Lower seroreactivity to European than to North American H3N2 swine influenza viruses in humans, Luxembourg, 2010

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Seroreactivity to H3N2 swine influenza viruses (SIVs) was evaluated in serum samples collected from 843 people aged 0 to 100 years in 2010 in Luxembourg. Sera were analysed by haemagglutination inhibition (HI) and virus neutralisation (VN) assays targeting a European H3N2 SIV, a North American H3N2 variant of swine origin (H3N2v) and human seasonal H3N2 viruses isolated in 1975, 1995 and 2005. HI antibodies (titre ≥ 10) against European H3N2 SIV were almost exclusively detected in those born before 1990, of whom 70% were seropositive. HI antibodies against H3N2v were predominantly found in those born before 2000, with 86% seropositive. Titres against the North American H3N2v were higher than against the European H3N2 SIV. VN patterns were similar, but with higher rates and titres. We also demonstrated lower seroreactivity to European H3N2 SIV than to North American H3N2v virus. Finally, we found a strong correlation between HI titres against the European H3N2 SIV and H3N2v and their respective human ancestors, A/Victoria/3/75 and A/Nanchang/933/95. This finding and the minimal contacts between humans and pigs in Luxembourg suggest that anti-SIV antibodies in human serum samples reflect serological cross-reactivity with historical human H3N2 viruses. Our findings help assess the pandemic risk of H3N2 SIV.

Introduction
Three swine influenza virus (SIV) subtypes, H1N1, H2N2 and H3N2, are enzootic throughout the world in regions with a high density of pigs. The haemagglutinin (HA) and neuraminidase (NA) genes of most, if not all, H3N2 SIVs have been derived from human seasonal influenza A(H3N2) viruses. In Europe, H3N2 SIVs are derived from descendants of the A/Hong Kong/1/68 pandemic influenza A(H3N2) virus, but they have evolved further through genetic reassortment with the endemic avian-like H3N2 SIVs present in western Europe since the late 1970s. This has resulted in H3N2 SIVs with human-like HA and NA genes and avian-like internal genes [1,2]. In North America, H3N2 viruses have become established in swine since 1998. They are known as ‘triple-reassortant’ viruses because their HA, NA and polymerase B1 genes stem from human seasonal H3N2 viruses and the remaining internal genes from avian influenza virus and classical H3N1 SIV [3]. Since 2009, novel reassortant H3N2 viruses with variable numbers of internal genes derived from the 2009 pandemic influenza A(H1N1)pdm09 virus have been reported frequently and this has further complicated the epidemiology of swine influenza in the United States (US) [4]. From 2009 to 2012, these novel influenza A(H1N1)pdm09 reassortants accounted for 54% of H3N2 SIVs isolated [4]. Reassortant viruses with seven genes from the triple-reassortant H3N2 SIVs and only the matrix (M) gene from the A(H1N1)pdm09 virus have become the dominant genotype. These viruses, called H3N2 variant or H3N2v when isolated from humans, have caused many zoonotic infections since 2011 [5].

Antigenic drift in the HA is generally slower in SIVs than in human influenza viruses, and pigs can therefore serve as reservoirs of older human HAs [6-8]. Swine-adapted viruses with an HA of human origin could initiate a pandemic once immunity within the human population has waned sufficiently to allow widespread infection, provided that the viruses also have the ability to spread efficiently from person to person. This was observed for the A(H1N1)pdm09 virus that contains the classical swine H1. Evolutionarily, the 1918 H1N1 pandemic influenza virus was the common ancestor of human seasonal and classical swine H1N1 influenza viruses; it has undergone significant antigenic drift in humans but remained largely in antigenic stasis in swine [9,10]. Consequently, only people born before the 1940s had been previously exposed to human seasonal H1N1 viruses with an H1 related to that of A(H1N1) pdm09, and a pandemic was possible because younger people lacked cross-reactive anti-H1 antibodies [11-14]. A similar situation could occur with human-adapted H3N2 SIVs in the future if they carry the HA of seasonal H3N2 viruses that have not circulated in decades.
Before 2011, only sporadic dead-end zoonotic infections with H3N2 SIVs had been reported, in humans in close contact with pigs. Recently, the H3N2v virus has caused 343 human infections in the US from August 2011 through October 2014 [15]. These infections occurred primarily in young children visiting agricultural fairs, and the H3N2v virus did not spread widely through the human population [16]. These zoonotic infections prompted serological investigations for cross-reactive antibodies against H3N2v in people of various ages in the US, Canada, Norway and England. These studies found that more than half of the adolescents and young adults tested had haemagglutination inhibition (HI) antibody titres ≥ 40, which is considered as seroprotective [17]. In contrast, younger children and older adults typically exhibited lower or negative antibody titres [18-22].

