

Whether the 10-fold difference is due to overestimation of the proportion of individuals with ILI who consulted a health-care professional during the first wave (assumed to be between 20% and 50% in the HPA method) and/or a greater than expected proportion of infections that were asymptomatic or atypical in their clinical presentation cannot be determined on the basis of the serological data alone. In a small household contact study, in Hong Kong, of 11 patients with serological evidence of H1N1 2009 infection (fourfold rise in MN titres between acute and convalescent sera) three (27%) had an ILI, although six (55%) had two or more respiratory symptoms.²⁵ These proportions are similar to those reported for infections with seasonal influenza viruses²⁶ and from a field study in a school in England in May and June 2009 (HPA unpublished data). There is little information, however, on propensity to consult among individuals with ILI in the pandemic in the UK, apart from a small web-based survey that was largely restricted to adults.²⁷ A large telephone survey in New Zealand suggested that only around 1 in 18 individuals with ILI consulted in the first pandemic wave in that country.²⁸ This estimate is considerably higher than that used to derive the HPA clinical case estimates.

Our seroincidence estimates show that the second wave of infection that started when the schools reopened in September 2009 was considerably larger than the first wave that occurred in the summer (Table 14). The relative magnitude of the two waves, as estimated from the serology, is thus more in line with that suggested by the mortality data than the HPA case estimates (Figure 1), which did not take account of likely changes in the propensity to consult over time. There is evidence both from likelihood-based estimates of seroincidence in the second wave and other surveillance data sources³ that the propensity to consult is likely to have been lower in the second than the first wave of infection in the UK.

The difference in magnitude between the two waves varied between regions. In the non-London regions, there was little evidence of infection outside the 5- to 14-year age group in the first wave (estimated seroincidence for this group 6.2%, 95% CI 0.3 to 13.4). In contrast, all age groups outside London showed evidence of infection in the second wave, ranging from 53.1% (95% CI 43.9 to 60.3) in 5- to 14-year-olds to 15.4% (95% CI 8.7% to 21.4) in those aged 65+ years (Table 15). The pattern was different in the London region where the first wave was larger than the second

(Table 16). The pandemic started earlier in the London region and reached the highest estimated rate of clinical cases/100,000 population of any region in the first wave⁸ before the national closure of schools took effect and temporarily reduced R to < 1 in all regions. The seroincidence data for the first wave are thus consistent with the regional clinical case estimates using the HPA method. Non-London regions then experienced their major wave of infection when the schools reopened after the summer holidays. The post-second-wave serology results provide some evidence that the final cumulative incidence was higher in London than elsewhere. Given the particular demographic structure of London with a smaller proportion of individuals from the older age groups²⁹ (i.e. those groups with pre-existing immunity), and more contacts due to population density, it can be hypothesised that the higher attack rates in London were due to a bigger R during the two waves. This, together with the likely higher number of imported infections in London than elsewhere, may explain why this region experienced an earlier start of the pandemic and larger overall incidence than other regions.

In addition to regional differences in the timing of the start of the pandemic, there were also differences in timing between age groups, with adults over 24 years in all regions being infected later than those in younger age groups. This is consistent with the key role of children in transmission in the early stages of the pandemic and the social mixing patterns between and within age groups.³⁰ Clearly, school children are a key target for intervention during a pandemic, especially if the aim is to delay its progression in order to buy time until pandemic strain vaccines become available. The widespread use of antiviral prophylaxis in schools as part of the UK containment policy³¹ did not appear to be effective in delaying progression of the first wave of infection. While antiviral prophylaxis may have been effective at an individual level¹ initial cases in schools were often not identified early enough for prophylaxis to have had a major impact on disease transmission.³² In contrast, school closures seem extremely effective in reducing transmission as seen by the termination of the first wave when all schools closed for the summer holidays. However, use of national school closure at other unplanned times as a pandemic control measure could result in a considerable economic and social burden. Localised school closures could potentially alleviate the burden on hospital intensive care units that are reaching capacity but a recent modelling

study³³ shows that, for a range of epidemiologically plausible assumptions, considerable local coordination of school closures would be needed to achieve a substantial reduction in the number of hospitals where capacity is exceeded at the peak of the epidemic. The heterogeneity in demand for intensive care beds means that even widespread school closures are unlikely to have an impact on whether demand will exceed capacity for many hospitals.³³ If school closure is to be used as an intervention strategy in a future pandemic, its deployment may need to be reserved for a more severe strain than the H1N1 2009 virus.

