition of influenza vaccine in the northern hemisphere took place in February 2012. Two influenza strains were changed in the vaccine composition. Influenza A/Perth/16/2009 (H3N2) was replaced by influenza A/Victoria/361/2011 (H3N2).

Influenza B/Brisbane/60/2008 strain, a Victoria-like lineage, has been replaced by a Yamagata-like lineage, the influenza B/Wisconsin/1/2010 strain.

Table 5 : Recommended composition of influenza vaccine for the 2012-2013 season[15]

	Vaccine strain 2012-2013
A (H1N1)pdm09	**A/California/7/2009**
A (H3N2)	**A/Victoria/361/2011**
B	**B/Wisconsin/1/2010**

9. Discussion

The season 2011-2012 constituted the third influenza season following the emergence of influenza A (H1N1)pdm09 virus in March 2009. By contrast to the past two seasons dominated by this new variant, a very low detection rate was observed this season, suggesting a progressive extinction of this virus. After playing a second role during the last two seasons, influenza A (H3N2) virus constituted more than 85% of influenza viruses characterized in the northern hemisphere reported to WHO.[16] In Switzerland, this virus constituted 77.5% of all viruses detected during the surveillance period. During previous seasons, influenza A (H3N2) predominated 10/17 epidemics and was present in three additional seasons, together with another virus. Although this virus is usually associated with the more severe influenza epidemics, the 2011-2012 epidemic can be classified as moderate. Medical consultation values remained in the range observed during many past seasons and the number of samples remained well below the number usually received by the NRCI during an epidemic with a high impact in the community.

Influenza A (H3N2) viruses affected preferentially the younger age groups (<14 years). These age groups were the same as those affected by influenza A (H1N1)pdm09.[12,13] However, individuals over > 65 years old were almost exclusively

affected by influenza A (H3N2) viruses. Whereas influenza A (H1N1)pdm09 and influenza B affected between 1% to 7% of the younger age groups, no such viruses were detected in those > 65 years old. A low antigen variation is observed for these two viruses in comparison with influenza A (H3N2) viruses. Indeed, the two past influenza seasons with influenza A (H1N1)pdm09 circulation, together with the regular circulation of influenza B viruses, induced most probably a cross-protection in those already exposed. As a consequence of a higher influenza A (H3N2) virus circulation in the elderly, an excess in the mortality rate of individuals > 65 years old was observed in 12/14 European countries, including Switzerland, that carry out weekly monitoring of all-cause mortality.[9]