Antibodies against the antigenically distinct European H3N2 SIV have been reported in ca 50% of humans in studies in Italy and Germany between 2008 and 2010 [23,24]. However, these studies sought to compare antibody prevalences in swine workers and non-swine workers with a mean age of 45 years, rather than using an age-stratified design to assess seropositivity in the general population. In this study, we primarily sought to compare the seroreactivity to a European H3N2 SIV with that to a North American H3N2v virus in people in various age groups in Luxembourg who were very unlikely to have been exposed to pigs. Importantly, we also examined the association between antibody titres against swine-origin and those against human seasonal H3N2 influenza viruses.

Methods

Serum samples

A total of 843 anonymised human serum samples were randomly selected from the Serum Bank of the Laboratoire National de Santé, Luxembourg. The sera were collected from patients admitted to hospital for various reasons in April or May 2010. The sera were from people born between 1910 and 2010 and were divided into 10 groups by birth decade. As an example, 1910s refers to people born between 1910 and 1919. Ca 10 sera per year of birth and 100 sera per birth decade were tested. Only the youngest (n = 40 samples) and oldest age group (n = 9 samples) had fewer samples. The sex ratio in each age group was ca 50:50, except for the participants born in the 1910s (two male vs seven females). No further personal data were collected due to ethical constraints.

H3N2 influenza viruses

We measured serum antibody titres against human seasonal H3N2 influenza viruses A/Victoria/3/75, A/Nanchang/933/95 and A/Wisconsin/67/05. These viruses were circulating worldwide during 1976–78, 1996–98 and 2006–08, respectively, and were recommended as the influenza vaccine strains by the World Health Organization during their time of circulation. We also measured antibody titres against sw/Gent/172/08, a virus representative of H3N2 SIVs that are currently circulating in western Europe, and against A/Indiana/08/11, which represents swine-origin H3N2v viruses isolated from humans in the US since 2011.

The HA1 amino acid sequences of these five viruses, a selection of human seasonal influenza A(H3N2) viruses (1968–2012), and European and North American H3N2 SIVs (1984–2012) were downloaded from GenBank. The sequences were compared using the MEGA programme with DNASTAR 5.01 software (DNASTAR, Inc., Madison, WI, US). A neighbour-joining phylogenetic tree was constructed to compare amino acid sequences with MEGA 5.05 software (http://www.megasoftware.net/). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are expressed as the number of amino acid substitutions per site.

Antigenic characterisation of the five viruses used for serology was performed by HI and virus neutralisation (VN) assays with hyperimmune swine serum (for sw/Gent/172/08) or post-infection ferret sera (for all other viruses). We used ferret serum against A/Wuhan/359/95 instead of A/Nanchang/933/95 because a ferret serum against the latter virus was not available. The two viruses have only two amino acid differences in the HA1, outside the antigenic region. Viruses used in HI assays were propagated in 10 day-old embryonated chicken eggs (≤ 4 passages); viruses used in VN assays underwent an additional passage in Madin-Darby canine kidney (MDCK) cells.

Serological assays

All sera were examined in HI assays against the three human seasonal H3N2 viruses and the two swine-origin H3N2 viruses. Since the VN assay is more sensitive than the HI assay and highly relevant for protection, sera from people born after 1940 were also tested in VN assays against the swine-origin viruses. HI and VN assays were performed following standard procedures [25,26]. Antibody titres were expressed as the reciprocal of the highest serum dilution that showed complete inhibition of HA of 4 hemagglutinating units of virus (HI assay), or 50% neutralisation of 10^2 50% tissue culture infectious doses (TCID_{50}) of virus in MDCK cells (VN assay). The starting serum dilution was 1:10 for both assays. Sera with titres ≥ 10 were considered as seropositive. HI titres ≥ 40 were considered as seroprotective.