It is not straightforward to disentangle to what the extent the low attack rate in older age groups (and conversely the very high attack rate in younger age groups) is explained by differing social mixing patterns or protective immunity from past infections. There are uncertainties around measuring mixing patterns, partly as a result of their survey origin and partly because it is not clear which type of contacts best describe influenza transmission (i.e. physical or conversational³⁰). There are also uncertainties regarding the interpretation of the high level of baseline cross-reactive antibodies among older persons found in this study in terms of protection against infection and disease. A modelling approach has been used to infer how well mixing patterns derived from the POLYMOD study³⁰ and the observed age-specific immunity profile as described in this study predict the observed pattern of infection.⁴ This showed that both factors contribute to reinforce the high attack rate in younger age groups. Some early predictions⁴ based on scenarios fitted to the HPA clinical case estimates up to October 2009 with a 10-fold scaling factor to derive the number of infections, indicated cumulative attack rates of around 42% in 5- to 14-year-olds and 4% in those aged 65+ years. Even if these numbers need to be revised in the light of the serological data generated in the second wave, these model predictions show that the difference in attack rates between school age children and the elderly can be explained easily by a combination of social mixing patterns and pre-existing immunity.

Study limitations

The serum samples in our study were not obtained as a result of a population screening approach whereby individuals or families are selected at random and asked to provide blood samples for a specific study. While such a method does allow additional clinical and epidemiological information

to be obtained with the serum sample, it generally suffers from a low participation rate, which may itself introduce bias and offset the advantages of the random sampling approach. In addition, young children are usually excluded for ethical reasons. For conducting a rapid H1N1 2009 seroincidence survey, the time required to obtain ethics approval and individual patient consent, together with the logistics and costs of such an approach, rendered a population-screening method impractical. The use of serum samples taken from individuals accessing health care for clinical reasons unrelated to a recent illness suggestive of influenza provided a convenient alternative method.

Construction of an HPA annual serum archive from residual aliquots of samples submitted to microbiology laboratories for screening or diagnostic testing has been in operation for over two decades, having been originally established to monitor the impact of the combined measles, mumps and rubella vaccine on age-specific population immunity.³⁴ The HPA archive has been extensively used for other seroepidemiological studies and has proven particularly useful for infections with a high incidence for which exposure is largely age dependent rather than determined by specific behavioural factors (such as for HIV) or associated with particular ethnic groups (such as hepatitis B). Because of the need for rapid generation of incidence data for H1N1 2009, serum collection was extended to chemical pathology laboratories – a new source for seroepidemiological studies. While there were no significant differences in H1N1 seroprevalence between samples from microbiology and chemical pathology laboratories in the logistic regression model (*Table 13*), patients undergoing regular chemical pathology testing may be more likely to have underlying morbidities (such as chronic cardiac, renal or respiratory conditions) than the general population. This could bias the results if such conditions affect the likelihood of being infected with the H1N1 2009 virus or the likelihood of having been vaccinated with the H1N1 2009 pandemic vaccine. Unless the underlying clinical conditions have a major impact on mixing patterns then bias in the estimates of H1N1 2009 infection through differential exposure would be unlikely. However, these clinical conditions are indications for seasonal and pandemic influenza vaccination, which is a potential source of bias that needs to be considered. As discussed earlier, it is unlikely that prior seasonal influenza vaccination will have generated cross-reactive antibody but the roll-out

[12b]

[12c] of pandemic strain vaccine in the UK for high-risk individuals from early November 2009³⁶ and for all children under 5 years of age from January 2010³⁷ may have biased the analysis, at least in the samples taken from November onwards.

