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Listeriosis outbreak caused by acid curd cheese 'Quargel', Austria and Germany 2009

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We report an outbreak of listeriosis in Austria and Germany due to the consumption of 'Quargel' cheese produced by an Austrian manufacturer. At the time of writing this report, the outbreak was known to account for 14 outbreak cases in 2009, including four cases with lethal outcome. On 23 January 2010, the cheese product was voluntarily withdrawn from the market.

On 14 August 2009, the binational Austrian-German Consiliar Laboratory for Listeria in Vienna noticed the occurrence of a new pulsed-field gel electrophoresis (PFGE) pattern in human isolates of *Listeria monocytogenes* serotype 1/2a. This consiliar laboratory receives all human isolates from Austria as required by law. In Germany, submission of isolates is voluntary. According to the available information at the time of writing this report, the outbreak clone accounted for 12 of the 46 Austrian cases in 2009 (serotype 1/2a (n=29), 4b (n=9), 1/2b (n=8)). Onset of illness is shown in the Figure. The 12 Austrian outbreak cases (two of them fatal) affected six of nine Austrian provinces. The mean age was 74.5 years (range: 58-88 years), eleven patients were male. In addition, two of 92 available human isolates from Germany in 2009 (total number of cases 389) showed this new PFGE-pattern. The German outbreak cases were two women in their 70s who died in November and December 2009 respectively. They had not visited Austria during the likely period of incubation (up to 70 days).

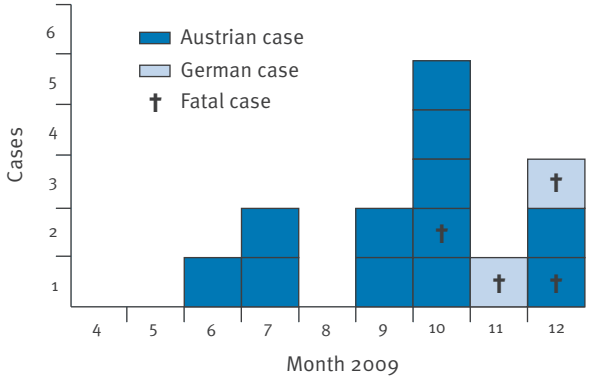
Since no reliable information was available on food consumed during the incubation period, all surviving Austrian outbreak cases were asked to collect grocery receipts for the three weeks after 3 December, i.e. after they were discharged from hospital, in order to collect information on routine food consumption behaviour. This epidemiological investigation revealed consumption of 'Quargel', a type of acid curd cheese available in different flavours, as a highly likely source of this outbreak. Three of seven outbreak cases providing receipts had bought product X produced by the Austrian manufacturer. Regular consumption of Quargel product X was confirmed by eight of nine participating outbreak cases, and consumption of Quargel cheese products was reported by heteroanamnesis for one German outbreak case (data on the second case remain unavailable).

Approximately 16 tons of Quargel per week are produced by the Austrian manufacturer. Fifty-three per cent of the product is exported to the German market and small amounts to the Czech Republic, Poland and Slovakia. This cheese is made of curdled milk, which ripens after addition of starter cultures for one day at 28°C, and after being sprayed with *Brevibacterium linens* for another two days at 14°C. The shelf life after packing and marketing is two months.

An environmental *L. monocytogenes* 1/2a isolate from the production plant, collected in December 2009, became available in January 2010 and proved indistinguishable from the outbreak strain by genotyping. Quargel cheese products sampled at the plant on January 13 yielded three different strains of

Figure

Outbreak cases of listeriosis by onset of illness, Austria and Germany, 2009 (n=14)



L. monocytogenes 1/2a, including the outbreak clone, in numbers of less than 100 colony-forming units (cfu) per gram. Food products collected on 18 January 2010 yielded greater than 100 cfu/g *L. monocytogenes*. The product was voluntarily withdrawn from the market on 23 January. On the same day, the public was informed about the incident and warned about cheese already bought. The plant stopped production. Investigation of the source of contamination is ongoing.

Conclusion

Industrial food production combined with international marketing of food and the low attack rate of *L. monocytogenes* hinder epidemiological outbreak investigation with traditional concepts [1]. Genotyping of *L. monocytogenes* isolates from clinical specimens can discriminate single-source clusters of food-borne infection and contribute to the identification and investigation of outbreaks. The outbreak described in this report probably would not have been identified without molecular typing [2]. The effectiveness of microbiological surveillance is entirely dependent upon the consistent and timely submission of all Listeria isolates from clinical laboratories to public health laboratories. In Austria, clinical laboratories are required by law to submit all clinical isolates of *L. monocytogenes* to AGES for PFGE analysis. In Germany, submission of *L. monocytogenes* isolates from clinical specimens by clinical laboratories is not required. The high case fatality ratio of listeriosis makes a strong case for the importance and priority of improved surveillance in Europe [3]. Our outbreak report underlines the value of routine molecular typing of Listeria isolates and also points out the considerable potential of cross-border cooperation for elucidating chains of infections.

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Higher all-cause mortality in children during autumn 2009 compared with the three previous years: pooled results from eight European countries

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The paper describes weekly fluctuations of all-cause mortality observed in eight European countries during the period between week 27 and 51, 2009, in comparison with three previous years. Our preliminary data show that the mortality reported during the 2009 influenza pandemic did not reach levels normally seen during seasonal influenza epidemics. However, there was a cumulative excess mortality of 77 cases (1 per 100,000 population) in 5-14-year-olds, and possibly also among 0-4-year-olds.

Introduction

Since the autumn 2009, monitoring of weekly all-cause mortality has been piloted in countries across Europe, as part of the *European monitoring of excess mortality for public health action* (EuroMOMO), a project funded by the European Union Health Programme [1]. The general objective of EuroMOMO is to develop and operate a routine public health mortality monitoring system aimed at detecting and measuring, as timely as possible, excess numbers of deaths related to influenza and other possible public health threats across European countries. A major task of the project was the development and implementation of a common statistical algorithm to estimate excess mortality. At the present, this algorithm is applied in 13 participating countries to generate weekly indicators for age-specific excess mortality that are comparable across countries. Data outputs from individual partner countries are compiled by the project coordination team at Statens Serum Institut, Copenhagen.

The current data outputs are still preliminary because the validity of the model and the added benefits and relevance of pooling data are under evaluation. Therefore, the results have until now only been available to a restricted audience. Nonetheless, bearing these limitations in mind, data from the pilot project have been used as one of many indicators to monitor the severity of the 2009 influenza A(H1N1) pandemic in Europe in terms of total and age specific all-cause mortality.

The aim of the present paper is to describe the weekly fluctuations of all-cause mortality observed in eight European countries during the period from week 27 to week 51 in 2009, in comparison with the deaths observed during the same period in the three previous years. This provides an early estimate of the impact of the 2009 pandemic on different age groups in Europe.

Methods

Countries acquired mortality data from the national sources (e.g. national death registries) and analysed their own data by applying a common algorithm in order to model the expected weekly all-cause mortality taking into account trend and seasonality. The algorithm, termed A-MOMO, is a time series Poisson regression model with number of weekly deaths as dependent variable and the time series decomposed with a trend and seasonal component (details available from the authors). Outputs were examined for consistency and errors using standardised methods such as control charts provided by the algorithm. Subsequently these national data outputs were submitted to Statens Serum Institut in Copenhagen where further pooled analysis using the same algorithm as the countries was carried out for countries that provided age-specific data numbers of weekly deaths. Of the 13 pilot countries in EuroMOMO, eight provided such datasets in week 1 of 2010. These datasets were included in the present analysis and preliminary assessment.

Deaths in week 52 and 53, 2009, were removed from the analysis because the delays in death registrations affect the completeness of weekly numbers of deaths. The variables "number of weekly deaths" and "number of deaths above and below the modeled baseline of expected deaths" were derived from the model output of the pooled analysis both for total mortality and mortality by age groups (0-4 years, 5-14 years, 15-64 years and 65 years and above).

The variables were plotted as crude weekly numbers and as weekly cumulative sums over four season-years from week 27 to week 26 the following year, covering the 2006-7, 2007-8, 2008-9, 2009-10 influenza seasons and allowing uninterrupted visualisation of winter seasons.

Crude weekly numbers with baseline of expected deaths and cumulative seasonal sums of the residual deaths relative to the modeled baseline of expected deaths were additionally analysed for each of the eight countries individually.