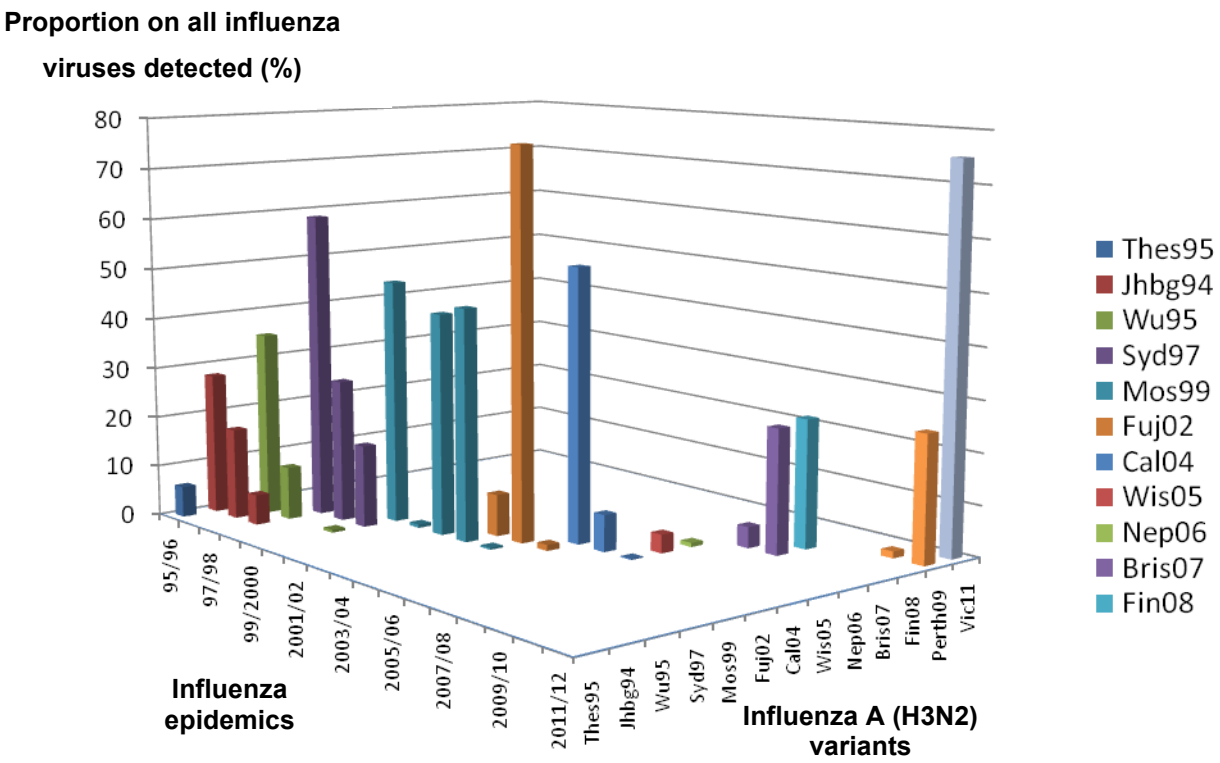


Figure 13: Influenza A (H3N2) variants detected according to surveillance seasons, 1995 to 2012 (%)

This year, we collected antigenic and genetic data indicating that influenza A (H3N2) viruses that circulated in Switzerland, had a decreased recognition rate by influenza A/Perth/16/2009 vaccine reference antiserum, as observed in other European countries. This observation was confirmed also by WHO[16] and the European WHO collaborating centre.[10] This information suggests that 2011-2012 influenza vaccine

induced a reduced immune protection against influenza A (H3N2) viruses this winter. During the 17 past surveillance seasons, 13 A (H3N2) variants could be detected (figure 13). Usually, the variant circulates between two to three years, with the exception of influenza A/Moscow/10/99 that circulated between 1999/2000 to 2002/03. Thus, after the past two epidemics without a massive circulation of this virus, a new drifted H3N2 virus was not unexpected. This strain could also persist for one or two more years.

Influenza B and A (H1N1)pdm09 viruses were also present, but at a lower rate. Each B variant can be detected from between two to five consecutive years (figure 14). This is somewhat longer than A (H3N2) that circulated for a maximum of three years (figure 12). B viruses are genetically and antigenically more stable. However, during the 2001-2002 season, a second B-lineage occurred in Asia, the B Victoria-like lineage. Since this period, it has co-circulated alternatively during the seasons with the B Yamagata-like lineage (figure 14) with some absences in 1999/2000 and 2006/07.

Detection rate on all influenza viruses detected (%)

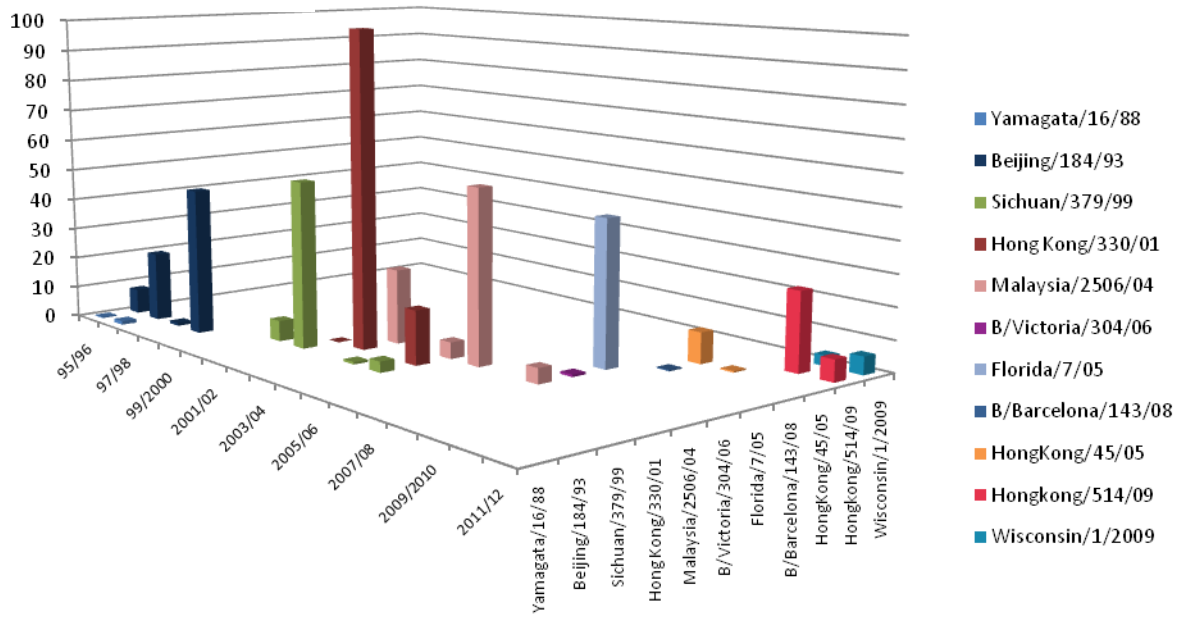
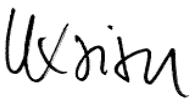