The pandemic vaccine uptake rates by age group in England, derived from database extracts from the 96 general practices enrolled in the RCGP network,³⁸ are shown in *Figure 14*. Given the relatively high uptake rates in the 65+ age group (26% overall and 41% in high-risk groups), and to a lesser extent in the 45- to 64-year age group (11% overall and 41% in risk groups) some of the increase in the prevalence of H1N1 2009 antibodies in these age groups between the 2008 baseline and the post-second-wave sera is likely to have been due to vaccination, even without selective inclusion of sera from high-risk individuals targeted for vaccination. Vaccine uptake among those aged between 5 and 45 years was much lower overall (< 5%) due to the lower proportion with underlying clinical conditions in these age groups, although coverage in risk groups was relatively high (30%, 19% and 27% for the groups aged 5–14, 15–24 and 25–44 years, respectively). The extent to which vaccination may have contributed to the change in antibody prevalence compared with the baseline in the sera collected from November onwards is therefore difficult to assess. However, even if sera in the 5- to 14-year-olds were exclusively from those in risk groups, the vaccine gave 100% seroconversion, and vaccination only occurred in persons not already infected, the increase in seroprevalence between the first and second wave (51.4%, as shown in *Table 14*) in this age group is substantially higher than the 30% observed uptake in 5- to 14-year-olds in a

risk group. For those aged < 5 years, vaccine was being delivered from January 2010 onwards (while the post-second-wave sera were being taken) and was targeted at all children not just those in risk groups so increases in seroprevalence would be expected from vaccination. It is perhaps surprising therefore that there was evidence of a decline in the proportion with HI titres ≥ 32 after January in this age group (*Figure 7*).

In estimating cumulative incidence across the two waves of infection, we assumed that HI and MN antibodies developed in response to infection remain at a titre of ≥ 32 or ≥ 40 , respectively, for around a year, such that that individuals infected early in the first wave (e.g. school-aged children in London in May 2009) would still be seropositive if tested in say March/April 2010). The data on antibody titres in confirmed cases did not allow this assumption to be tested, as there were few samples taken later than 90 days after onset and few overall from children. It is possible that the decline in proportions with HI titres ≥ 32 in the March 2010 sera, which was statistically significant only in London, may have been the result of waning antibody levels following infection early in the second wave in this region.

Ideally, to generate information on the serological response in laboratory-confirmed cases of H1N1 2009 infection, a cohort would have been prospectively recruited and those with a PCR-confirmed infection would be followed up with sequential serology samples. Such an approach would be expensive and time consuming and there is no reason to assume that our method of using single serum samples taken from different individuals at various time after confirmation of

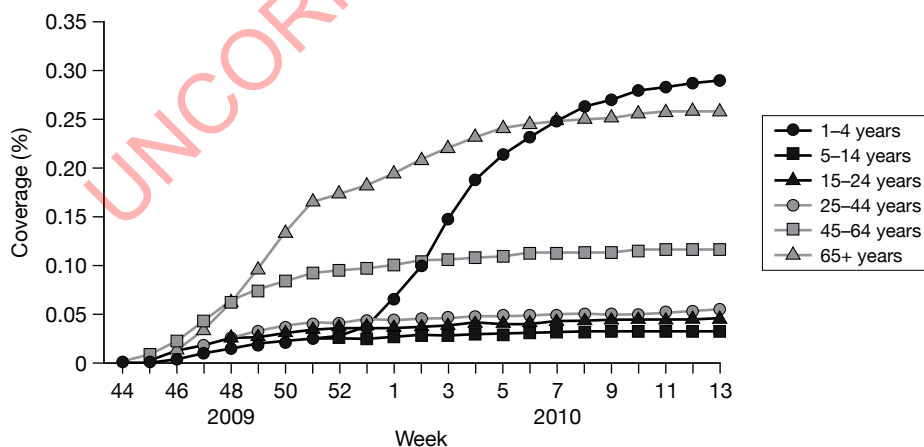


FIGURE 14 Percentage of populations vaccinated with pandemic strain vaccine by age group and week, 2009 and 2010 (data from RCGP).

infection would have produced biased results. The serum samples from confirmed cases that were used to estimate the temporal distribution of the seroconversion interval distribution were obtained from a variety of sources. Many were obtained from the follow-up of the first few hundred confirmed cases and their contacts (the so called FF100 database¹). These were actively sought to try and identify seroconversions in contacts of confirmed cases to derive secondary attack rates. Although few paired samples were obtained it is difficult to envisage why those that were obtained from PCR-positive contacts or index cases were somehow biased with respect to the serological response they developed. Other samples were obtained from subjects investigated as part of outbreaks while some were individuals for whom the clinician decided that a serological test for H1N1 2009 was indicated. The distribution for the seroconversion intervals obtained with this approach was consistent with what would be expected for seasonal influenza.

