 doi:10.20944/preprints202010.0100.v1

Article

Serologically-based evaluation of cross-protection antibody responses among different A(H1N1) influenza strains

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Abstract: After the influenza H1N1 pandemic of 2009, the seasonal A/Brisbane/59/2007 strain was replaced by the A/California/07/2009 strain for the influenza virus vaccine composition. After several seasons with no indications on the occurrence of antigenic drift, A/Michigan/45/2015 was chosen as the H1N1 vaccine strain for season 2017/2018. Since the immune response to influenza is shaped by the history of exposure to antigenically similar strains, the potential cross-protection between seasonal human influenza vaccine strains and the emerging pandemic strains was investigated. Human serum samples were tested by haemagglutination inhibition and single radial haemolysis assays against A/Brisbane/59/2007, A/California/07/2009, and A/Michigan/45/2015 strains. Strong cross-reactions between A/California/07/2009 and A/Michigan/45/2015 strains were observed in 2009/2010, most likely induced by the start of the 2009 pandemic, and the subsequent post-pandemic seasons from 2010/2011 onwards when A/California/07/2009 becomes the predominant strain. In 2014/2015 season, population immunity against A/California/07/2009 and A/Michigan/45/2015 strains increased again, associated with strong cross-reactions. While haemagglutination inhibition assay has a higher sensitivity for detection of new seasonal drift, the single radial haemolysis assay is an excellent tool to determine the presence of pre-existing immunity, allowing a potential prediction on the booster potential of influenza vaccines against newly emerging drifted strains.

Keywords: influenza virus; H1N1; immunity; antigenic drift.

1. Introduction

The immune response to influenza is shaped by the individual history of exposure to related antigens. Individuals who have been exposed to a greater number of influenza strains, through natural infection and/or vaccination, may be likely to harbor pre-existing memory responses that cross-react with vaccine strains.

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A new A/H1N1 influenza virus began circulating in humans in spring of 2009 and has caused the first pandemic of the 21st century. The new virus (A/California/07/2009 H1N1pdm09) was the result of a triple reassortment from human, swine and Eurasian avian influenza viruses and affected mostly children and young adults [1, 2]. This could be due to the similarity of the H1N1 pdm09 strain to the viruses circulating in humans between the 1930s and 1950s suggesting that adults, in particular those over 60 years old, could have some cross-reactive antibody responses against the pandemic virus [3-6], as proven by several studies [3, 4, 7-11].

The recommendation for the H1N1 component for the influenza vaccines changed from A/New Caledonia/20/1999-like strain in seasons 2005/2006 and 2006/2007 to A/Solomon Island/03/2005-like strain in season 2007/2008 and subsequently to A/Brisbane/59/2007-like strain in seasons 2008/2009 and 2009/2010. However, in 2009 A/California/07/2009 emerged as a pandemic strain and replaced A/Brisbane/59/2007 as the H1N1 circulating strain. Consequently, the World Health Organization (WHO) vaccine recommendation provided for A/California/07/2009 as the seasonal H1N1 vaccine strain after the end of the pandemic. In 2017/2018 season, WHO recommendation changed to A/Michigan/45/2015-like (Table 1) [12]. Here we refer to the recommendations until 2009/2010 as "pre-pandemic", 2009 and 2010 as "pandemic", and from season 2010/2011 onwards as "post-pandemic".

Table 1. Influenza A/H1N1 strain egg-based vaccine composition Northern Hemisphere (NH) seasons 2005/2006 – 2020/2021.

NH SEASON	A/H1N1 STRAIN
2005/2006	A/New Caledonia/20/99-like
2006/2007	A/New Caledonia/20/99-like
2007/2008	A/Solomon Island/3/2005-like
2008/2009	**A/Brisbane/59/2007-like**
2009/2010	A/Brisbane/59/2007-like
2010/2011	**A/California/7/2009-like**
2011/2012	A/California/7/2009-like
2012/2013	A/California/7/2009-like
2013/2014	A/California/7/2009-like
2014/2015	A/California/7/2009-like
2015/2016	A/California/7/2009-like
2016/2017	A/California/7/2009-like
2017/2018	**A/Michigan/45/2015-like**
2018/2019	A/Michigan/45/2015-like
2019/2020	A/Brisbane/02/2018-like
2020/2021	A/Guangdong-Maonan/SWL1536/2019-like

Haemagglutinin (HA) and neuraminidase (NA) are the two major surface glycoproteins of influenza viruses, both recognizing the sialic acid (SA). HA binds to SA on the host cells allowing the virus entry, while the NA has enzymatic activity removing SA and facilitating the release of progeny virus [13]. The HA is the major targets of humoral immune responses and rapid antigenic variations due to the accumulation of mutations result in antigenic drift. In addition, periodic reassortment between antigenically distinct influenza viruses can lead to antigenic shift and the emergence of

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pandemic strains [14]. Most antibody responses against HA are strain-specific, targeting the globular head (HA1) of HA. Alternatively, the stalk (HA2) of HA is highly conserved when compared with the globular head, making it a strong target for vaccine development to induce broadly and protective immune responses [15].