Results

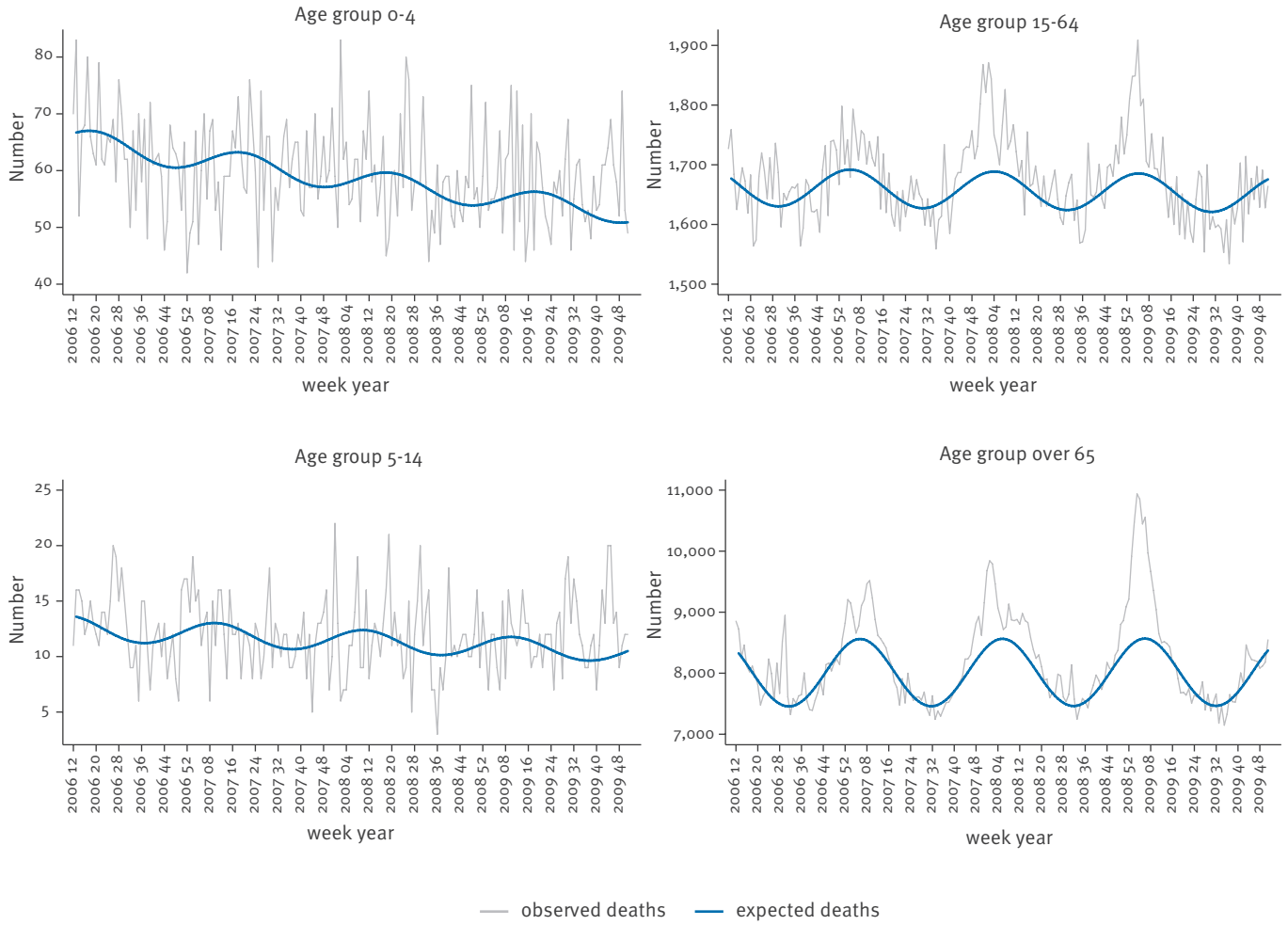
Eight countries (including one region) were included in the pooled analysis: Belgium, Denmark, Greece, Hesse (region of Germany), Malta, Netherlands, Sweden and Switzerland. The population size of the countries including the German region, ranges from 0.5 - 16.4 million, with a total of 66.8 million inhabitants [2].

Figure 1 shows the crude number of deaths by week and age group from week 12 in 2006 to week 51 in 2009 over a season-year covering the three influenza seasons 2006-7, 2007-8 and 2008-9. The time series show typical patterns with seasonal increases and excess mortality peaks during winter in the two older age groups; for the autumn of 2009, there is no apparent increase in mortality for the two older age groups. In the 0-4 and 5-14-year-olds seasonal patterns are not clearly evident. In 2009, there are spikes above the baseline for these age groups, but it is difficult to see any consistent patterns due to the small numbers and the large random variation around the baseline.

Figure 2 shows the cumulative residuals of weekly deaths, i.e. the sum of the positive and negative

Figure 1

Observed and expected number of deaths by week and age group, week 12 2006 - week 51 2009, pooled data from eight European countries*



* Belgium, Denmark, Greece, Hesse (region of Germany), Malta, the Netherlands, Sweden, Switzerland.

variations around the individually modeled baseline of expected deaths, for each season-year. The figure shows an excess of mortality in the 5-14-year-olds. For this age group, the model estimated a total excess of 77 deaths (corresponding to a cumulative excess mortality risk of 1 per 100.000 population) between week 27 and week 51 in 2009. The steep rise of deaths after week 41 coincided with widespread pandemic influenza activity in the participating countries. In the previous three years the number of excess deaths that had occurred by week 51 varied between nine and 16. Deaths in 5-14-year-old children are not common. On average, 275 (range 272 to 279) annual deaths have been observed in the period from week 27 to week 51 in the previous three years. In other words, an excess number of 77 deaths corresponds roughly to a 28% increase in mortality among children 5-14 years old coinciding with the pandemic.

There was a similar tendency of excess mortality in children less than 5 years old. In the age groups 15-64 and over 65, a more or less prominent lack of deaths can be observed, however this may be partly attributed to incomplete reporting of deaths.

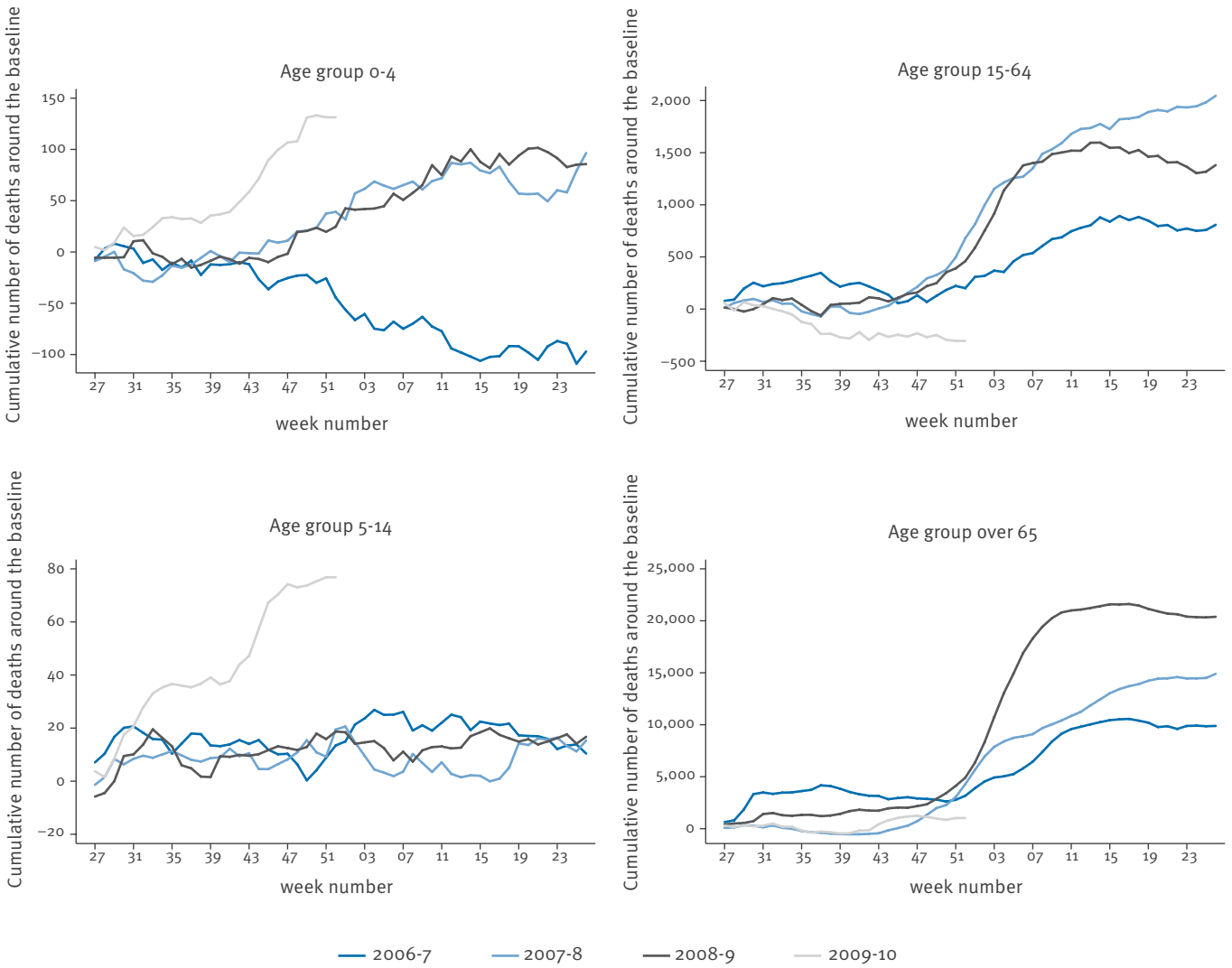
Discussion

The present analysis is subject to some limitations and results presented should thus be interpreted with caution.

A major limitation is that the expected numbers of deaths in the younger age groups are low overall, and therefore a few reported deaths, unrelated to the pandemic, in each of several countries may generate signals in the analysis. From a statistical point of view, the baseline for comparison is less precise when fitted on small numbers. By summing the residuals over time, artefacts may appear because the baseline due to random errors sits below or above the observed data. Another limitation relates to the fact that the influenza A(H1N1) pandemic started early in summer 2009 and peaked in most European countries around week 44

Figure 2

Cumulative numbers of deaths relative to the expected mortality by influenza season and age, influenza seasons 2006-7, 2007-8, 2008-9, 2009-10, pooled data from eight European countries*



* Belgium, Denmark, Greece, Hesse (region of Germany), Malta, the Netherlands, Sweden, Switzerland.

to 50, whereas seasonal influenza peaks usually occur after Christmas. This means that 2009 is not comparable with the previous years in a simple week-by-week analysis.

The aim of EuroMOMO is to provide near to real-time monitoring of excess mortality, however, delays in reporting are inevitable and vary between countries and possibly age groups. Although we removed the two most recent weeks (week 52 and 53, 2009) from our analysis, mortality during weeks 40 to 51 2009 has to be considered an underestimation of the true weekly mortality with increasing incompleteness over time. This also has to be taken into account in the interpretation of the results: delays in registration may mask excess deaths in recent weeks compared with the same weeks in previous years. Adjustment for known delay is an important target for further EuroMOMO research.