Statistical analysis

Geometric mean titres (GMTs) of antibody with 95% confidence intervals (CI) were calculated for each age group against each of the five influenza viruses. A numeric value of 5 was assigned to samples with antibody titres < 10. Antibody titres between age groups
Figure 1
Neighbour-joining phylogenetic tree of HA1 amino acid sequences from human seasonal influenza A(H3N2) viruses and European and North American swine influenza A(H3N2) viruses

The viruses used for the present serological studies are indicated in bold.
were compared using the nonparametric Wilcoxon signed-rank test, and differences in the proportion of sera with HI titres ≥ 40 were analysed using Fisher’s exact test. A p value < 0.05 was considered statistically significant. Pearson correlation tests were used to compare HI antibody titres against human and swine-origin viruses. All analyses were performed with Graphpad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, US).

Results

Relationships between human seasonal and swine-origin influenza A(H3N2) viruses

Phylogenetic relationships between the HA1 amino acid sequences of human seasonal H3N2 viruses and H3N2 SIVs from Europe and North America are shown in Figure 1. European and North American H3N2 SIVs formed separate clusters, and branched off from human viruses at different apparent time points. The HA1 of the human A/Victoria/3/75 virus was most closely related to the European H3N2 SIVs (87.0% identity to sw/Gent/172/08). The HA1 of the human A/Nanchang/933/95 virus was most closely related to North American H3N2 SIVs (89.7% identity to A/Indiana/08/11).

Antigenic relationships between human seasonal and swine-origin H3N2 viruses are shown in Table 1. All viruses reacted with their homologous antisera at HI and VN titres ≥ 160. There was minimal cross-reactivity between the H3N2v virus A/Indiana/08/11 and the European H3N2 SIV sw/Gent/172/08 using hyperimmune swine serum against sw/Gent/172/08, and ferret serum against A/Indiana/08/11 failed to cross-react with sw/Gent/172/08. Sw/Gent/172/08 showed cross-reaction with antiserum against the human A/Victoria/3/75 virus in both HI and VN assays (titre = 80). A/Indiana/08/11, on the other hand, reacted with antiserum against the human A/Wuhan/359/95 virus, which is similar to A/Nanchang/933/95, in the VN assay (titre = 240), but not in the HI assay. Both swine-origin viruses had negligible cross-reactivity with A/Wisconsin/67/05.

Serological status to human seasonal influenza A(H3N2) viruses

HI antibody titres ≥ 10 against the A/Victoria/3/75 virus were detected in 78% of individuals born before 1990 (Figure 2). In contrast, only 10% of those born in the 1990s and none of the children born after 2000 had detectable antibodies. HI titres ≥ 40 were most common in persons born in the 1950s, 1960s and 1970s. A larger proportion of people had detectable HI antibodies and titres ≥ 40 against the A/Nanchang/933/95 virus. Detectable HI antibodies against the latter virus were observed in 85% of individuals born before 2000, but only in 20% of the children born after 2000. More than half of those born in the 1970s, 1980s and 1990s had HI titres ≥ 40. Seroprevalence rates against the A/Wisconsin/67/05 strain had the least variation across different age groups. More than half of the people in each age category had detectable antibodies, except for those born in the 1960s (42%). HI titres ≥ 40 were most common in those born after 1990.

GMTs of HI antibodies against the three human seasonal H3N2 viruses showed similar age-dependent trends as the seroprevalence rates (Table 2). Antibody titres against A/Victoria/3/75 were highest in people born in the 1950s, 1960s and 1970s. Antibody titres against A/Nanchang/933/95 and A/Wisconsin/67/05, on the other hand, were highest in people born in the 1980s and 1990s and those born in the 1990s, respectively. Peak antibody titres against A/Nanchang/933/95 were higher than those against either of the other viruses.

Table 1

Cross-reactivity between human seasonal A(H3N2) viruses (A/Victoria/3/75, A/Nanchang/933/95 and A/Wisconsin/67/05) and swine-origin A(H3N2) viruses (sw/Gent/172/08 and A/Indiana/08/11) in haemagglutination inhibition and virus neutralisation assays, Luxembourg, 2010 (n = 843)

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Antibody titres with serum against</th>
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<tbody>
<tr>
<td></td>
<td>A/Victoria/3/75</td>
</tr>
<tr>
<td></td>
<td>HI</td>
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<tr>
<td>A/Victoria/3/75</td>
<td>160</td>
</tr>
<tr>
<td>A/Nanchang/933/95</td>
<td>&lt;10</td>
</tr>
<tr>
<td>A/Wisconsin/67/05</td>
<td>&lt;10</td>
</tr>
<tr>
<td>sw/Gent/172/08</td>
<td>80</td>
</tr>
<tr>
<td>A/Indiana/08/11</td>
<td>20</td>
</tr>
</tbody>
</table>

HI: haemagglutination inhibition; VN: virus neutralisation.