The immunological response to influenza vaccine and/or natural infection is mainly evaluated by two serological techniques, the haemagglutination inhibition (HI) and the single radial haemolysis (SRH) which still are the most widely used and officially recognized serological assays by regulatory authorities [16]. In recent years, the importance of neutralization assays for serological evaluations has been increased and the analysis of neuraminidase-specific antibodies has also become a topic of increasing interest [17].

However, the HI assay is still considered the gold standard for antigenic and serological analysis [18]. In addition, it still widely serves as a correlate of protection for influenza vaccines in detecting antibodies able to inhibit the interaction between red blood cells (RBCs) and the head domain of HA i.e. blocking of receptor-binding activities and subsequent inhibition of infection events. The detected antibodies seem to be strain-specific and not protective against mismatching influenza strains [17, 19].

Alternatively, the SRH assay measures complement fixation antibodies, mainly IgG (IgG1 and IgG3), not only against the surface glycoproteins HA and NA, but also against internal antigens, leading to a potential lack of specificity to HA antibodies [16, 17, 20, 21].

For decades, an HI titer ≥40 and a SRH haemolysis area of 25 mm2 or greater have been acknowledged as correlates of protection and were considered as the protective threshold level beyond which the probability of contracting influenza infection was reduced by 50% or more. Since February 2017, the revised guidelines on influenza vaccines by the European Medicine Agency (EMA) have withdrawn the traditional concept of HI and SRH as correlates of protection [22], in contrast to other representative regulatory authorities such as United States Food and Drug Administration (US FDA). However, this debate on HI and/or SRH representing a "correlate of protection" (CoP) or at least a "surrogate of protection" (SoP) is still ongoing [17].

The aim of this study was to investigate the specificity of the HI and SRH assays with respect to influenza virus antigenic drift and shift variants and potential cross-reactivity between seasonal human influenza H1N1 vaccine strains and the emerging pandemic H1N1 strain of swine origin. Therefore, A/Brisbane/59/2007 has been chosen as the last recommended H1N1 strain of the pre-pandemic seasons until 2008/2009, representing the H1N1 viruses circulating in the human population since then. The pandemic strain A/California/07/2009 has been chosen as the representative strain for the pandemic season 2009/2010 and the post-pandemic seasons from 2010/2011 onwards, including A/Michigan/45/2015 as a drift variant of A/California/07/2009, recommended by WHO as the H1N1 vaccine strain of seasons 2017/2018.

2. Materials and Methods

2.1 Virus antigens

The infectious influenza A H1N1 viruses used for serological assays were seasonal influenza strains obtained from NIBSC: A/Brisbane/59/2007 (H1N1, 09/276), A/Michigan/45/2015 (H1N1, 16/354) and A/California/07/2009 (H1N1, 09/216). All viruses used were egg-grown.

2.2. Serum samples

Human serum samples were obtained from the Sera Bank of the Laboratory of Molecular Epidemiology, Department of Molecular and Developmental Medicine, University of Siena, Siena, Italy. The samples were anonymously collected and stored in compliance with Italian ethics law.

Serum samples were collected in Italy between January 2005 and August 2017, and randomly selected in order to have 100 samples for each season included in this study (2005/2006, 2008/2009, 2009/2010, 2010/2011, 2013/2014, 2014/2015, 2015/2016, 2016/2017) balanced between two age groups, 18-65 years old (younger adults) and >65 years old (elderly adults).

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2.3. Haemagglutination Inhibition assay

All serum samples have been pre-treated with receptor destroying enzyme (RDE) (ratio 1:5) from Vibrio Cholerae (Sigma Aldrich, St. Louis, MO, USA) for 18 hours at 37°C in a water bath and then heat inactivated for 1 hour at 56°C in a water bath with 8% sodium citrate (ratio 1:4).

Turkey RBCs (TRBCs) were centrifuged two times, washed with 0.9% saline solution and adjusted to a final dilution of 0.35%.

From an initial dilution of 1:10, serum samples were 2-fold diluted in duplicate with 0.9% saline solution in a 96-well plate. Twenty-five microliters (◎l) of standardized viral antigen were added to each well and the mixture was incubated at room temperature for one hour. TRBCs were added and after one hour of incubation at room temperature, the plates were evaluated for presence of agglutination inhibition.

The antibody titer is expressed as the reciprocal of the highest serum dilution showing complete inhibition of agglutination. Since the starting dilution is 1:10, the lower limit of detection (LoD) for antibody titer is 10. When the titer is under the detectable threshold, the results were conventionally expressed as 5 for calculation purposes, half the lowest detection threshold.