With these limitations in mind, our preliminary data indicate that there was no major excess of deaths during the 2009 influenza pandemic in the participating countries. Compared with excess mortality of the three previous years the mortality observed during the autumn wave of the 2009 pandemic did not reach levels normally seen during seasonal influenza epidemics when mainly senior citizens die. However, there was excess mortality in the 5-14-year-olds compared with excess levels of the previous three years. This estimate is probably conservative due to delay in reporting. An early estimate of a death toll of less than hundred deaths that may be attributable to the pandemic in a population of 7.4 million 5-14-year-olds [2], corroborates the notion that the overall burden on mortality remains low also among children [3,4]. It is not known how many of these children belonged to groups at risk for severe illness from influenza, but such a relatively limited number of deaths may be ascribed to deaths primarily in groups at risk of severe illness following influenza. We also found a similar pattern in children 0-4 years of age with an excess of almost the same magnitude as the older children. It is possible that this in part may be due to a declining baseline-trend among children 0-4 years (see Figure 1). Further research is necessary to disentangle the mortality pattern among children, in particular in light of the pandemic.

Modeling excess deaths during influenza epidemic periods based on historical baselines can capture excess mortality that otherwise would be missed [5]. However, the method does not permit to attribute any excess of death to influenza with certainty, and one has to be cautious in the interpretation of the present observations. For the period of interest, no competing risks are known to the authors that could explain the increase in child mortality during autumn 2009. We hope that our data can stimulate European countries to review causes of deaths particularly in children and to explain and clarify the present observation. In the United States, a substantially higher number of influenza deaths in children were reported during the present pandemic than in recent influenza seasons [4].

The results presented here demonstrate the potential usefulness of timely mortality monitoring to assess the severity of the pandemic and the impact on different age groups in Europe, and underscores the added value of pooling data to detect possible deviations from baseline that may have gone unnoticed in analyses in individual small countries.

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Estimating the impact of the 2009 influenza A(H1N1) pandemic on mortality in the elderly in Navarre, Spain

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We analysed mortality among people aged 65 years or older in Navarre, Spain in 2009 and compared it with the mean for the same period of time in the previous three years. In the pandemic weeks 24 to 52 2009 we observed 4.9% more deaths than expected (p=0.0268). Excess mortality occurred during the circulation of seasonal influenza (8.0%, p=0.0367) and the first wave of pandemic influenza (9.9%, p=0.0079). In the second wave of pandemic influenza there was a non-significant excess of deaths (5.2%, p=0.1166). Surveillance of laboratory-confirmed severe influenza cases detected only one death in this age group.

Introduction

Influenza has usually been associated with increased mortality in the elderly [1-4]. However, the large majority of influenza cases are not laboratory-confirmed, and only a small part of the excess deaths occurring in periods when influenza virus is circulating are registered as deaths caused by influenza [5].

The 2009 influenza A(H1N1) pandemic coincided with increased use of laboratory techniques to confirm influenza cases, although confirmation was limited to only a small proportion of all possible cases [6]. In Spain, as in other developed countries, influenza testing has been systematically incorporated for all patients hospitalised with acute respiratory disease [7]. From the standpoint of epidemiological surveillance, this was interpreted to mean that the large majority of severe cases and of deaths from the influenza virus can now be confirmed [8]. Based on this information, the 2009 pandemic influenza A(H1N1) virus has been related to a relatively low number of deaths in patients with laboratory-confirmed results, a small proportion of which occurred in elderly people [9-11].

The objective of this study was to evaluate the possible repercussions of the circulation of the 2009 pandemic influenza on mortality in adults aged 65 years and older.

Methods

The study was carried out in Navarre, Spain (a region of 620,000 inhabitants). Influenza surveillance in this region is based on automatic reporting of cases of influenza-like illness diagnosed in primary healthcare and hospitals. Virological surveillance includes weekly reports from all three laboratories that perform influenza tests to confirm the causative agent. Through a sentinel network composed of a representative sample of 76 general practitioners and primary healthcare paediatricians covering 18% of the population, nasopharyngeal swabs were taken from systematically selected patients with influenza-like illness, after receiving informed consent from the patients or their parents. Hospitalised patients with acute respiratory disease were swabbed for influenza virus testing in accordance with hospital protocols until week 20 of 2009. Swabbing was subsequently extended to all patients who were treated in emergency rooms or hospitalised with acute severe respiratory disease possibly caused by influenza. Swabs were processed in the laboratory by reverse transcription polymerase chain reaction (RT-PCR) and virus culture. Positive samples were characterised for influenza A (subtypes H1 and H3) and influenza B virus using immunofluorescence and RT-PCR. Starting in June, real-time RT-PCR for detection of the 2009 influenza H1N1 virus was performed on all swabs.

Based on the incidence of reported influenza-like illness and the type of influenza virus in circulation in the region, we distinguished six periods in 2009. Between weeks 1 and 8, a wave of seasonal influenza was identified, with 94% of the circulating strains corresponding to type A(H3N2). From week 9 to 15, there was only sporadic circulation of influenza B, with very low incidence. Between weeks 16 and 23, no influenza virus was detected (inter-seasonal period). From week 24 to 35, influenza activity re-emerged with all 98 positive swabs corresponding to the 2009 pandemic influenza A(H1N1) virus (first wave of pandemic influenza). From week 36 to 39, sporadic detection of this strain continued, with low incidence of influenza-like illness (pandemic remission period). From week 40 to 49 there was a large wave of influenza with high incidence and all circulating strains corresponding to the 2009 pandemic influenza virus (second wave of pandemic influenza). Finally, from week 50 to 52 there was a new remission

period with low incidence and only sporadic detection of 2009 pandemic influenza A(H1N1).

In the present study we used information from computerised mortality registers, which cover approximately 70% of the population (76,201 adults aged 65 years or older) and of the deaths in the region and provide information in real time. We analysed all deaths reported in adults aged 65 years or older in 2009 and compared them with the expected number of deaths, calculated as the average of deaths for the same periods of the three preceding years (2006, 2007 and 2008). For comparison we used the periods defined according to the incidence and the type of influenza virus in circulation in Navarre in 2009. Poisson distribution was used to compare observed and expected deaths.

Rates were calculated with the population covered by the computerised registers on 1 January of each year taken as the denominator. Standardised mortality ratios were obtained using the population on 1 January 2009 as the reference population. Available age groups were 65-69, 70-74, 75-79, 80-84 and ≥85 years.

Results

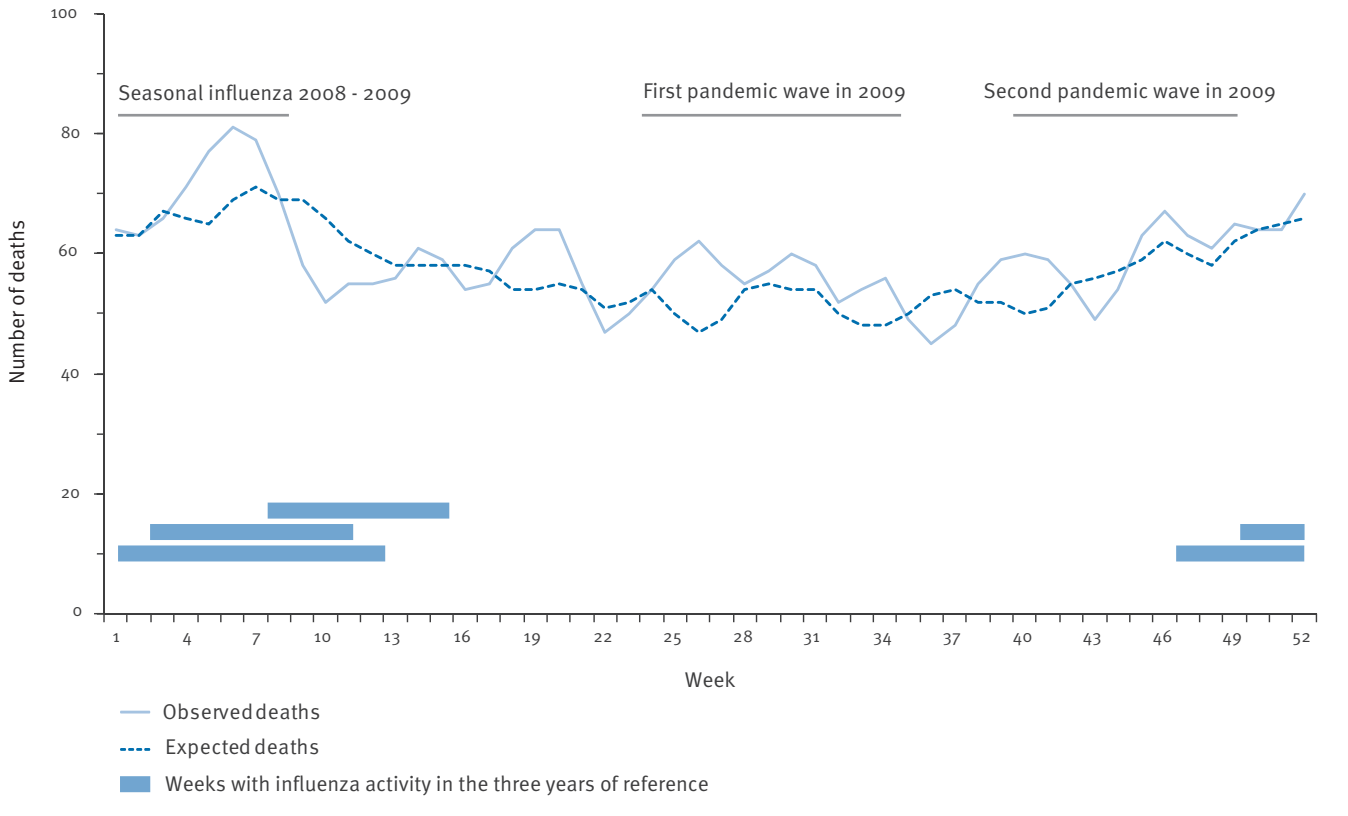
Active epidemiological and virological surveillance of influenza detected the first laboratory-confirmed case of 2009 pandemic influenza A(H1N1) in Navarre in week 24 of 2009. Between that date and the end of 2009, 3,190 swabs were tested for influenza, and 933 cases of laboratory-confirmed 2009 pandemic influenza A(H1N1) were detected. The number of cases of influenza-like illness that received medical attention reached 37 cases per 1,000 population (n=22,374). During 2009, 223 patients diagnosed with 2009 pandemic influenza in Navarre required hospitalisation, 17 of them required admission to intensive care units, and four died. In adults aged 65 years and older, 829 tests for influenza were performed, 28 patients with confirmed 2009 pandemic influenza required hospital admission, two in intensive care units, and one person died.