Figure 14: Influenza B variants detecting according to surveillance seasons, 1995-2012

Blue and green: influenza B Yamagata-like lineage
Red and yellow: influenza B Victoria-like lineage

This alternance between both lineages raises difficulties to select the next B strain choice for influenza vaccine composition. Interestingly, for the first time since influenza surveillance in Switzerland, two B lineages co-circulated at approximately similar rates. Since no cross-reaction can be observed between Victoria-like antiserum and Yamagata-like strains, half of all influenza B viruses were not well covered by the 2011-2012 influenza vaccine containing an influenza B/Brisbane/60/2008, a Victoria-like lineage. Together with influenza A (H3N2) viruses that drifted from the vaccine strain, most circulating influenza viruses during this epidemic were less covered by the 2011-2012 vaccine. For this reason, two influenza strains have been adjusted in the 2012-2013 vaccine.[4]

Geneva, 12 July 2012



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Annex 1: Influenza virus detection according to weeks and nature of the virus

Sentinel Surveillance Hiver 2011-12																		
Weeks	Dates		‰ ILI	Total sample	Flu. A undet.	A/H1N1pdm09			A (H3N2) seasonal				Influenza B				Total virus (n)	% pos
						undet.	HK10	Total	undet	Perth09	Vic11	Total	undet	BHK09	Bwiscon	Total		
40	01-oct-11	07-oct-11	1.5	7													**0**	
41	08-oct-11	14-oct-11	2.7	10													**0**	
42	15-oct-11	21-oct-11	2.4	11													**0**	
43	22-oct-11	28-oct-11	3.1	14													**0**	
44	29-oct-11	04-nov-11	2	14													**0**	
45	05-nov-11	11-nov-11	3	10													**0**	
46	12-nov-11	18-nov-11	2.4	10													**0**	
47	19-nov-11	25-nov-11	3.3	15										1		**1**	**1**	**7**
48	26-nov-11	02-déc-11	2.3	22													**0**	**0**
49	03-déc-11	09-déc-11	3.6	17													**0**	**0**
50	10-déc-11	16-déc-11	3.7	15													**0**	**0**
51	17-déc-11	23-déc-11	3	27							2	**2**					**2**	**0**
52	24-déc-11	30-déc-11	4.2	11								**0**					**0**	**0**
1	31-déc-11	06-janv-12	6.2	11							2	**2**					**2**	**18**
2	07-janv-12	13-janv-12	5.1	22					1		5	**6**					**6**	**27**
3	14-janv-12	20-janv-12	6.6	25					1		1	**2**		1		**1**	**3**	**12**
4	21-janv-12	27-janv-12	6.7	36			1	**1**	1		4	**5**		1		**1**	**7**	**19**
5	28-janv-12	03-févr-12	9.8	31				**0**	4		3	**7**			1	**1**	**8**	**26**
6	04-févr-12	10-févr-12	11.6	59	**3**			**0**	19		3	**22**		1		**1**	**26**	**44**
7	11-févr-12	17-févr-12	18.1	55	**3**	1		**1**	17		3	**20**		1	2	**3**	**27**	**49**
8	18-févr-12	24-févr-12	25.2	41			2	**2**	22		2	**24**		3		**3**	**29**	**71**
9	25-févr-12	02-mars-12	26.7	56	**2**	3		**3**	32		2	**34**			1	**1**	**40**	**71**
10	03-mars-12	09-mars-12	19.3	35	**2**	2		**2**	17			**17**			1	**1**	**22**	**63**
11	10-mars-12	16-mars-12	15.9	42		2		**2**	17			**17**			4	**4**	**23**	**55**
12	17-mars-12	23-mars-12	11.8	22				**0**	8			**8**	1			**1**	**9**	**41**
13	24-mars-12	30-mars-12	10.1	36	**1**			**0**	17			**17**	1	5	1	**7**	**25**	**69**
14	31-mars-12	06-avr-12	5.2	26				**0**	4			**4**		2	2	**4**	**8**	**31**
15	07-avr-12	13-avr-12	4.1	9				**0**	3			**3**				**0**	**3**	**33**
16	14-avr-12	20-avr-12	1.4	10				**0**				**0**		2	2	**4**	**4**	**40**
				699	**11**	**8**	**3**		**163**	**0**	**27**		**2**	**17**	**14**		**245**	
						11			**190**				**33**					