2.4. Single Radial Haemolysis assay

Serum samples were heat inactivated at 56°C for 30 minutes in a water bath.

Fresh TRBCs were centrifuged and washed with phosphate buffer saline (PBS) twice. Diluted virus antigen was added to the TRBCs suspension at a concentration of 2000 haemagglutinin units (HAU) per millilitres (ml). In order to allow the adsorption of viral antigen to the TRBCs, the suspension was incubated at 4°C for 20 minutes. A solution of Chromium Chloride (CrCl3) 2,5mM was added to the previous suspension and incubated at room temperature for 10 minutes to increase the binding affinity between the TRBCs and the viral antigen. The suspension was carefully mixed and centrifuged. The supernatant was removed and PBS was added to the pellet, which was then carefully re-suspended. A stock solution of 1.5% agarose in PBS containing 0.1% sodium azide and 0.5% low gelling agarose was prepared. The agarose stock solution was kept at 45°C in a water bath.

Each SRH plate consisted of TRBCs - viral antigen suspension and guinea pig complement in the agarose mixture. The final suspension was homogenized by shaking and spread onto each plate, incubated at room temperature for 30 minutes and then stored at 4°C for 30 - 90 minutes in order to set the agarose. Holes were punctured in each plate with a calibrated punch and 6 µl of serum samples and controls added into each hole. The plates were stored in a humid box and incubated at 4°C for 16 – 18 hours in the dark. After overnight incubation, the plates were furtherly incubated in a water bath at 37°C for 90 minutes and then the diameters of haemolysis areas read in millimetres with a calibrating viewer [21].

2.5. Statistical analysis

For the purpose of this study, only samples with HI titer ≥ 40 and SRH haemolysis area ≥25 mm2 were considered as positive.

The results from the HI and SRH assays were reported as proportion of positive samples along different seasons for the three strains and for two age groups (18-65 years old and > 65 years old), and were calculated with 95% confidence intervals (CI) (Wilson method) using the DescTools R-package. Chi-square test was used to compare proportions of positives. Statistical significance was set at $p<0.05$, two tailed. All statistical analyses were performed using Microsoft R-Open version 3.5.0 (R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/). Venn diagrams reporting proportions of cross-protection were prepared using the VennDiagram R-package.

2.6. Influenza haemagglutinin multiple sequence alignments

Multiple sequence alignments were performed using BLASTp server [23]. HA sequences of A/Brisbane/59/2007 (Genbank accession: CY030234) and A/Michigan/45/2015 (Genbank accession: MK622940) were compared against A/California/07/2009 (Genbank accession: GQ280797) and the similarity of the sequences were expressed as percentages.

 doi:10.20944/preprints202010.0100.v1

3. Results

Results by HI and SRH assays against human influenza H1N1 vaccine strains A/Brisbane/59/2007 (referred as A/Brisbane), A/California/07/2009 (referred as A/California), and A/Michigan/45/2015 (referred as A/Michigan) are shown in figures 1 and 2.

Between 2005/2006 and 2008/2009 seasons, A/Brisbane strain showed a significant increase in the proportion of positive subjects (39.0%, 95% CI 30.0-49.0 for HI and 79.0%, 95% CI 70.0-86.0 for SRH; $p<0.0001$ for both assays). In the following years, the proportion of HI positive subjects against A/Brisbane strain decreased, with the exception of a peak observed for the season 2015/2016 (43.0%, 95% CI 34.0-53.0; $p=0.0002$ vs 2014/2015 for HI results). In contrast, variations in the proportion of positive subjects were lower in SRH.

The A/California strain showed a trend characterized by two different peaks, the first in 2010/2011 (49.0%, 95% CI 39.0-59.0; $p<0.0001$ vs 2009/2010 for HI results) and the second one in 2014/2015 (53.0%, 95% CI 43.0-62.0; $p=0.004$ vs 2013/2014 for HI results). As observed for the A/California strain, also A/Michigan strain showed a peak in 2010/2011 (36.0%, 95% CI 27.0-46.0; $p=0.012$ vs 2009/2010 for HI results) and in 2014/2015 (38.0%, 95% CI 29.0-48.0; $p=0.002$ vs 2013/2014 for HI results) seasons. Considering SRH results, both strains showed a significant increase in seasons 2009/2010 (67.0%, 95% CI 57.0-75.0 for A/California and 63.0%, 95% CI 53.0-72.0 for A/Michigan; $p<0.0001$ and $p=0.002$ vs 2008/2009, respectively), while for season 2014/2015 only an increase for A/Michigan was observed (70.0%, 95% CI 60.0-78.0; $p=0.002$ vs 2013/2014). In general, proportions of positive subjects for A/Michigan were lower than for A/California in both assays.