The Figure shows the number of deaths per week observed in persons aged 65 years or older compared with the number of expected deaths, and indicates the periods with influenza activity in 2009 and in the reference years. In the pandemic period (weeks 24 to 52) 1,671 deaths were registered in persons aged 65 years or older, 4.9% more than expected (p=0.0268). In contrast, in the weeks without circulation of pandemic virus (weeks 1 to 23), there was no significant difference between observed and expected deaths (Table 1).

Table 2 shows the mortality in short periods based on incidence of influenza-like illness and the type of virus circulating in the region. Statistically significant excess mortality among adults aged 65 years or older

Figure

Number of deaths per week registered and expected (mean of the three previous years) in the population aged 65 years or older covered by computerised death registers, Navarre, 2009



The data represented are moving averages calculated over three values with weights 0.25, 0.5, 0.25.

Table 1

Mortality among adults aged 65 years or older in the population covered by computerised death registers in Navarre, 2006-2009

	Non-pandemic period weeks 1 to 23	Pandemic period weeks 24 to 52
Expected deaths (annual average for 2006-2008)	1,390	1,593
Observed deaths in 2009	1,410	1,671
Percentage of change	+1.4%	+4.9%
P value	0.2993	0.0268
Annual average mortality rate for 2006-2008[a]	4,255	3,867
Mortality rate in 2009[a]	4,198	3,946
Percentage of change	-1.3%	+2.0%
P value	0.6785	0.4753
Standardised mortality ratio	0.96	1.00

[a] Rates per 100,000 person-years.

was observed in periods with circulation of seasonal or pandemic influenza virus, but not in periods when influenza activity was low or absent. There was an excess mortality of 8.0% (p=0.0367) in the seasonal influenza period compared with the expected number of deaths. There was also an excess mortality of 9.9% (p=0.0079) coinciding with the first wave of pandemic influenza, and again an excess mortality of 5.2% in the second wave, although the latter did not reach statistical significance (p=0.1166) (Table 2).

During the study there were no other known causes that could explain the excess mortality observed. In the summer of 2009, the heat alert threshold (minimum temperature of 21.5 ºC) was not exceeded at any time in Navarre, and the days with highest mortality did not coincide with the hottest days.

When we repeated the comparisons, taking as the reference the mean number of deaths in the same period in the previous five years, the main study findings were not affected.

Table 2

Epidemiological and virological surveillance of influenza in all ages, and mortality in the population aged 65 years or older, in six periods with circulation of different influenza viruses, Navarre, 2009

	Weeks 1 to 8	Weeks 9 to 15	Weeks 16 to 23	Weeks 24 to 35	Weeks 36 to 39	Weeks 40 to 49	Weeks 50 to 52
Medically-attended influenza-like illness							
Number of cases	6,683	680	182	2,495	1,385	17,578	1,175
Incidence rate per 1,000 inhabitants	10.9	1.1	0.3	4.1	2.3	28.2	1.9
Laboratory-confirmed influenza in sentinel network							
Swabs from sentinel network patients	107	19	27	245	64	419	62
Number of laboratory-confirmed cases (%)	57 (53%)	12 (63%)	0 (0%)	98 (39%)	2 (3%)	213 (51%)	14 (23%)
Circulating influenza strains	A(H3N2)[a]	B	None	2009 A(H1N1)	2009 A(H1N1)	2009 A(H1N1)	2009 A(H1N1)
Laboratory-confirmed influenza among hospitalised patients							
Hospitalised patients	12	3	0	52	14	143	14
Intensive care units	2	0	0	6	3	7	1
Deaths	0	0	0	0	0	4	0
Deaths in population aged 65 years or older							
Expected number (annual average for 2006-2008 period)	528	432	431	612	216	569	197
Observed number in 2009	570	395	445	673	207	598	193
Percentage of change	+8.0%	-8.6%	+3.3%	+9.9%	-4.0%	+5.2%	-1.9%
P value	0.0367	0.0346	0.2563	0.0079	0.2748	0.1166	0.6217
Mortality rates in population aged 65 or older							
Annual average mortality rate for 2006-2008 period**	5,305	4,344	3,789	3,591	3,795	4,002	4,614
Mortality rate in 2009[b]	5,576	3,864	3,809	3,840	3,544	4,095	4,405
Percentage of change	+5.1%	-11.0%	+0.5%	+6.9%	-6.6%	+2.3%	-4.5%
Standardised mortality ratio	1.03	0.87	0.98	1.05	0.91	1.00	0.93

[a] Influenza A(H3N2): 94%; seasonal influenza A(H1N1): 2%; influenza B: 4%.

[b] Rate per 100,000 person-years.

Discussion

Our results suggest excess mortality affecting older adults that coincided with the time when the 2009 pandemic influenza was in circulation. Similar excess mortality has also been observed to coincide with circulation of the seasonal influenza virus, but not in periods with little or no influenza activity. Most of the excess deaths in our study were not identified as influenza deaths, despite systematic testing during the pandemic period of cases with acute respiratory disease requiring hospital admission, as well as a substantial number of patients in primary and emergency care. By focusing concern on deaths occurring in persons with laboratory-confirmed 2009 pandemic influenza A(H1N1) [8-11] this additional mortality may not be detected. It is possible that some of these deaths occurred outside the hospital, or that the influenza was hidden by another underlying pathology. Previous studies have suggested that influenza can trigger or exacerbate non-infectious pathologies such as cardiovascular diseases [12].

Our study was ecological in design; therefore causes other than the influenza pandemic could explain the detected excess mortality. However, the fact that the excess mortality coincided with periods of viral circulation, whether seasonal or pandemic, lends some strength to this hypothesis. Moreover, we reviewed and ruled out other causes that are most frequently associated with excess mortality in the older population. Carrying out the study in a relatively small geographic area allowed us to discriminate between periods of clear influenza activity and those in which influenza was absent. This made it possible to establish more precisely the temporal coincidence between influenza circulation and excess deaths, an association that may be diluted when larger geographic areas are analysed.

Some possible biases have to be considered. The population aged 65 years or older increased by 3% from the period between 2006 and 2008 to 2009, and the population aged 85 years and over increased by 10%. This explains the differences between the results in absolute numbers and rates, with predominance of general upward trends in the absolute number of deaths and downward trends in crude and adjusted mortality rates. These changes in the population produce a greater number of deaths, regardless of other factors, but do not affect adjusted mortality rates. On the contrary, if the influenza has produced an increase in mortality, it may be partially hidden by the descending secular trend in the general mortality. Therefore, similar comparisons of observed versus expected mortality in periods with and without influenza activity allow us to estimate the impact of the influenza on mortality, avoiding the mentioned biases.

The source of mortality data represents a large and stable proportion of the population of Navarre. The registers include deaths occurring in the major municipalities, but do not include deaths in the Navarre population that have occurred outside these municipalities. This probably has little consequence when analysing the population aged 65 years or older, given their low geographic mobility. In any case, it seems appropriate to evaluate the association between influenza circulation (acute disease) and deaths occurring in the same place, regardless of whether or not it is the usual residence.

The method of analysis used probably does not allow us to show the impact of influenza on mortality in all its magnitude. Other factors affecting mortality could have been present in the reference periods of the previous three years, which would tend to attenuate the excess mortality shown in 2009. For example, waves of seasonal influenza also occurred in the reference years, which affected the periods between the first fifteen and last six weeks in the year (Figure). This could explain why the deaths coinciding with the summer wave of pandemic influenza were clearly shown as excess mortality, whereas excess mortality during the times of the year when influenza is usually present may have been partially hidden by including in the reference periods weeks in which seasonal virus was circulating in previous years.

In conclusion, these results suggest that the 2009 pandemic influenza A(H1N1) may have been accompanied by increased mortality in older persons, which would not have been detected by looking for cases of acute respiratory disease in hospitals, either because the death could have occurred outside the hospital or because influenza infection was not suspected.

Acknowledgements

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Association of ciprofloxacin prescriptions to outpatients to Clostridium difficile infections

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To study if antibiotic treatment of outpatients had triggered *Clostridium difficile* infections (CDI), prescription numbers were compared with CDI-affected patient numbers. A strong correlation was observed for ciprofloxacin (R=0.917), suggesting that increased use of ciprofloxacin by outpatients contributed to increased numbers of CDI. These findings deserve further investigation as they may have an impact on future decisions regarding antibiotic prescribing.

Increasing numbers of *Clostridium difficile* infections (CDI) have been reported in North America and Europe over the past years. Use of antibiotics is widely acknowledged as a risk factor for hospitalised as well as outpatients [1]. In Germany increasing numbers of CDI are reported, while prescriptions of antibiotics for outpatients in general stayed constant. However, shifts from lower prescription rates of narrow-spectrum antibiotics to higher prescription rates of other antibiotics (e.g. quinolones, oral cephalosporins, combinations of aminopenicillins and beta-lactamase inhibitors) are obvious [2].

To study whether increased use of these antibiotics has contributed to increased numbers of CDI, antibiotic prescriptions to outpatients in southern Germany (Bavaria) were examined and compared to the number Tcd-positive patients identified in our laboratory. Here we describe that increased use of ciprofloxacin correlated with the number of Tcd positive patients suggesting that increased use of this drug might has contributed to increased numbers of CDI.

The number of antibiotic prescriptions was examined as described previously [3]. Prescription data from outpatients registered at statutory health insurances in Bavaria were obtained from the AOK Research Institute (WIdO) (http://www.wido.de). 83% of the Bavarian population are members of the statutory health insurances. Information on prescriptions for outpatients covered by all German statutory health insurances is collected by WIdO. Prescription data for the years 2000 to 2006 were calculated according to the World Health Organization's (WHO) Anatomical Therapeutic Chemical (ATC) classification system (http://www.whocc.no). Prescriptions numbers are given in defined daily doses (DDD) per outpatient per three months.