Annex 2: Microneutralization assay

Viral suspension recovered from cell supernatant of 13 influenza A (H3N2) grown on SIAT cells have been analysed by MN assay. The tested strain is inhibited by the tested serum within a range of dilutions (no colour) and start to grow again (yellow) when no sufficient antisera can still inhibit viral culture. a) Homologue titre obtained with 5, 10, and 20 $TCID_{50}$ of the A/Perth/16/2009 strain in the presence of serial dilution of corresponding antiserum is represented. b) 10 $TCID_{50}$ of 13 influenza A (H3N2) viruses were incubated with a two-fold dilution of influenza A/Perth/16/2009 (H3N2) ferret antiserum for a MN assay c) MN test obtained with dilution of human antiserum vaccinated with the A/Perth/16/2009 strain.

a) Microneutralization of influenza A/Perth/16/2009 (H3N2)

Plate 5	A/Perth/16/2009 AS dilution											
Strains	32	64	128	256	512	1024	2048	4096	8192	16384	32768	C-
Perth 20 $TCID_{50}$	0.198	0.267	0.329	0.465	0.528	0.528	0.595	0.515	0.454	0.530	0.489	0.091
	0.216	0.241	0.509	0.489	0.663	0.530	0.552	0.506	0.408	0.467	0.454	0.066
Perth 10 $TCID_{50}$	0.151	0.141	0.216	0.302	0.341	0.462	0.515	0.599	0.546	0.627	0.551	0.043
	0.151	0.152	0.209	0.323	0.369	0.363	0.381	0.377	0.376	0.452	0.377	0.118
Perth 5 $TCID_{50}$	0.112	0.066	0.085	0.148	0.177	0.190	0.198	0.214	0.269	0.286	0.217	0.076
	0.113	0.115	0.140	0.197	0.241	0.234	0.236	0.244	0.355	0.296	0.293	0.089
C-	0.020	0.020	0.020	0.023	0.025	0.019	0.019	0.018	0.022	0.016	0.036	0.071
	0.025	0.021	0.022	0.019	0.026	0.030	0.023	0.025	0.029	0.024	0.022	0.025
Cut off	0.152991											

b) Microneutralization

Plate 1	A/Perth/16/2009 AS dilution											
Samples	32	64	128	256	512	1024	2048	4096	8192	16384	32768	C-
5920857	0.347	0.332	0.329	0.296	0.341	0.333	0.364	0.331	0.370	0.358	0.350	0.095
5952619	0.415	0.398	0.396	0.424	0.386	0.391	0.407	0.358	0.412	0.409	0.376	0.065
5952760	0.369	0.482	0.476	0.527	0.481	0.534	0.561	0.602	0.564	0.564	0.572	0.04
5952968	0.204	0.182	0.161	0.174	0.164	0.197	0.208	0.222	0.226	0.221	0.211	0.024
6015981	0.159	0.179	0.194	0.216	0.237	0.266	0.262	0.260	0.305	0.359	0.317	0.019
6048351	0.205	0.187	0.239	0.258	0.230	0.292	0.341	0.318	0.339	0.355	0.321	0.026
6114397	0.092	0.076	0.059	0.080	0.079	0.103	0.121	0.144	0.167	0.174	0.114	0.021
6124837	0.114	0.105	0.108	0.128	0.125	0.213	0.210	0.244	0.215	0.230	0.183	0.029
Cut-off	0.114372											