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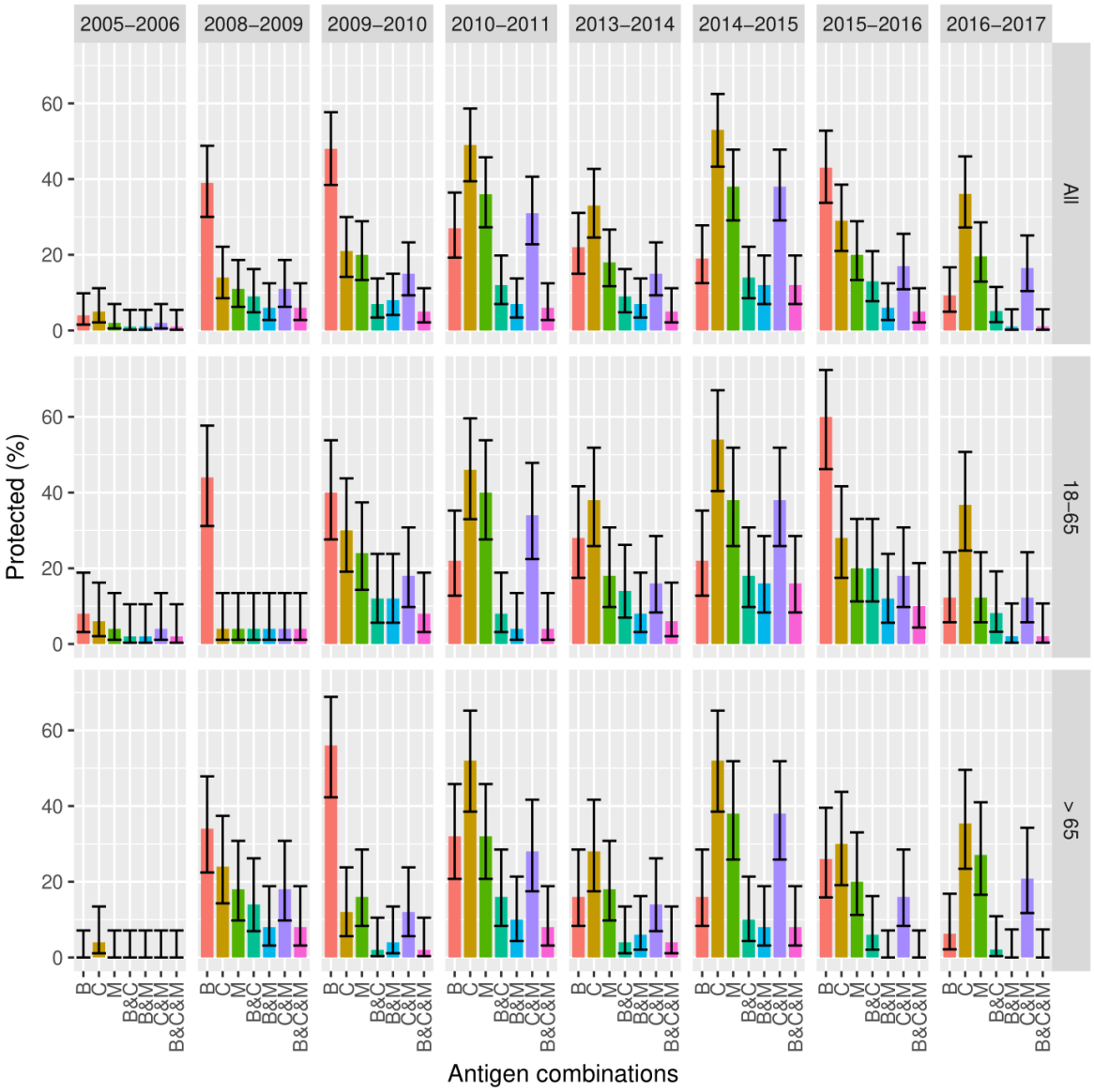

Figure 1. HI proportions of subjects with protective titers with 95% CI by strain (A/Brisbane, B; A/California, C; A/Michigan, M) and combinations, by age group and season.