* Ferret serum against A/Wuhan/359/95 was used because ferret serum against A/Nanchang/933/95 was not available. The two viruses are antigenically identical.

* Serum against sw/Gent/172/08 was obtained by hyper-immunisation of swine; the other sera were post-infection ferret sera.
Prevalence of haemagglutination inhibition antibodies against three human seasonal influenza A(H3N2) viruses, an endemic European H3N2 swine influenza virus and a North American H3N2v virus, by decade of birth, Luxembourg, 2010 (n = 843)

Serological status to European and North American swine-origin influenza A(H3N2) viruses
Rates of seroprevalence against the European H3N2 SIV sw/Gent/172/08 and North American H3N2v virus A/Indiana/08/11 were similar to those against the human seasonal viruses A/Victoria/3/75 and A/Nanchang/933/95, respectively (Figure 2). HI antibodies (titres ≥ 10) against sw/Gent/172/08 were almost exclusively found in those born before 1990, of whom 70% were seropositive. HI antibodies against A/Indiana/08/11 were mainly detected in people born before 2000, of whom 86% were seropositive. HI antibodies against A/Indiana/08/11 were mainly detected in people born after 2000 (p > 0.05). Higher VN antibody titres were detected against A/Indiana/08/11 than against sw/Gent/172/08 in children born after 2000 (p > 0.05). Higher VN antibody titres were detected against A/Indiana/08/11 than against sw/Gent/172/08 (p < 0.05).

Correlations between antibody titres against human seasonal and swine-origin influenza A(H3N2) viruses
There was a strong correlation between HI antibody titres against sw/Gent/172/08 and A/Victoria/3/75 viruses (r = 0.71), and between those against A/Indiana/08/11 and A/Nanchang/933/95 viruses (r = 0.69) (Table 4). Correlations were low between HI
titres against A/Wisconsin/67/05 and sw/Gent/172/08 (r = 0.25), as well as between those against A/Wisconsin/67/05 and A/Indiana/08/11 (r = 0.42) (all p < 0.01).

Discussion

The present study was designed to investigate the extent to which prior exposure, through infection or vaccination, to earlier antigenic variants of seasonal influenza A(H3N2) viruses was associated with the presence of antibodies against swine-origin H3N2 viruses from Europe and North America in people in Luxembourg born between 1910 and 2010. Our results demonstrate that as many as 70% of people in the study born before 1990 had detectable HI antibodies against the European H3N2 SIV, whereas such antibodies were generally lacking in those born after 1990. The prevalence of antibodies against the antigenically distinct H3N2v swine-origin virus was also age-dependent: antibodies were predominantly found in people born before 2000, of whom 86% were seropositive. Our data are consistent with previous studies [18-23,27], but we have for the first time demonstrated a lower level of seroreactivity to the European H3N2 SIV than to the H3N2v virus. Although HI titres of ≥ 40 are generally considered as seroprotective, people with lower antibody titres may also have some protection against H3N2 viruses from swine. Indeed, HI assays do not measure mucosal antibodies, antibodies against NA and cell-mediated immunity, which will also contribute to protection [28]. The VN assay yielded higher seroprevalence rates and antibody titres against

Table 3

| Virus-neutralising antibody titres against swine-origin influenza A(H3N2) viruses sw/Gent/172/08 and A/Indiana/08/11 in people born after 1940, Luxembourg, 2010 (n = 643) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Birth decade    | n               | sw/Gent/172/08  | A/Indiana/08/11 |
|                 | % sera with titre | GMT (95% CI) | % sera with titre | GMT (95% CI) |
|                 | ≥ 10            | ≥ 40           | ≥ 10            | ≥ 40           |
| 1940s           | 100             | 77             | 36              | 24.0 (18.6–31.0) * | 98             | 71              | 80.6 (62.6–103.6) * |
| 1950s           | 100             | 94             | 68              | 76.4 (56.8–102.7) * | 96             | 69              | 90.1 (67.3–120.6) * |
| 1960s           | 100             | 93             | 62              | 62.6 (46.6–84.0) * | 100             | 82              | 112.4 (87.7–144.2) * |
| 1970s           | 100             | 99             | 79              | 90.4 (70.9–115.1) * | 100             | 94              | 247.2 (199.7–306.0) * |
| 1980s           | 104             | 79             | 30              | 22.7 (18.4–28.1) * | 100             | 99              | 488.1 (410.4–580.5) * |
| 1990s           | 99              | 30             | 9               | 8.0 (6.7–9.7) * | 97             | 89              | 235.4 (172.3–321.7) * |
| 2000s           | 40              | 10             | 0               | 5.4 (5.0–5.7) | 58             | 15              | 13.3 (9.2–19.2) * |

CI: confidence interval; GMT: geometric mean titre.