Other limitations of our method of estimating incidence by measuring differences in prevalence between two time points are that it does not take account of the variable time to seroconversion between individuals and the fact that a small proportion of infected individuals do not appear to seroconvert by methods used in this study. It also relies on grouping by calendar month yet incidence may be changing rapidly over this time period. The likelihood-based estimation was developed to overcome some of these limitations and allowed the generation of a continuous cumulative incidence curve by region and age group over the first and second waves. However, to accommodate complexities such as vaccination, or a non-random serum sampling strategy with the potential for oversampling in risk groups for whom vaccination was recommended, or changes in the propensity to consult over time, a more complex set of parameters would need to be estimated. Additionally, the method can incorporate other data sets, such as the vaccine uptake data by age and risk groups or results from immunogenicity trials. For this, Monte Carlo Markov Chain (MCMC) could be used with the combined likelihood of the different data sets to draw jointly a sample from the parameter space and then the parameters can be evaluated from the sampled distribution. Such a model could also incorporate the effect of waning antibody levels if this is shown to be important using additional data on antibody persistence after infection or vaccination.

Our study did not provide the timely incidence estimates to inform the parameterisation of 'real-time' predictive models as originally envisaged, due to the necessary lag time in H1N1 2009 assay development and, to a lesser extent, the collection and testing of the seroincidence samples. However, had the pandemic been with an H5N1 virus for which serological assays had already been developed then generation of baseline data on the age-specific prevalence of cross-reactive antibodies and insight into the incidence of infection would have been obtained more rapidly. Indeed the HPA serum archive has already been used to measure the prevalence of cross-reactive antibodies to the H5N1 virus. Nevertheless the data generated on the prevalence of cross-reactive antibodies to H1N1 2009, which was available prior to the second wave, did assist in the parameterisation of the HPA real-time model used to evaluate the likely impact of vaccination.⁴ It also helped validate the scaling factor used in that model to convert the HPA clinical case estimates to infections.⁸ For future pandemics, the availability of new serological tools that can detect incident infection in a single sample (e.g. analogous to an IgM test) rather than relying on changes in seroprevalence over time, and the availability of non-invasive techniques, such as oral fluid testing, would greatly facilitate the rapid generation of seroincidence data through a random population-screening approach.

Finally, our study was geographically limited to England. As the pandemic was UK wide, although with some differences in timing and magnitude,³⁹ it would have been informative if a pan-UK study had been undertaken that included Scotland, Wales and Northern Ireland. A proposal for a similar seroepidemiological study was funded in Scotland, for which we shared our protocol and testing standard operating procedures (SOPs), and work is under way to provide data to compare with our study, although is not available at present. Seroepidemiology from Northern Ireland would also have been useful to compare with England, particularly with the different timing and impact of first wave in Northern Ireland. Given the difference between the UK and other European countries in the timing of the first wave of infection, useful epidemiological insights might have been gained if comparable studies had been conducted in those countries. Although serological data are emerging from a range of European Union (EU) countries, the complexity of health systems and variability of studies undertaken makes developing a regional European picture of impact of the pandemic unfeasible at present.

Implications for the NHS

1. The current low levels of susceptibility to the H1N1 2009 virus in the population of England after the second wave, particularly in school-aged children who are the main transmitters of infection, together with the early decline in clinical cases in November 2009, at a time when seasonal influenza is usually increasing, imply that there has been sufficient infection of susceptibles in the population such that a third wave of infection in the 2010–11 influenza season is not to be expected. This interpretation would be consistent with the HPA real-time model that correctly predicted that the second wave would peak in early November when the R fell below '1' due to the exhaustion of susceptibles.⁴ The situation could change, however, if there was emergence of an antigenically drifted strain for which antibody generated through infection or vaccination with the H1N1 2009 strain did not provide good cross-protection. Nevertheless, sporadic cases of H1N1 are likely to continue to occur, some of which may arise in particular risk groups and be associated with severe illness. The inclusion of pregnant women in groups recommended for influenza vaccination in winter 2010–11 is a recognition of increased risk of severe illness from influenza H1N1 2009, and clinicians should remain alert to the possibility of influenza in this and other risk groups, ensuring early access to antiviral drugs and laboratory confirmation where appropriate.
2. Continued virological surveillance of the strains causing influenza is therefore essential

during the 2010–11 season. This requires the ongoing cooperation of NHS colleagues in the enhanced surveillance schemes run by the HPA, whereby patients presenting with suspected influenza in general practice or other health-care facilities are investigated virologically whenever possible, followed by full virus characterisation, ensuring that priority is given to analysis of viruses from severe illness. The use of sentinel hospital trusts for surveillance and weekly reporting of laboratory-confirmed hospitalised cases of influenza H1N1 during winter 2010–11 is intended to provide information about ongoing severe illness.