The number of CDI-affected patients was assessed from the number of patients with *C. difficile* toxin (Tcd)-positive stool samples tested in our microbiological department in Weiden in the period from 2000 to 2006, which serves about 40 hospitals and 2,500 ambulatory care settings (approximately 161,000 microbiological samples in 2006) [4].

The comparison of the number of Tcd-positive patients with the number of antibiotic prescriptions resulted in a negative correlation for class I cephalosporines (ATC index J01DB; Spearman's rank correlation coefficient R=-0.446), beta-lactamase-sensitive penicillins (J01CE; R=-0.480), combinations of sulfonamides and trimethoprim including derivatives (J01EE; R=-0.563), and macrolides (J01FA; -0.063). A positive correlation was noticed for second generation cephalosporins (J01DC; R=0.711), combinations of penicillins including beta-lactamase inhibitors (J01CR; R=0.767), and for quinolones (J01M; R=0.709).

In contrast to second generation cephalosporins and combinations of penicillins with beta-lactamase inhibitors, certain quinolone derivatives are commonly used for the treatment of defined infections (e.g. moxifloxacin and levofloxacin for respiratory infections and ciprofloxacin for urinary infections). Therefore, a possible association of quinolone prescriptions with the number of Tcd-positive patients was analysed in more detail. From 2000 to 2006, a continuous increase was noticed in the number of Tcd-positive patients, with an obvious peak in the first months of 2006 (Figure 1).

This course is paralleled by the prescription numbers of ciprofloxacin (Figure 2) resulting in a strong positive correlation coefficient of R=0.917, p<0.0001 (Figure 3). Comparably low positive correlation coefficients were calculated for moxifloxacin (R=0.382) and levofloxacin (R=0.553; data not shown).

In contrast to moxifloxacin and levofloxacin, prescription numbers of ciprofloxacin exhibited only minor seasonal alterations suggesting that ciprofloxacin was not primarily used for the treatment of respiratory infections (Figure 2). The prescription rate of ciprofloxacin and folic acid antagonists taken together stayed fairly constant during the observation period [5]. So it appears obvious that ciprofloxacin is replacing trimethoprim-sulfamethoxazole (co-trimoxazole) and other folic acid antagonists for the treatment of urinary infections.

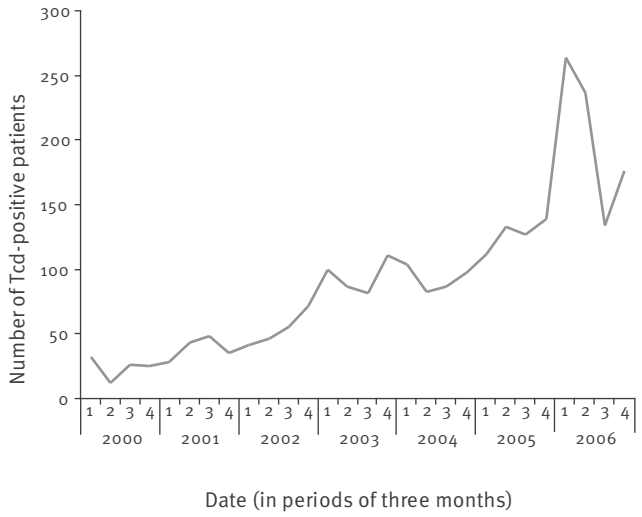
Recently it has been shown that consumption of co-trimoxazole by outpatients did not increase the risk of developing CDI but the use of quinolones actually did [1,6]. Beside the well-known deleterious effects of CDI, application of quinolones seems to act as an up-regulating mediator for the production of toxins and germination of spores thus contributing to the epidemic spread of *C. difficile* [7,8]. Resistance to ciprofloxacin in *C. difficile* isolates from European patients is common and independent of bacterial ribotype, while resistance to moxifloxacine is restricted to defined ribotypes [4,9], suggesting that the increasing use of ciprofloxacin has indeed contributed to elevated numbers of CDI.

A limitation of our study might be that antibiotic prescriptions to outpatients were compared to the number of CDI-affected in- and outpatients. On the other hand, it is difficult to distinguish between community-acquired and hospital-acquired CDI. In a previous study, 44% of CDI-affected hospitalised patients developed symptoms and exhibited Tcd-positive stool samples within 48 hours after admission. Many of these patients had been hospitalised previously and were probably colonised with *C. difficile* at that time. When re-admitted later for infections other than CDI and treated with antibiotics, the treatment may then have induced the development of CDI [10]. Therefore, antibiotic treatment of outpatients, who acquired *C. difficile* at previous hospital stays could result in hospitalised and also in non-hospitalised patients suffering from CDI, which is a reason to include hospitalised CDI patients in the study. Moreover, even when restricting the analysis to CDI-affected outpatients, a positive correlation with ciprofloxacin prescription was observed (R=0.744).

Nevertheless, also ciprofloxacin treatment of inpatients may be contributing to CDIs. Unfortunately, data about the use of antibiotics in German hospitals are not easily accessible, but there seems to be a parallel

Figure 1

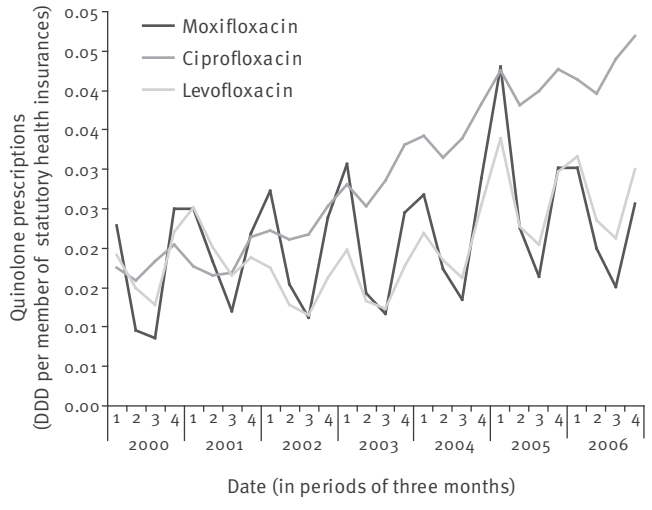
Clostridium difficile toxin-positive patients (in- and outpatients), Bavaria, 2000-2006 (per three-month period) (n=2,536)



Tcd: Clostridium difficile toxin.

Figure 2

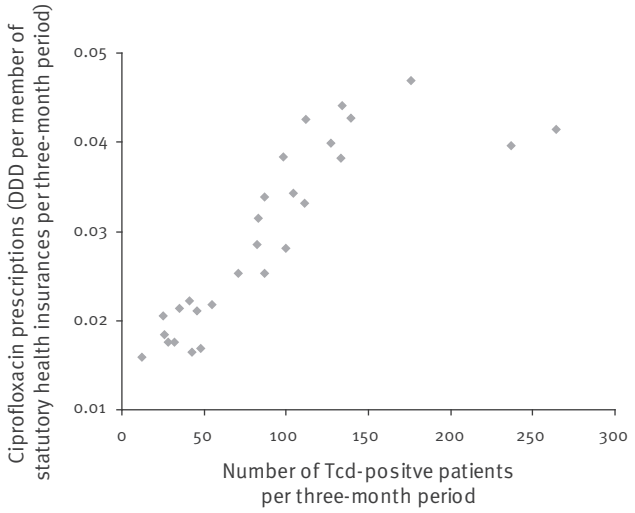
Prescription of quinolone antibiotics to outpatients, Bavaria, 2000-2006 (per three-month period)



DDD: defined daily dose.

Figure 3

Correlation of ciprofloxacin prescriptions to the number of Tcd-positive patients, Bavaria, 2000-2006 (per three-month period)



DDD: defined daily dose.

development to that observed in ambulatory settings, with quinolones as the second most prescribed antibiotics in the hospital setting [11].

In summary, the present study shows that increasing consumption of ciprofloxacin by outpatients correlates with increasing numbers of CDI. Therefore, replacement of folic acid antagonists with ciprofloxacin for the treatment of urinary infections seems to be an underestimated mechanism for the propagation of *C. difficile* infections. This needs to be investigated further as it may have an impact on future decisions regarding antibiotic prescribing.

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High frequency of cross-reacting antibodies against 2009 pandemic influenza A(H1N1) virus among the elderly in Finland

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Since May 2009, the pandemic influenza A(H1N1) virus has been spreading throughout the world. Epidemiological data indicate that the elderly are underrepresented among the ill individuals. Approximately 1,000 serum specimens collected in Finland in 2004 and 2005 from individuals born between 1909 and 2005, were analysed by haemagglutination-inhibition test for the presence of antibodies against the 2009 pandemic influenza A(H1N1) and recently circulating seasonal influenza A viruses. Ninety-six per cent of individuals born between 1909 and 1919 had antibodies against the 2009 pandemic influenza virus, while in age groups born between 1920 and 1944, the prevalence varied from 77% to 14%. Most individuals born after 1944 lacked antibodies to the pandemic virus. In sequence comparisons the haemagglutinin (HA) gene of the 2009 pandemic influenza A(H1N1) virus was closely related to that of the Spanish influenza and 1976 swine influenza viruses. Based on the three-dimensional structure of the HA molecule, the antigenic epitopes of the pandemic virus HA are more closely related to those of the Spanish influenza HA than to those of recent seasonal influenza A(H1N1) viruses. Among the elderly, cross-reactive antibodies against the 2009 pandemic influenza virus, which likely originate from infections caused by the Spanish influenza virus and its immediate descendants, may provide protective immunity against the present pandemic virus.

Introduction

In March and April 2009, a previously unknown variant of influenza A(H1N1) virus was able to cause sporadic infection clusters and epidemics in North America [1] and Mexico [2]. Rapid identification of the virus indicated that it was a novel H1N1-reassortant influenza A virus that originated from a triple reassortant North American swine influenza A virus that had acquired two virus genes (NA and M) from a Eurasian swine influenza A virus; the virus thus contains genetic material from avian (PA and PB2), human (PB1) and two lineages of swine influenza A viruses [3,4]. Compared with seasonal H1N1 viruses the novel virus is genetically and antigenically very different from human H1N1 viruses that have been circulating during the last 60 to 70 years [5]. Since the majority of the world's population is lacking immunity against this new virus, it has been spreading throughout the world with an unprecedented speed. On 11 June 2009, the World Health Organization (WHO) declared the first pandemic of the 21st century to have started, caused by the 2009 pandemic influenza A(H1N1) virus.