* GMTs significantly higher (p<0.05, by Wilcoxon signed-rank test) in the virus neutralisation than in the haemagglutination inhibition assay.
both swine-origin influenza viruses than the HI assay. This is not surprising, because the VN assay detects a broader range of antibodies than the HI assay [29].

We have several reasons to believe that antibodies against current swine-origin H3N2 viruses result from exposure to historical human H3N2 strains rather than from infection with SIVs. Firstly, Luxembourg has a low pig density compared with other European regions and only 0.07% of the population are employed in the swine industry. Furthermore, only 0.99% of the Luxembourg swine population tested seropositive against H3N2 SIV in 2013 [30]. Secondly, H3N2 SIVs from North America have never been detected in swine in Europe. Finally, we found a strong correlation between antibody titres against swine-origin viruses sw/Gent/172/08 and A/Indiana/08/11 and their respective human ancestor A/Victoria/3/75 and A/Nanchang/933/95. Similar correlations have previously been reported between antibody titres against the European H3N2 SIV and A/Port Chalmers/1/73 [27], and between the H3N2v virus and A/Wisconsin/67/05 and A/Sydney/5/97 viruses [18,20]. In contrast, younger people who had not been exposed to these human H3N2 strains in past decades, were generally seronegative against the swine-origin viruses. In addition, our phylogenetic and antigenic analyses, in agreement with previous studies [31,32], confirm the close relationship between these swine-origin viruses and their human H3N2 ancestors.

Despite lower seroreactivity to the European H3N2 SIV than to the North American H3N2v virus, only three human infections with the European H3N2 SIV have been reported between 1993 and 2014 [33,34]. The H3N2v viruses, in contrast, have caused 343 human infections in the US between 2011 and 2014. It is possible that the H3N2v virus is more infectious for humans than other H3N2 lineages from swine or that more people in the US may have opportunities for exposure to pigs. The H3N2v is the only North American H3N2 SIV genotype that has caused widespread infections in humans, and although some believe this is due to the presence of the pandemic M gene segment, this has not been firmly proven [35]. Most H3N2v cases reported exposure to pigs at agricultural fairs [16,36]. Thousands of fairs are held in summer and autumn in North America, and these fairs provide unique settings where pigs from numerous sources can come into contact with millions of persons, which may facilitate interspecies transmission of influenza viruses. Such large-scale pig shows are rare in Europe. Furthermore, human cases of animal influenza have been notifiable in the US since 2007, which is not the case in Europe, and there is much more extensive surveillance for influenza in humans and swine in the US than in Europe.

Our data further support the notion that pigs serve as reservoirs for older human H3 HAs against which immunity in the human population will gradually decrease over time. Experimental infection studies in pigs and in ferrets have shown that prior infection with recent seasonal H3N2 viruses offers limited or no protection against challenge with the European H3N2 SIV or H3N2v [31,37]. As such, H3N2 SIVs, and the European strains in particular, could potentially contribute to pandemic viruses in the future as seroreactivity to the respective HAs wanes over time in the human population. Yet, it is highly likely that the current substantial immunity in people born before 1990 would prevent extensive spread of H3N2 SIVs. As for the swine-origin A(H1N1)pdm09 virus, pre-pandemic antibodies against A(H1N1)pdm09 were present in most individuals born before 1944, but were low or absent in younger people [11-14]. It may take nearly 50 years before a substantial proportion of the human population would be fully susceptible to swine-origin H3N2 viruses. To assure the best preparation for swine-origin influenza virus pandemics, surveillance of influenza in pigs should be expanded and integrated with human public health surveillance efforts, and additional studies on the cross-reactivity between human and swine influenza viruses are warranted.

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Conflict of interest

None declared.

Authors' contributions

Yu Qiu and Kristien Van Reeth conceived and designed the experiments. Yu Qiu performed the experiments and analysed the data. Yu Qiu and Kristien Van Reeth wrote the paper. Claude P Muller provided sera, read and revised manuscript.

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