3. Measurement of the HI and MN titres to any drifted strains in sera generated by infection or vaccination with the H1N1 2009 virus would be essential for the rapid assessment of the potential for a third wave of infection. There is also lack of information on persistence of antibody levels after an influenza infection, especially in children, and thus the degree of protection that can be expected from an infection or vaccination a year ago against the same strain.
4. Opportunities to collaborate with NHS partners to study persistence antibodies after vaccination or infection should therefore be pursued, as well as further investment in pandemic preparation within the NHS to ensure that robust mechanisms for future serosurveillance in different sectors of acute care delivery are in place and can be rapidly activated.

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Chapter 6

Research recommendations

The research recommendations are as follows:

- The authors consider that investment in seroepidemiological studies for seasonal influenza would improve understanding of its epidemiology and the impact of vaccination. Investing in infrastructure for storage and investigation of alternative modalities of collection, such as dried blood spots, would enable more rapid execution of research to inform the management of future epidemics.
- Collaboration between the devolved administrations in the UK in the preparation of pandemic plans to ensure a common approach to generating comparable seroepidemiological data.
- Detailed analysis of surveillance data from H1N1 2009 to ensure legacy systems that can provide information about propensity to consult are developed for use in seasonal influenza.
- Development of more rapid serological assays that can measure recent infection in a single acute sample and do not require collection of convalescent sera.
- Further research into key cross-reacting antibodies, their genesis and implications for immunity in older people.
- Further snapshot of population immunity at regular intervals during the next 5 years to track the waning of immunity to pandemic influenza in the affected ages and investigate the interplay with immunity arising from seasonal circulating viruses.
- Further development of statistical methods, such as likelihood-based estimation, which can facilitate the rapid interpretation of serological data for real-time model parameterisation.

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Contribution of authors

Dr Pia Hardelid (Statistician) conducted the statistical analyses of seroprevalence, seroincidence and assay comparisons, and contributed to drafting the results section of the paper.

Nick Andrews (Senior Statistician) helped with the study design, data analysis and drafting the paper.

Dr Katja Hoschler (Clinical Scientist) was responsible for the assay development and validation, as well as performance of serological analysis, and contributed to drafting these sections of the paper.

Elaine Stanford (Clinical Scientist) was responsible for the collection and storage of serum samples and demographic data, and contributed to the drafting of this section of the paper.

Marc Baguelin (Mathematical Modeller and Health Economist) carried out the analysis using the likelihood-based method, and helped with the selection of samples for testing and drafting the paper.

Pauline Waight (Senior Scientist) linked the confirmed H1N1 2009 cases and serology databases, managed these databases and contributed to the drafting of these sections of the paper.

Professor Maria Zambon (Director of the CFI) contributed to the study design and directed the validation and interpretation of laboratory data, analysis and drafting of the paper.

Professor Elizabeth Miller (Consultant Epidemiologist) designed the study, submitted the grant application, directed the analysis and contributed to drafting the paper.

All authors commented on, and reviewed, the final paper.

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Appendix I

Protocol for the serological methods

Microneutralisation

The microneutralisation (MN) will be performed in 96-well format according to previously described protocols (Nicholson KG, Colegate AE, Podda A, Stephenson I, Wood J, Ypma E, *et al.* Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. *Lancet* 2001;**357**:1937–43) and standard operating procedures (SOPs) developed at the Respiratory Virus Unit (RVU).

Serum pre-treatment

Elimination of complement (e.g. from fetal calf serum in culture medium) by incubation of the sera and appropriate quality control sera (provided and chosen according to test virus by the RVU – usually serum of ferret, sheep or human, with/without neutralisation activity) at +56°C/30 min. This step will be performed simultaneously for all study samples and control sera.

Microneutralisation test

The analysis with the NIBRG122 virus will be performed. In the early stage of the outbreak the assumption can be made that most sera will show no evidence of antibody to H1N1 2009 virus. Therefore, sera could be screened at a limited dilution range or one dilution only (1:10). Only sera that inhibit virus growth at this dilution will be titrated further: a twofold dilution series will be set up for each of the samples and control sera. After addition of a pre-titred virus (usually around $100 \times \text{TCID}_{50}$ per well or 0.1–1 virus particle per cell – input might vary according to the virus used in the assay) neutralisation will be performed by incubation of the virus–serum mixture at room temperature for 1 hour.