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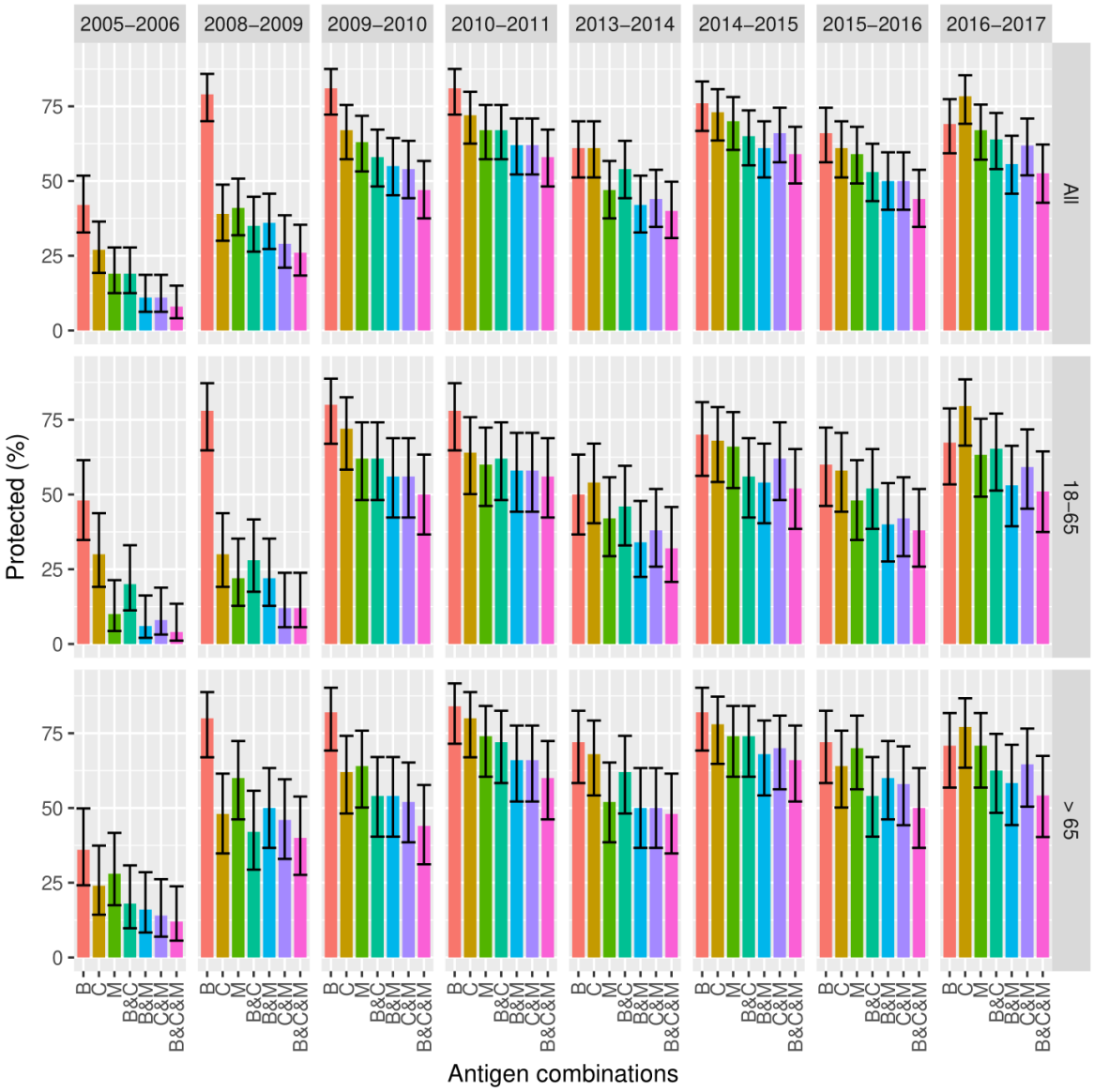

Figure 2. SRH proportions of subjects with protective titers with 95% CI by strain (A/Brisbane, B; A/California, C; A/Michigan, M) and combinations, by age group and season.

Considering proportions of cross-protection (Figure 3), in 2008/2009 season HI positives were mostly positive only for A/Brisbane (30.0%, 95% CI 21.9-39.6). In 2009/2010 season, the higher value was still represented by A/Brisbane only (38.0%, 95% CI 29.1-47.8), with higher proportion of positives in the >65 year old age group (52.0%, 95% CI 38.5-65.2 vs 24%, 95% CI 14.2-37.5 in 18-65 year old age group, p=0.004). In the same season, the second higher proportion was for A/California and A/Michigan (10%, 95% CI 5.3-17.6 of cross-protection), and from 2010/2011 season onwards, the higher proportions were for A/California and A/Michigan together and/or for A/California only, with the exception of season 2015/2016.

Considering, SRH results in season 2005/2006, 42.0% (95% CI 33.0-52.0) of the total population already showed protective titers against A/Brisbane, 27.0% (95% CI 19.0-36.0) for A/California, and 19.0% (95% CI 13.0-28.0) for A/Michigan. The highest proportion was for positives to A/Brisbane only (20.0%, 95% CI 13.3-29.0), followed by proportion of positives for A/California and A/Brisbane (11.0%, 95% CI 6.1-18.8). Considering the >65 year old age group, the second higher proportion was for all three strains together (12.0%, 95% CI 5.2-24.2). In 2008/2009 season, 34.0% (95% CI 25.4-43.7) of the subjects showed positivity to A/Brisbane only. However, when considering age groups, 40% (95% CI 27.6-53.8) of >65 years old showed protective titers for all of the three strains. This was also reflected

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in the results for the total population, where 26 (95% CI 18.4-35.4) of subjects had protective titers against all of three strains. Starting from 2009/2010 season onwards, the highest proportion of subjects had protective titers against all three strains, in both age groups.