Epidemiological analyses initially from Mexico [6] and the United States (US) [4], and later from Europe [7] and the southern hemisphere [8,9] revealed that the disease is affecting children, young adults and the general population under 65 years of age. Recent reports from Japan [10], the US [11,12] and the United Kingdom (UK) [13] have suggested that pre-existing antibodies and thus cross-protection against the pandemic virus exist in some individuals, especially those that are currently over 65 years old. At present only limited data on this is available from Europe.

Rapid isolation and characterisation of the 2009 pandemic influenza virus, the fast dissemination of the early virus isolates to laboratories around the world, and the swift generation of reassortant and recombinant vaccine viruses enabled vaccine manufacturers to start mass production of pandemic virus vaccines rapidly. Presently, many vaccine producers have been successful in preparing functional vaccines and mass vaccinations are ongoing in a number of countries. Following recommendations by the WHO, the European Centre for Disease Prevention and Control (ECDC), and other relevant agencies, many countries have made their own vaccination prioritisations, in which health professionals, pregnant women, people with chronic underlying diseases, and children and young adults are among the first groups to be vaccinated. In order to have a better view of the possible pre-existing cross-reactive immunity against the 2009 pandemic influenza virus in the Finnish population we measured

pre-existing antibodies to this virus in more than 1,000 serum samples collected in 2004 and 2005, long before the present pandemic, from individuals born between the years 1909 and 2005.

Methods

Serum specimens

The study had been approved by the ethical committee of the Helsinki University Central Hospital. The serum specimens analysed were obtained from the virus diagnostic unit at the Central Laboratory Services of the Helsinki University Hospital (HUSLAB). The sera had been collected in 2004 and 2005 from persons representing different age groups and from different parts of the country. Only the age and sex of the individual and the collection date of the sample were known. Altogether 1,031 serum specimens were analysed.

Viruses and laboratory methods

We isolated a number of 2009 pandemic influenza A(H1N1) viruses from patients suffering from an acute respiratory infection. Nucleotide sequence analysis of

Table 1

Cross-reactive antibody levels against 2009 pandemic influenza A(H1N1) and recent seasonal H1N1 and H3N2 influenza viruses in Finnish individuals born 1909-2005 (n=1,031)

2009 pandemic H1N1 influenza A/Finland/554/09				
Year of birth (age in 2009)	n	Titre mean +/- SD (range)	% ≥10	% ≥40
1909 – 1919 (90-100)	27	27.0 +/- 2.3 (<10 – 160)	96.3	55.6
1920 – 1929 (80-89)	104	11.8 +/- 2.7 (<10 – 320)	56.7	21.2
1930 – 1939 (70-79)	125	5.6 +/- 1.4 (<10 – 40)	13.6	1.6
1940 – 1949 (60-69)	116	5.4 +/- 1.3 (<10 – 20)	9.5	0.0
1950 – 1969 (40-59)	119	5.2 +/- 1.2 (<10 – 20)	3.4	0.0
1970 – 1989 (20-39)	120	5.2 +/- 1.3 (<10 – 40)	2.5	0.8
1990 – 1999 (10-19)	144	5.0 +/- 1.0 (<10)	0.0	0.0
2000 – 2005 (4-9)	276	5.0 +/- 1.0 (<10)	0.0	0.0
Seasonal H1N1 influenza A/Finland/814/01 (New Caledonia-like)				
Year of birth (age in 2009)	n	Titre mean +/- SD (range)	% ≥10	% ≥40
1909 – 1919 (90-100)	27	15.0 +/- 3.1 (<10 – 160)	66.7	25.9
1920 – 1929 (80-89)	104	11.1 +/- 2.4 (<10 – 80)	57.7	17.3
1930 – 1939 (70-79	125	12.8 +/- 3.0 (<10 – 320)	56.0	24.8
1940 – 1949 (60-69	116	7.1 +/- 2.1 (<10 – 160)	24.1	6.9
1950 – 1969 (40-59)	119	7.0 +/- 2.2 (<10 – 160)	21.8	6.7
1970 – 1989 (20-39)	120	8.7 +/- 2.4 (<10 – 80)	32.5	13.3
1990 – 1999 (10-19)	144	10.9 +/- 2.8 (<10 – 160)	42.4	22.9
2000 – 2005 (4-9)	276	5.7 +/- 1.6 (<10 – 80)	9.1	2.2
Seasonal H3N2 influenza A/Finland/715/00 (Panama-like)				
Year of birth (age in 2009)	n	Titre mean +/- SD (range)	% ≥10	% ≥40
1909 – 1919 (90-100)	27	57.0 +/- 6.0 (<10 – 2560)	85.2	66.7
1920 – 1929 (80-89)	104	27.7 +/- 4.0 (<10 – 640)	73.1	51.9
1930 – 1939 (70-79)	125	37.2 +/- 4.2 (<10 – 1280)	76.0	60.8
1940 – 1949 (60-69)	116	17.5 +/- 3.5 (<10 – 1280)	66.4	35.3
1950 – 1969 (40-59)	119	15.7 +/- 3.3 (<10 – 2560)	59.7	33.6
1970 – 1989 (20-39)	120	34.0 +/- 3.7 (<10 – 1280)	82.5	57.5
1990 – 1999 (10-19)	144	89.4 +/- 3.7 (<10 – 2560)	89.6	86.1
2000 – 2005 (4-9)	276	13.7 +/- 4.0 (<10 – 320)	39.1	31.2

SD: standard deviation.

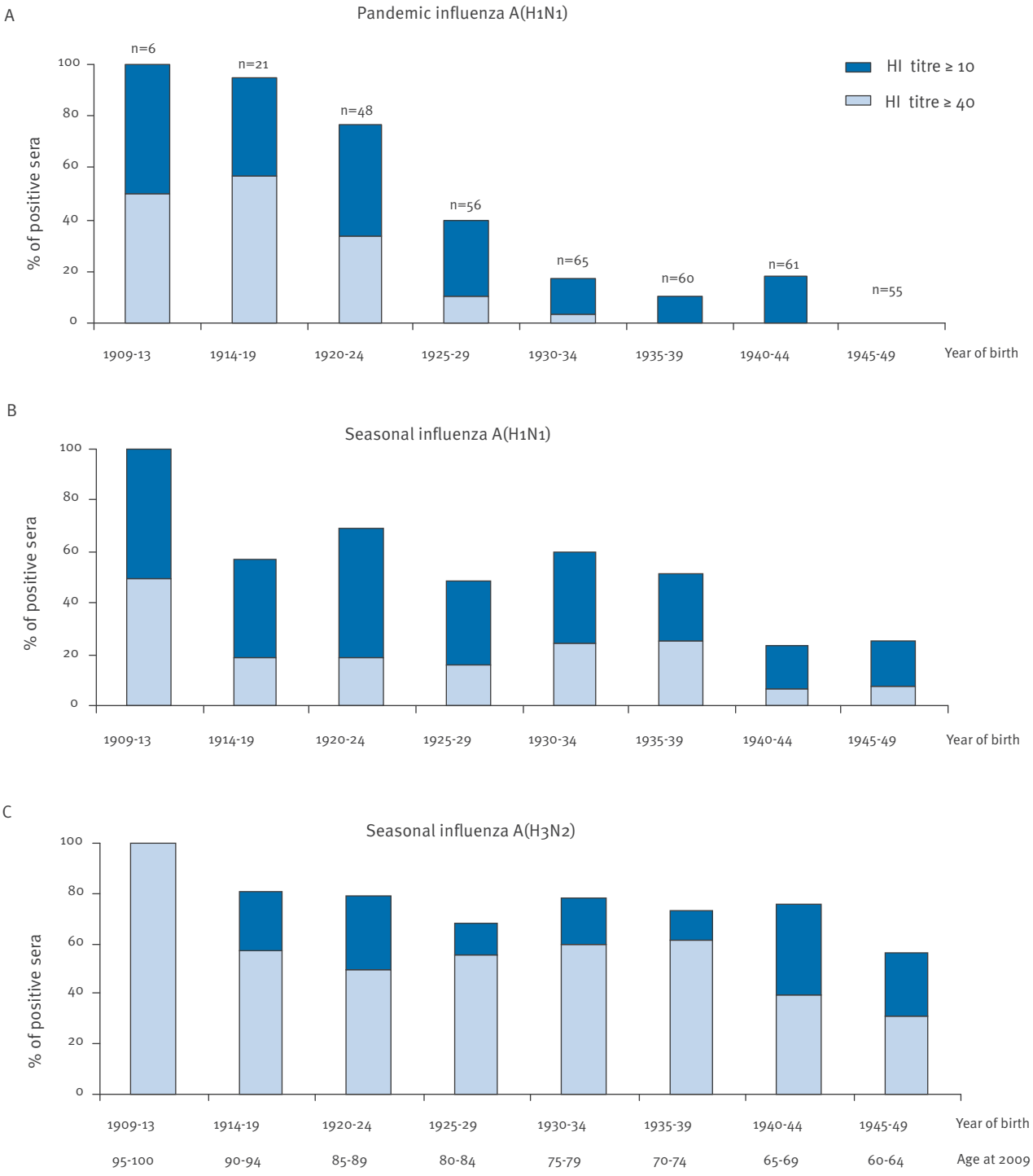
The serum specimens were split by year of birth in groups of ten or twenty years. The geometric mean titres and standard deviations were calculated, and the range of HI titres as well as the percentage values of HI titres ≥10 or ≥40 are presented for each age group.

almost the entire genome of three early Finnish isolates, and haemagglutinin (HA) and neuraminidase (NA) sequence data from 25 viruses subsequently identified in our laboratory revealed that the Finnish viruses were almost identical to the prototype pandemic vaccine virus (A/California/07/2009) and represented typical North American/European pandemic virus strains.