As discussed earlier, the dilution range for the study samples might vary depending on the development of the prevalence of antibody in the population over time. We will routinely perform a six-step dilution (covering titres 10–320).

After neutralisation, a suspension of Madin–Darby Canine Kidney (MDCK) cells will be added and the plates will be incubated for 16 hours at 37°C

in a CO₂ incubator. The remaining infectivity of virus after neutralisation is determined in an enzyme immunoassay (EIA) format using a monoclonal antibody to detect expression of viral nucleoprotein. The amount of nucleoprotein expression is determined photometrically [optical density (OD) 450] using a plate reader.

Reading

An OD reading for each dilution step for each sample will be used to calculate the titre. The titre will be reported as the reciprocal dilution at which 50% of the virus is neutralised (e.g. titre of 100). The MN analysis will be performed in duplicate (in separate runs on 2 days) for each sample.

The two titres for each sample must not differ by more than a twofold serial dilution. In cases, where samples do not fall within this limit, a third analysis is performed and the two closest titres (which must be within a twofold serial dilution) will be reported.

Haemagglutination inhibition

The principle of the haemagglutination inhibition (HI) test is based on the ability of specific anti-influenza antibodies to inhibit haemagglutination of red blood cells (RBCs) by influenza virus HA. The sera to be tested have to be previously treated to eliminate the non-specific inhibitors and the anti-species HAs. The experiment will be performed in accordance to protocols and SOPs established by the RVU.

Serum pretreatment

Elimination of non-specific inhibitors by incubation of the unknown serum samples and quality control sera (serum of ferret or human immunised with influenza virus) with neuraminidase [receptor-destroying enzyme (RDE) II: 18 hours/+36°C followed by heat inactivation 1 hour/+56°C].

Preparation will be performed simultaneously for serum obtained pre-vaccination and post vaccination.

Haemagglutination inhibition test

Sera screened in the early phase of the study in a limited dilution range (e.g. 8- to 32-fold dilution)

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using the NIBRG122 virus. Samples that show activity in this range will be further titrated: eight twofold dilutions, starting at a 1 : 8 dilution of serum sample (or quality control sera) are performed and incubated with the haemagglutinin (HA) antigen suspension [previously titrated to adjust the dilution at eight haemagglutination units (HAUs)/25 μ l]. The HA antigen is not added to the well that is dedicated to the RDE quality control.

The mixture is incubated for 1 hour at room temperature and 25 μ l of the 0.5% RBC suspension (turkey blood) is added. The reaction is left for 1 hour at room temperature before reading.

Reading

The serum titre is equal to the highest reciprocal dilution, which induces a complete inhibition of haemagglutination. The titre of each quality control serum is close to the previously assigned value (within one serial twofold dilution limits).

The RBC controls (RBC suspension without antigen) and the RDE controls do not produce any agglutination.

Each serum sample is titrated in duplicate and individual titres will be reported (two for each sample). These must not differ by more than a twofold serial dilution. In cases, where samples do not fall within this limit, a third analysis is performed and the two closest titres (which must be within a twofold serial dilution) will be reported. For laboratory details of the HI and MN assays, see Appendix 1. In accordance with laboratory procedure, each sample was tested twice by each assay and the geometric mean titre (GMT) of the two results was used in the analysis. The starting dilution for the HI assay was 1 : 8 and for the MN was 1 : 10.

Reporting

The collaborator(s) will receive results for both assays in form of an EXCEL table by e-mail.