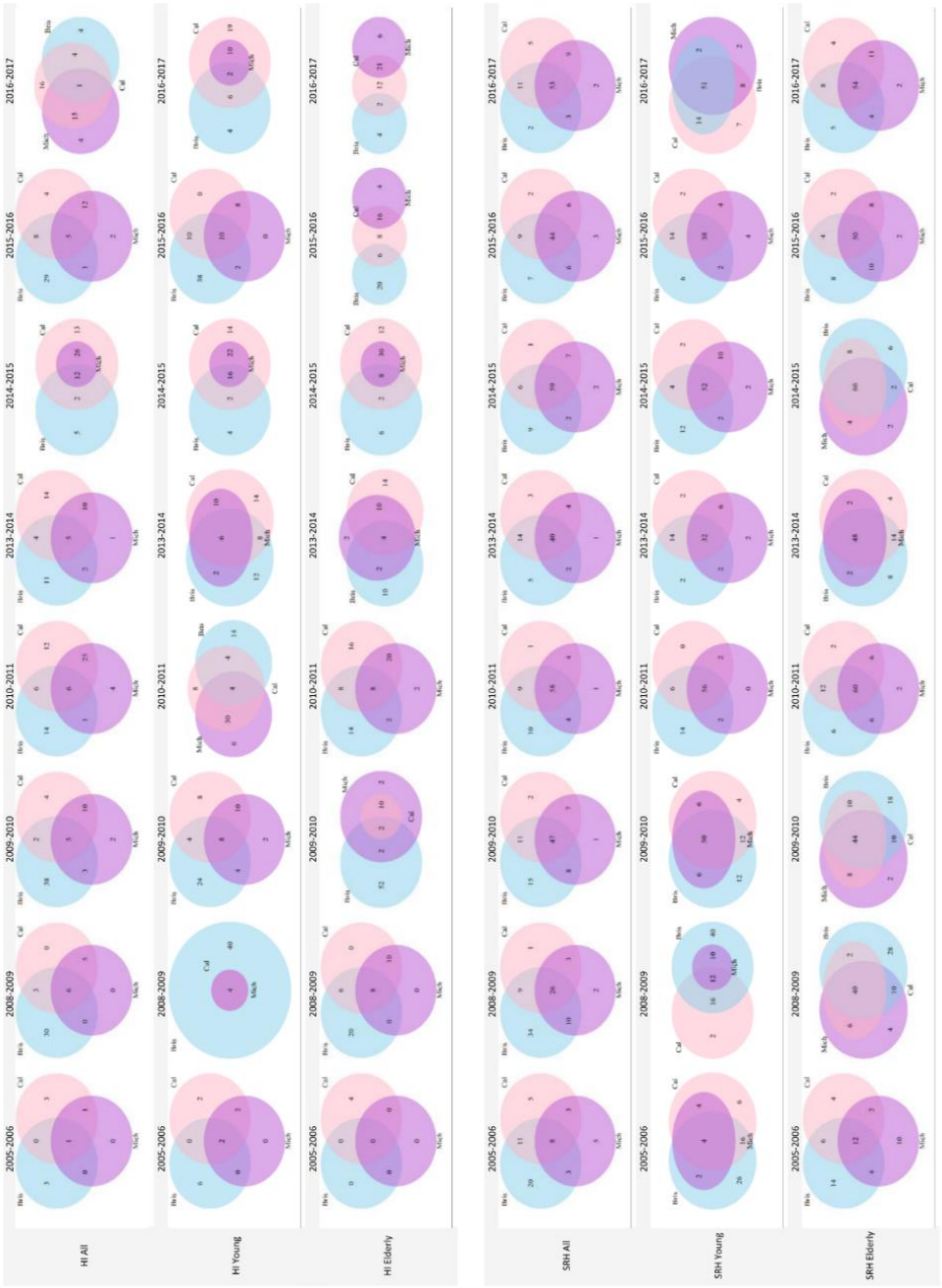

Figure 3. Venn diagrams for HI and SRH proportions of subjects with protective titers (A/Brisbane, blue circle; A/California, pink circle; A/Michigan, purple circle) and combinations, by age group and season.

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Overall, proportions of subjects with protective titers were significantly higher for SRH assay across strains, seasons, and age groups than HI assay ($p<0.0001$) (Table 2). The proportion of SRH positives was higher in the >65 years old age group in comparison to the 18-65 years old one.