For the present study we selected the pandemic influenza A/Finland/554/2009 H1N1 isolate (GenBank

Figure 1

Frequency of cross-reactive virus-specific antibodies against 2009 pandemic influenza A(H1N1) in people born between 1909 and 1949 in Finland (n=372)



HI: haemagglutination-inhibition.

Cross-reacting antibodies against (A) 2009 pandemic H1N1 (A/Finland/554/09) virus, (B) recent seasonal H1N1 (A/Finland/814/2001) and (C) seasonal H3N3 (A/Finland/715/2000) influenza A strains.

The percentage of individuals with detectable (dark blue bars) and high (light blue bars) antibody levels are shown in five-year subgroups based on their year of birth.

accession numbers GQ328866-7 and GQ283487-91) as a representative virus for the immunological analysis. The virus was derived from a nasopharyngeal aspirate obtained from a patient who had recently returned from Chicago. The virus was isolated in Madin-Darby canine kidney (MDCK) cells. The pandemic influenza A/Finland/554/2009 H1N1 (HA-titre 32), and the seasonal influenza A/Finland/814/2001 H1N1 (New Caledonia-like, HA-titre 64) and A/Finland/715/2000 H3N2 (Panama-like, HA-titre 64) viruses were grown in MDCK cultures. For safety reasons the pandemic virus was inactivated with β-propiolactone (Ferrak, Berlin, Germany), which did not reduce the antigenicity of the virus.

Antibody responses to the pandemic and seasonal influenza A viruses were determined with the haemagglutination-inhibition (HI) test using standard microtitre procedures [14]. Serum specimens were pretreated with Vibrio cholerae filtrate (Denka Seiken, Tokyo, Japan) to remove non-specific inhibitors and with packed guinea pig erythrocytes to remove non-specific agglutinins. In the analysis four HA units of virus and 0.75% guinea pig erythrocytes were used.

Statistical analyses

The mean antibody levels in different age groups against the three viruses were calculated as geometric mean titres and standard deviations (SD) of the means. The results are presented as the geometric means with one SD unit. The ranges of antibody titres are also given. For HI titre values under 10 an arbitrary value of 5 was assigned in order to enable the calculation of geometric means.

Amino acid sequence comparisons of viral HA gene

The HA sequences of the 2009 pandemic influenza A (H1N1) viruses (A/California/7/2009 and A/Finland/554/2009) were compared with those of three Spanish influenza viruses (GenBank accession numbers AF116575-6, AF117241) genes as well as with six other seasonal human influenza A(H1N1) viruses from 1933 to 2007 (CY009284, CY009612, CY021053, CY030230, CY033577, CY045756 and FJ969540) and with the swine influenza A(H1N1) virus that caused a human outbreak in Fort Dix in the US in 1976 (CY039991). Comparisons were done only for the HA1 region (327 of 566 amino acids) because this region contains the major antigenic epitopes of the molecule (see Figure 2). Sequence comparisons at amino acid level were conducted in MEGA4 (Molecular Evolutionary Genetics Analysis software version 4.0 [15]). In pairwise sequence comparisons the gaps were included.

Structural analysis of amino acid differences in the HA molecule

Three-dimensional structures of several influenza A virus HA proteins have been determined. Of the human H1 virus subtypes, the HA structures of A/South Carolina/1/18 and A/Puerto Rico/8/34 have been resolved by crystallography [16]. For our analysis we selected the structure of A/South Carolina/1/18

Table 2

Amino acid sequence comparisons between haemagglutinin proteins of Spanish influenza 1918, swine influenza 1976, and pandemic and seasonal H1N1 influenza A viruses

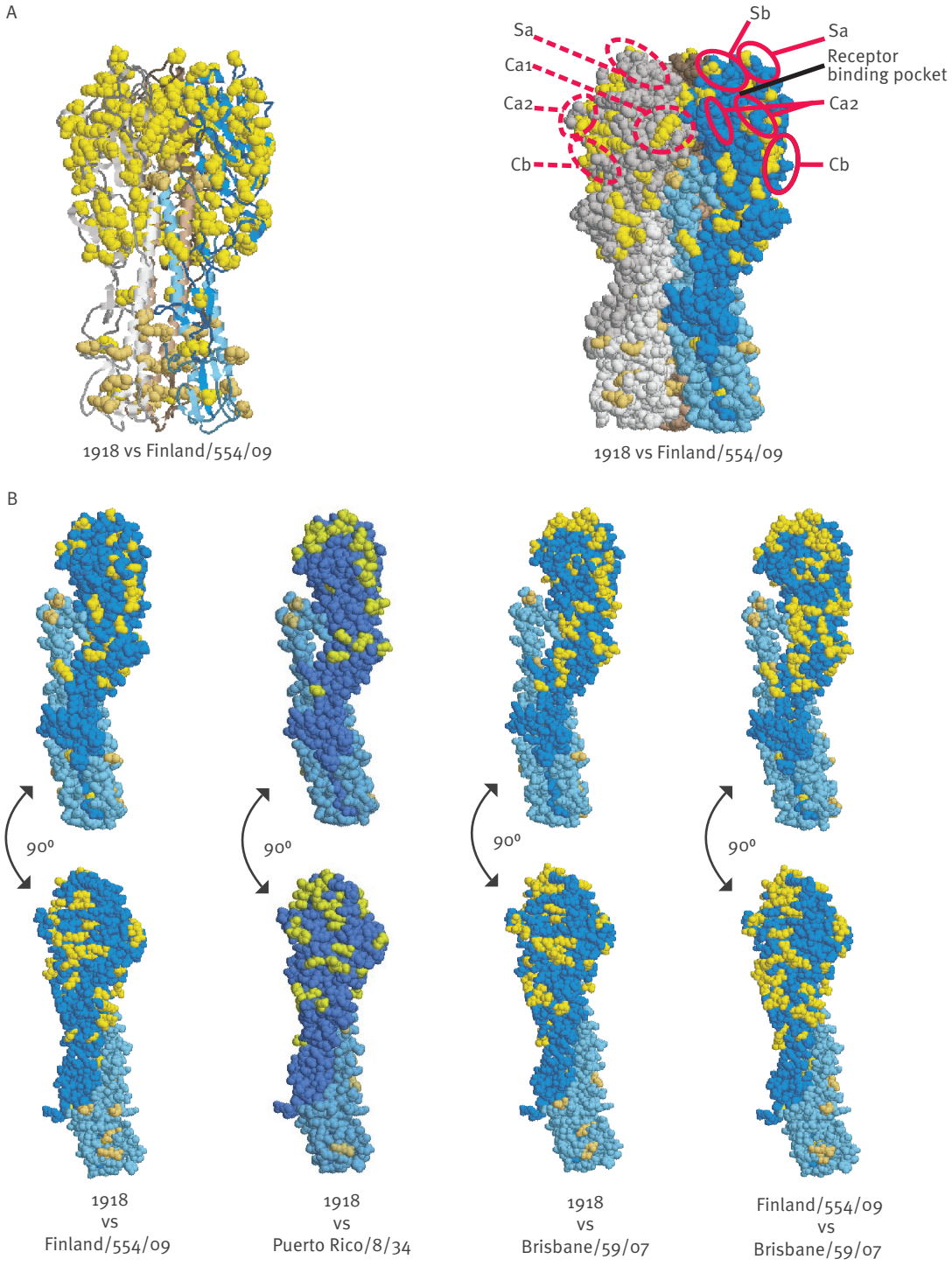
A(H1N1) HA1	Brevig Mission/1/1918	South Carolina/1/1918	New York/1/1918	United Kingdom/1/1933	Puerto Rico/8/1934	Forth Monmouth/1/1947	Malaya/302/1954	New Jersey/8/1976	USSR/92/1977	Brisbane/59/2007	Finland/554/2009 v
South Carolina/1/1918	100.0										
New York/1/1918	**99.7**	**99.7**									
United Kingdom/1/1933	87.5	87.5	87.2								
Puerto Rico/8/1934	85.0	85.0	84.7	89.3							
Forth Monmouth/1/1947	84.1	84.1	84.4	86.9	87.8						
Malaya/302/1954	82.0	82.0	82.3	85.6	86.5	95.4					
New Jersey/8/1976	**90.5**	**90.5**	**90.8**	80.1	79.4	78.8	76.1				
USSR/92/1977	82.6	82.6	82.9	84.7	86.2	95.4	92.0	76.7			
Brisbane/59/2007	80.1	80.1	79.8	80.4	82.0	85.6	83.8	74.5	87.2		
Finland/554/2009 v	**83.2**	**83.2**	**82.9**	76.5	75.2	75.2	74.0	**89.0**	72.5	72.2	
California/7/2009 v	**83.1**	**83.2**	**83.1**	77.0	75.8	76.1	74.9	**89.2**	73.3	72.7	**99.4**

Percentage of amino acid identity in the HA1 region (327 amino acids). Viruses from 1918 are from the Spanish influenza, New Jersey/8/1976 represents the swine virus causing the outbreak in Fort Dix, United States, Finland/554/2009 and California/7/2009 are 2009 pandemic influenza A(H1N1) viruses, and other viruses in the table are representative for seasonal H1N1 influenza viruses from 1933 to 2007. The percentages of homology between 1918, Fort Dix and 2009 pandemic sequences are shown in bold.

HA (RCSP Protein Data Bank accession number 1ruz) as the basis for analysing the locations of amino acid differences between the HA structures of the Spanish influenza, the pandemic influenza A/Finland/554/2009 H1N1, the seasonal influenza Puerto Rico/8/1934 H1N1 and the seasonal vaccine A/Brisbane/59/2007 H1N1 virus. The representations were done using RasMol Molecular Graphics software version 2.7.3 [17].

Figure 2

Three-dimensional structure of the 1918 Spanish influenza haemagglutinin molecule and amino acid changes between the Spanish influenza 1918, the 2009 pandemic influenza and seasonal influenza viruses from 1934 and 2007



HA: haemagglutinin.