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Appendix 2

No. of seroincidence samples collected by age group, month and region: 2009–10

August 2009

Age group (years)	East	East Mids	London	NE	NW	SE	SW	West Mids	Y&H
<5	26	9	25	0	24	6	19	10	0
5–14	26	22	26	0	30	6	41	15	0
15–24	8	17	10	0	21	24	60	11	0
25–44	18	27	55	0	28	9	74	14	0
45–64	23	38	48	0	31	0	102	25	0
65+	24	41	53	0	87	23	101	24	1

September 2009

Age group (years)	East	East Mids	London	NE	NW	SE	SW	West Mids	Y&H
<5	25	15	25	0	39	1	4	1	0
5–14	27	24	25	0	68	13	26	10	0
15–24	15	6	12	0	83	34	20	9	0
25–44	17	14	53	0	73	0	87	0	0
45–64	22	31	78	0	71	0	86	0	0
65+	23	34	62	0	111	12	71	0	0

October 2009

Age group (years)	East	East Mids	London	NE	NW	SE	SW	West Mids	Y&H
<5	0	9	25	1	29	0	1	0	0
5–14	0	11	25	0	59	0	24	0	0
15–24	0	4	12	0	3	0	9	0	0
25–44	0	9	13	0	21	0	16	0	0
45–64	0	18	24	0	27	0	25	0	0
65+	0	20	26	0	74	0	47	0	0

November 2009

Age group (years)	East	East Mids	London	NE	NW	SE	SW	West Mids	Y&H
<5	0	17	43	24	27	0	1	7	0
5-14	0	16	32	19	69	0	31	19	0
15-24	0	10	5	5	77	0	16	17	0
25-44	0	31	21	21	60	0	35	20	0
45-64	0	0	0	2	0	0	0	0	0
65+	0	0	0	0	0	0	0	0	0

December 2009

Age group (years)	East	East Mids	London	NE	NW	SE	SW	West Mids	Y&H
<5	0	11	19	3	10	0	1	5	0
5-14	0	9	17	8	16	0	31	25	0
15-24	0	11	11	0	10	2	19	7	0
25-44	0	15	18	0	43	0	31	0	0
45-64	0	0	0	0	0	0	0	0	0
65+	0	0	0	0	0	0	0	0	0

January 2010

Age group (years)	East	East Mids	London	NE	NW	SE	SW	West Mids	Y&H
<5	0	12	23	14	46	3	6	0	0
5-14	0	10	22	23	82	11	31	0	0
15-24	0	15	6	3	94	49	9	0	0
25-44	0	25	21	22	55	12	19	122	0
45-64	0	45	24	25	54	3	24	39	0
65+	0	43	25	25	105	2	26	50	0

February 2010

Age group (years)	East	East Mids	London	NE	NW	SE	SW	West Mids	Y&H
<5	0	8	25	14	0	0	1	0	0
5-14	0	10	26	8	0	0	24	0	0
15-24	0	2	7	3	8	0	14	0	0
25-44	0	21	18	12	17	0	13	27	0
45-64	0	29	25	25	24	0	24	10	0
65+	0	41	25	25	76	0	27	0	0

March 2010

Age group (years)	East	East Mids	London	NE	NW	SE	SW	West Mids	Y&H
<5	0	3	24	11	0	0	2	0	0
5–14	0	7	25	17	0	0	25	0	0
15–24	0	2	5	1	0	0	19	0	0
25–44	0	4	20	9	0	0	47	0	0
45–64	0	16	24	0	0	0	39	0	0
65+	0	33	24	0	0	0	31	0	0

April 2010

Age group (years)	East	East Mids	London	NE	NW	SE	SW	West Mids	Y&H
<5	0	0	0	0	0	0	4	0	0
5–14	0	0	0	0	0	0	3	0	0
15–24	0	0	0	0	0	0	3	0	0
25–44	0	0	0	0	0	0	9	0	0
45–64	0	0	0	0	0	0	5	0	0
65+	0	0	0	0	0	0	2	0	0

UNCORRECTED PROOFS

UNCORRECTED PROOFS

Appendix 3

Framework for undertaking seroepidemiological studies at population level, with specific reference to influenza



Background

These notes set out the main considerations when seeking to undertake seroepidemiological studies to provide real-time rapid response to an emerging infection, such as pandemic influenza. Under these circumstances, serological data provide important information, such as levels of cross-protective immunity in different population groups and incidence rates of infection.

A seroepidemiological study can provide information on:

1. age-specific incidence as the disease emerges in the population
2. prevalence of existing cross-protective immunity
3. cumulative prevalence to inform future incidence predictions, for example through disease modelling where seroepidemiological data can be combined with age-specific morbidity data to predict the likely burden of illness and the impact of a novel infection such as pandemic influenza.

These data can be used to complement other descriptive epidemiological data or to inform disease transmission models used to predict the future course of the pandemic.