Table 2. Proportions of positives (%) by strain, age group and assay.

age group	*assay*	*A/Brisbane*	*A/California*	*A/Michigan*
18-65	HI	29.50	30.00	20.25
>65	HI	23.25	29.75	20.75
18-65	SRH	66.17	58.95	46.91
>65	SRH	72.17	63.19	61.43

A comparison between the proportion of HI and SRH positives was performed between pre- and post-outbreak seasons for each strain, with reference to the WHO vaccine strain recommendation (Table 1). Seasons 2005/2006 (pre-outbreak) and 2008/2009 and following seasons (post-outbreak) were compared for A/Brisbane strain, and seasons 2005/2006 and 2008/2009 (pre-outbreak) were compared to seasons 2009/2010 and following (post-outbreak) for A/California strain. In contrast, there was no clear definition of pre- and post-outbreak seasons for A/Michigan strain when comparing the years before the WHO recommendation. Therefore, based on the serological results, the most reasonable season for the appearance has been set as season 2014/2015.

HI and SRH results for A/Brisbane and A/California strains, showed a clear distinction between the number of subjects with positive titers between pre- and post-outbreak seasons ($p<0.0001$ for both assays). For A/Michigan strain, differences between pre- and post-outbreak seasons were more evident for SRH results ($p<0.0001$) than HI ($p=0.01$) (Figure 4).

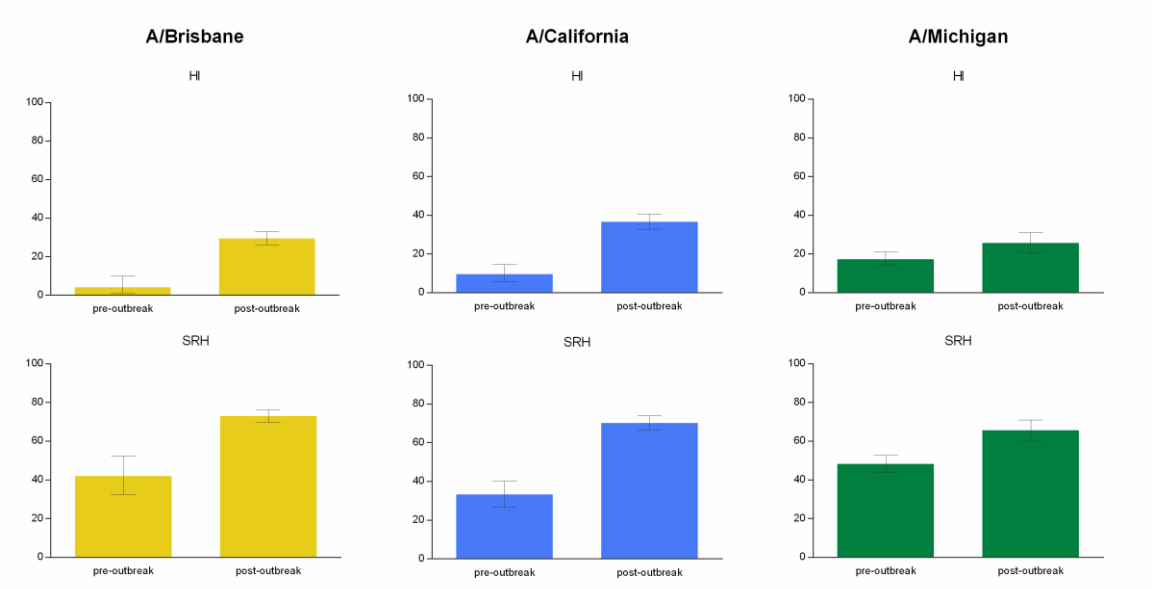

Figure 4. Pre- and post-outbreak seasons by strain, HI results (above) and SRH results (below).

4. Discussion

This study provides a serologically based evaluation and interpretation of the levels of cross-reactive (and cross-protective) antibodies against H1N1 viruses circulating in the human population since 2008.

In the 2005/2006 season, low HI proportion of subjects with protective titers were observed in both age groups, as none of the H1N1 strains included in this study was circulating. SRH results showed some protection, most probable correlating with pre-existing immunity, which was higher for A/Brisbane (42.0%) and lower for A/California and A/Michigan (27.0% and 19.0%, respectively). However, elderly adults showed higher cross-protective titers against all the three strains (12.0%)

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than younger adults. In season 2008/2009 (considered as pre-pandemic) HI results clearly show A/Brisbane as the predominant strain, especially in the elderly adults (34.0%). With respect to SRH, 34.0% of the total population had protective titers against A/Brisbane, however, 40.0% of elderly adults showed cross-protective titers against all three strains, probably because of the presence of pre-existing cross-reactive antibodies against former H1N1 strains that may have contributed to protection in this age group [3]. In 2009/2010, the higher proportion of protective antibodies is still A/Brisbane specific, however cross-protective antibodies between A/California and A/Michigan are already detectable, most likely induced by the spread of the 2009 pandemic strain. Starting from 2009/2010 season onwards, the higher proportion of SRH positive subjects show cross-protection for all the three strains. In 2010/2011 (pandemic/post-pandemic) season and onwards, the higher proportions of protective antibodies were against A/California and A/Michigan together, or A/California only, also in seasons when possibly A/Michigan was circulating.