A) Cartoons and spacefill diagrams of the trimer of human 1918 HA with locations of amino acid differences. Different monomers are coloured in blue, grey and brown. HA1-regions are shown in darker and HA2-region in lighter colours. Amino acid changes between the HA molecules are compared and the changes in the HA1-region are shown in yellow and in the HA2-region in gold. In the spacefill model antigenic sites of H1 HA (Ca1, Ca2, Cb, Sa and Sb; [21]) are marked in red circles and the receptor binding pocket with a black line.

B) The differences between viruses are shown in the monomer structure in two different orientations with 90 degree rotation.

Results

Antibody levels

Antibody levels against a representative 2009 pandemic influenza virus, A/Finland/554/2009 showed that the oldest individuals (born between 1909 and 1919) had a very high prevalence of antibodies to the 2009 pandemic influenza A(H1N1) virus. More than 96% of these individuals had detectable antibodies (titres ≥10), and in more than 55% of them, antibody titres of ≥40 were detected, which is generally considered to be a protective level. In younger age groups the prevalence of detectable antibodies against 2009 pandemic influenza virus decreased gradually with increasing year of birth (Table 1). Only some (10-14%) of the individuals born between 1930 and 1949 had cross-reactive antibodies against the pandemic virus. It is of note that some individuals in the oldest age-group had very high antibody levels with HI titres ranging between 160 and 320. Antibodies against seasonal H1N1 and H3N2 influenza viruses were found in 9-67% and 39-90% of all the individuals, respectively, depending on the age group (Table 1).

When the older age groups were divided into subgroups of five birth years (Figure 1), a gradual decrease in the frequency and levels of antibodies against the 2009 pandemic influenza virus was observed with increasing year of birth, indicating that high antibody levels were only found in individuals born in the mid 1920s or earlier. A relatively large proportion of individuals in these age groups also had antibodies against seasonal H1N1 and H3N2 influenza viruses.

Sequence comparisons

Amino acid sequence comparisons between different HA sequences (Table 2) revealed that the three 1918 influenza HA1 proteins are almost identical (99.7% identity). More than 99% sequence identity is also shown between the 2009 pandemic viruses, which can be verified by sequence comparison between any of the pandemic influenza HA genes submitted to GenBank (data not shown). It is of note that the seasonal influenza viruses from the 1930s had already significantly drifted from the Spanish influenza virus HA1 sequences and approximately 15% of the amino acids had been mutated giving an estimated evolutionary rate of 1% amino acid changes per year. The HA1 proteins of the seasonal influenza A(H1N1) virus, A/Brisbane/59/2007, shows approximately 20% divergence from that of the Spanish influenza virus. Comparison of the 2009 pandemic influenza virus HA1 sequences revealed that the most closely related human H1N1 influenza viruses were in fact the viruses of the Spanish influenza (16.8 to 17.1 divergence) with the exception of the swine virus that caused the 1976 outbreak in Fort Dix (10.8 to 11.0 divergence). The seasonal H1N1 influenza viruses isolated between 1933 and 2007 showed 23.0 to 27.8% sequence differences to the pandemic H1N1 2009 virus (Table 2). Genetic data thus clearly indicate that the closest relatives of the 2009 pandemic virus are the Spanish influenza and Fort Dix virus strains.

Molecular and structural analysis

Figure 2 visualises the differences in the amino acids between the HA sequences of the 1918 Spanish and the 2009 pandemic influenza (A/Finland/554/2009), the 1918 Spanish and the 1934 seasonal influenza (Puerto Rico/8/1934), the 1918 Spanish and the 2007 seasonal influenza (A/Brisbane/59/2007) and between the 2009 pandemic and the 2007 seasonal influenza virus. The three-dimensional structure of a trimeric HA molecule is shown in Figure 2A, where the amino acids changes between the 1918 and the 2009 pandemic virus are marked. There are several changes on the surface of the HA molecule, and some of the changes are accumulated in the antigenic epitopes. However, other comparisons (Figure 2B) indicate that the changes between the HA molecules of the 1918 and the 1934 seasonal, the 1918 and the 2007 seasonal, and especially between those of the 2009 pandemic and 2007 seasonal virus are more numerous. Most of the amino acids at the distal end of the molecule, around the sialic acid receptor binding pocket, and the antigenic epitopes on the sides of the HA molecule are altered. Since the surface structure of the 2009 pandemic influenza (H1N1) virus HA molecule is dramatically different from that of seasonal H1N1 influenza viruses, it can be expected that immunity induced by seasonal H1N1 viruses (from strains isolated later than the 1930s to 1940s) does not provide significant cross-protection against infection with the present 2009 pandemic virus.

Discussion and conclusion

This study demonstrates that in Finland, individuals born between 1909 and 1924 and to a lesser extent those born between 1925 and 1944 have pre-existing humoral immunity against the 2009 pandemic H1N1 influenza A virus. Genetic and structural analyses also revealed that the 2009 pandemic virus is more closely related to the 1918 Spanish influenza and to the 1976 Fort Dix outbreak swine viruses than to any other seasonal H1N1-type influenza viruses that have been isolated since the 1930s. It is highly likely that immunity induced by the Spanish influenza virus, as seen in the oldest individuals included in this study, provides cross-protection against the currently circulating 2009 pandemic influenza virus.

The sera selected for this study represent very well the general population in Finland, since the diagnostic laboratory received samples from all over the country and different age groups (0-96 years) were included. Historical records also indicate that the Spanish influenza was prevalent practically all around the world. In Finland the Spanish influenza was highly prevalent and found in almost all corners of the country including the most northern parts [18]. In this respect our serum material covers the Spanish influenza history in Finland very well, so that our results are likely to be representative and informative for the general situation in Europe. Recent studies from Japan, the US and the UK also describe the presence of cross-reactive antibodies to the 2009 pandemic influenza virus among the oldest

age groups (born in 1930 or earlier) [10,11,13]. In order to obtain a clearer picture on the prevalence of cross-reactive antibodies in different age groups in Europe, there is a need to study retrospective serum materials collected from different European countries.

In case the cross-reactivity against the 2009 pandemic influenza virus is indeed due to infections caused by the Spanish influenza and/or its immediate descendant viruses in the late 1910s and the 1920s, this would seem to suggest that specific anti-influenza immunity can last for an extremely long time, even a lifetime. The 33-55% of individuals who were born between the years 1909 and 1924 had relatively high antibody levels (≥40 HI titres) against the 2009 pandemic influenza virus and are thus likely to be protected against infection with this virus. Antibody levels ≥40 as measured by the HI method are generally considered as protective and such post-vaccination antibody levels are an indication of an efficient vaccine-induced humoral immune response. There was also a very good correlation between the level of cross-reactivity in the older age groups and the evolution of the Spanish influenza virus descendants. Even if there is a considerable gap in available virus isolates and HA sequences between the years 1918 and 1933 we can estimate the evolutionary speed of the virus to be at least 1% of HA1 amino acids changes per year. Apparently, the evolution was so fast that the viruses circulating in the 1930s and 1940s were already quite distinct from the initial Spanish influenza virus (see Table 2 and Figure 2) and thus infections caused by those viruses were unable to induce significant cross-reactivity against the 2009 pandemic influenza virus.

Based on HA sequence data, the 2009 pandemic influenza A(H1N1) virus is more closely related to the Spanish influenza virus than to the present day seasonal influenza A(H1N1) viruses. It is thus likely that the Spanish influenza virus was transmitted from the human to the swine population after the first wave of 1918 pandemic and the evolution of the viral HA gene in pigs went on independently from that in humans [19]. However, since the HA proteins of the Spanish influenza and the 2009 pandemic influenza virus show 17% amino acid divergence, this gives an estimated evolutionary rate of approximately 0.2% amino acid changes per year, which is considerably slower than usually seen among human seasonal influenza A(H1N1) viruses. It is likely that the shorter life span of domestic pigs and their lack of pre-existing immunity allowed the virus to spread in swine populations without significant evolutionary pressure. In many ways this may reflect the present situation with the 2009 pandemic virus, which shows extremely low rates of evolution due to the lack of protective immunity in the majority of the world's population. At present, the amino acid changes from the HA and NA gene sequences of the prototype pandemic strain and the and vaccine strain A/California/7/2009 to those of currently circulating 2009 pandemic influenza strains are less than 1% and 0.5%, respectively. Thus, basically any 2009 pandemic influenza A(H1N1) isolate can at present serve as a suitable strain for immunological analyses and vaccine production.

The availability of the three-dimensional structure of the 1918 influenza virus HA molecule allowed us to seek for molecular and immunological explanations of the humoral cross-reactivity between the Spanish influenza and the 2009 pandemic influenza viruses. The analysis clearly revealed that there are a number of amino acid differences in the important antigenic epitopes on the surface of the HA molecule, but these differences are far fewer between the 1918 Spanish influenza and the 2009 pandemic influenza virus HA molecules as compared to the differences seen between the 1918 Spanish influenza and seasonal viruses from 1934 or 2007 or between the 2009 pandemic and the 2007 seasonal influenza HA molecules. Even though the comparison was done by modelling, it can be assumed that the overall structure of the HA molecule of H1 influenza viruses is highly conserved. All in all we can say that the critical antigenic epitopes between the 1918 Spanish influenza and the 2009 influenza viruses are at least partially conserved, which probably explains the observation that people who have been infected with the Spanish influenza virus or a closely related virus have good cross-reactive immunity against the 2009 pandemic virus.

The present study, as well as a previous study [20] showing the existence of B cell clones specific for the Spanish influenza HA in the elderly, indicate that immunological memory may last a whole lifetime. These observations also suggest that the driving force of human influenza A virus evolution is the host's immune response that stimulates antigenic drift. Epidemiological analyses from North America, Europe and Australia [4,6-9] of the underrepresentation of the elderly in population groups contracting 2009 pandemic influenza suggest that persisting immunity against the Spanish influenza virus and its early variants may in fact give life-long immunity against the same or a very closely related virus strain such as the 2009 pandemic influenza A(H1N1) virus.

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