Plate 2	A/Perth/16/2009 AS dilution											
Samples	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	**4096**	**8192**	**16384**	**32768**	**C-**
6125030	**0.246**	0.484	0.613	0.587	0.611	0.574	0.532	0.538	0.629	0.601	0.462	0.098
6146939	**0.304**	0.523	0.392	0.412	0.395	0.332	0.367	0.356	0.335	0.361	0.335	0.059
6157769	**0.245**	0.413	0.465	0.522	0.448	0.338	0.398	0.390	0.423	0.397	0.416	0.038
6215618	**0.631**	0.602	0.638	0.668	0.645	0.573	0.586	0.626	0.589	0.621	0.634	0.032
6246188	**0.464**	0.407	0.418	0.476	0.459	0.383	0.361	0.425	0.369	0.408	0.432	0.027
Tneg	**0.025**	0.017	0.019	0.013	0.018	0.019	0.017	0.017	0.014	0.015	0.047	0.036
Tneg	**0.025**	0.019	0.020	0.019	0.018	0.020	0.017	0.020	0.018	0.016	0.112	0.046
Tneg	**0.052**	0.028	0.033	0.028	0.025	0.030	0.034	0.032	0.027	0.073	0.168	0.136
Cut-off	**0.16417**											

c) Microneutralization with vaccinated individual

Plate 7	A/Perth/16/2009 vaccinated individual (1-004)											
Samples	**1/8**	**1/16**	**1/32**	**1/64**	**1/128**	**1/256**	**1/512**	**1/1024**	**1/2048**	**1/4096**	**1/8192**	**C- AS**
5920857	0.135	0.083	0.094	0.112	0.176	0.213	0.267	0.254	0.253	0.250	0.245	0.074
5952619	0.142	0.100	0.086	0.132	0.319	0.301	0.276	0.256	0.287	0.225	0.224	0.050
5952760	0.132	0.109	0.105	0.182	0.311	0.348	0.357	0.361	0.417	0.376	0.363	0.069
5952968	0.122	0.057	0.048	0.053	0.081	0.100	0.17	0.149	0.183	0.157	0.144	0.102
6048351	0.122	0.186	0.163	0.116	0.137	0.198	0.212	0.249	0.321	0.238	0.292	0.153
6157769	0.116	0.083	0.114	0.145	0.172	0.141	0.155	0.134	0.166	0.133	0.161	0.067
6125030	**0.115**	0.070	0.118	0.137	0.102	0.115	0.095	0.217	0.111	0.086	0.069	0.024
Perth/16/2009	0.128	0.081	0.053	0.047	0.069	0.089	0.133	0.144	0.148	0.113	0.128	0.025
cut-off:	0.136094											

Annex 3: Hemagglutination inhibition of influenza B

		Influenza B antisera							
		B/Brisbane 60/08	B/Hong Kong 514/09	B/Paris 1762/09	B/Florida 04/06	B/Banglades h 3333/07	B/Wisconsin 01/10	Malaysia 2506/04	England 145/08
		Victoria-like			Yamagata-like			Victoria-like	
Victoria-like strains	B/Brisbane/60/2008	512	256	128	<8	<8	<8	ND*	ND
	B/Hong Kong/514/2009	128	512	128	<8	<8	<8	ND	ND
	B/Paris/1762/2009	128	512	128	<8	<8	<8	ND	ND
Yamagata-like strains	B/Florida/4/2006	32	<8	<8	4096	512	256	ND	ND
	B/Bangladesh/3333/07	16	<8	<8	1024	512	256	ND	ND
	B/Wisconsin/01/10	<8	<8	<8	512	256	256	ND	ND
Samples	HI result								
5632623	B/Hong Kong/514/2009	64	256	64	<16	<16	<16	ND	ND
5867933	B/Hong Kong/514/2009	64	256	64	<16	<16	<16	ND	ND
6026851	B/Hong Kong/514/2009	128	128	128	ND	ND	ND	ND	ND
5953332	Victoria-like (Asia)	<16	16	16	<16	<16	<16	32	<16
5932008	Poor recognition by antisera available directed against the vaccine strain	<16	<16	<16	128	64	32	ND	ND
5984661		ND	ND	ND	128	64	32	ND	ND
6124882		ND	ND	ND	128	64	32	ND	ND

* ND : Not done

Annex 4: Hemagglutination inhibition of influenza A (H1N1)pdm09

		Influenza A (H1N1)pdm09			
		A/California/7/09	A/Bayern/69/09	A/Hong Kong/2212/10	A/Brisbane/10/07 (seasonal)
Strains	A/California/7/09	1024	256	256	<8
	A/Bayern/69/09	256	512	128	<8
	A/Hong Kong/2212/10	1024	512	2048	<8
	A/Brisbane/10/07 (Seasonal)	<8	<8	<8	1024
Samples	HI result				
5307666	A/Hong Kong 2212/2010	1024	ND	2048	<8
5899843	A/Hong Kong 2212/2010	1024	1024	2048	<16
6048812	A/Hong Kong 2212/2010	2048	ND	2048	<16