To determine an explanation for the observed cross-protective antibody responses, the amino acid sequences of HA have been compared between the strains. The HA amino acid sequence of A/California was aligned with the HA amino acid sequences from A/Brisbane and A/Michigan. The HA molecule comprises a membrane-distal HA1 and a membrane-proximal HA2. A/California exhibits the highest sequence identity to A/Michigan (97.1% of homology). In particular, the HA1 chain of A/California share 96.0% of homology with A/Michigan, 98.6% for the HA2 chain. In contrast, HA sequence identity to A/Brisbane was lower (79.8%), with 71.6% of homology for HA1 and 91.8% for HA2. Despite some antigenic differences, A/California and A/Michigan strains share common epitopes. Although WHO recommended A/Michigan as H1N1 vaccine component in season 2017/2018, replacing A/California, the population show protection against A/Michigan strain already in the first seasons of A/California circulation. It could be concluded that there has been a co-evolution of both strains over the seasons, associated with a cross-reactivity between A/California and A/Michigan that does not allow a discrimination of the exact season when A/Michigan strain has become predominant. The data described here indicate that antibodies raised against A/California are cross-protective against A/Michigan. As previously observed [24, 25], the antigenic drift of HA of 2009 pandemic H1N1 strains was not observed with ferret antisera, although more recent 2009 pandemic H1N1-like strains, such as A/Michigan, are antigenically different to the vaccine strain (A/California).

In the elderly adults, protective antibody titers against A/Brisbane strain are associated with cross-protective antibodies against A/Michigan and A/California strains as well. One possible explanation is that, despite the HA from the pandemic H1N1 virus is not antigenically similar to any previous human seasonal influenza virus [26], the adaptation to human hosts leads to common conserved epitopes, which can be recognized by the broader antibody repertoire of elderly adults and as such results in higher titers in this age group. This is supported also by the observation that elderly may show lower titers than younger adults against homologous strains, but they showed higher cross-reactions (and cross-protective titers) against heterologous strains (drifted strains). These findings were supported by other studies [9, 11] which have shown that immunological priming by previous influenza strains exposure participates in the immune response to an antigenically related strain and increases with age.

The analysis performed in this study supports the use of the two different assays, HI and SRH, in parallel as it allows investigations on the antigenic nature of three different virus types in two distinct populations, the younger and the elderly adults in an unusual scenario; (i) a seasonal influenza H1N1 strain of human origin as a representative of classical drifted A/H1N1 strains, A/Brisbane; (ii) an influenza H1N1 virus of animal origin (swine) which has caused a pandemic in humans in 2009/2010 and which then has become a classical seasonal human influenza virus replacing the former seasonal human H1N1 strain, A/California; and (iii) a drift variant of the original pandemic strain due to antigenic changes because of frequent circulation in the human population over several seasons, A/Michigan. This study has shown that the HI assay is an optimal assay for determination and detection of new seasonal drift strains as shown in the pre/post-outbreak A/Brisbane and pre/post-outbreak A/California seasons. Based on its specificity, HI assay is able to discriminate which new strain is circulating following vaccine recommendation. In accordance with

HI result of this study, it is possible to recognize peaks of prevalence through the seasons, that in the majority of cases are in accordance with virological surveillance data. On the other hand, SRH immunity seems to accumulate over the seasons, with minor variations in the proportion of positive subjects. Antibodies against viral epitopes, recognized by SRH, are more stable between seasons and strains, as SRH assay is able to detect antibodies directed against potentially more conserved epitopes between different strains, such as antibodies against HA2 [15], allowing the detection of a broader range of functional antibodies that contribute to previous immunity. The SRH assay, in addition, is an excellent tool to determine the presence of pre-existing immunity against drifted and actual circulating seasonal strains which allows a potential prediction on booster ability against newly emerging drifted strains.

Author Contributions: Conceptualization, C.M.T. and O.K.; formal analysis, S.M., P.P., and E.J.R.; investigation, C.M.T., I.M., S.M., A.M., F.B. and T.C.; resources, E.M.; writing—original draft preparation, S.M. and C.M.T.; writing—review and editing, S.M., C.M.T., O.K., A.M., F.B., I.M., G.L., and E.M.; visualization, S.M.; project administration, C.M.T.. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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