The requirements are different from those when setting up a seroepidemiological programme, the main focus of which is to monitor levels of immunity to particular diseases within a population to assist in evaluation of the impact of a vaccination programme or to inform the need for other public health interventions.^{1,2}

For any seroepidemiological study, it is essential that recent, age-stratified baseline sera are available to inform the interpretation of data. Such sera cannot be collected retrospectively so long-term investment by member states is essential to ensure access to population-based stored sera. The absence of recent baseline sera is a significant limiting factor in the interpretation of data for newly emerging infections. For example, knowledge of the baseline prevalence of antibodies to pandemic influenza AH1N1 in 2009 was essential for understanding the epidemiology and informing vaccine policy, as it indicated that lower clinical attack rates in the elderly were the result of pre-existing cross-reactive antibody, thereby reducing the clinical benefit and cost-effectiveness of delivering vaccine to this group. This required access to sera obtained prior to the pandemic.

It is essential that appropriate personnel (microbiologists, epidemiologists, mathematical modellers and statisticians) are involved in the establishment of seroepidemiological studies from the outset.

When considering region-wide seroepidemiology (e.g. EU wide) it is necessary to form teams with broad representation, and establish a limited network of laboratories or a central laboratory for testing to ensure maximum generalisability. Comparisons between countries may be confounded by differences in assay performance, sensitivity and specificity.^{3,4}

Populations and sampling methodologies

Serum source

There are two basic methods for obtaining serum samples from populations:

1. Opportunistically available residual samples from routine biochemistry, microbiology or other sources (e.g. blood transfusion service) conducted on outpatient or inpatient populations. While serum samples submitted to diagnostic laboratories may not be entirely representative of the population they are readily accessible and therefore cheap to collect. Special arrangements may be needed for paediatric samples.
2. Establishment of specific studies to achieve sampling of population-based cohorts. This can be sampling of particular groups, for example schoolchildren, or targeted sampling [e.g. the Tecumseh study,⁵ Flu Watch⁶ or the National Health and Nutrition Examination Survey (NHANES) studies⁷]. While this is important for evaluating the effect of public health interventions in specific groups, it can be difficult to achieve high levels of compliance and will not always give generalisable results. This method provides the opportunity to collect more detailed demographic data on the participants.

Documentation

The minimum data set for each specimen should be date of birth or age, sex, date of specimen collection, geographic location of specimen collection, and specimen source (e.g. laboratory, hospital, community).

It is usually a condition of ethics approval that samples will be irreversibly unlinked to any possible patient-identifying information to ensure anonymity.

Ethics and approvals

Issues of patient confidentiality and obtaining ethical approval will differ in each member state and may cause significant difficulties in some circumstances. In some member states, implementing an 'emergency public health response' or 'service evaluation' may invoke an emergency clause that bypasses normal regulatory mechanisms.

Member states should give attention to how to achieve rapid sampling as part of preparedness planning and should establish a rapid ethics approval framework as part of their pandemic plan.

Population

Ideally, sera should be taken from both sick and healthy subgroups in the population to establish

levels of cross-protective immunity and levels of seroconversion; however, opportunistic sampling may not permit this.

Emerging infections that have a high clinical attack rate and do not depend on specific behavioural risk factors will cause infection across the population. In these cases, the necessity to sample different populations may not be so critical. It is essential, however, in the event of newly emerging infections (e.g. pandemic influenza, SARS) to ensure the ability to establish seroprevalence in a population 'baseline sample' that has been taken or archived prior to the disease emergence.

Immunosuppression and HIV

Samples from patients who are known to be immunosuppressed should be excluded due to difficulties in interpreting these data.

Timing of samples

Samples must be timed appropriately to give interpretable data:

1. Sequential sampling of opportunistic cohorts (e.g. women attending antenatal care) are extremely informative but generalisability to the general population may be limited.
2. Repeated cross-section samples with the same sampling methodology can generate cumulative age-specific prevalence from which infection incidence over time can be derived.⁸

Ideally, information should be obtained on whether the sample has been taken post natural infection or post vaccination.

Age stratification

It is essential that serum samples are stratified by age in equal numbers for male and female to provide information about the cross-protective immunity or specific effects in different age groups. All age groups in the population should be represented (e.g. useful age groups include < 5, 5–14, 15–44, 45–64 and ≥ 65 years). It is important to have background information about the age composition of the population to inform interpretation of data, and provide ability to perform predictive analyses.

Geographical representation

It is important to have samples from both urban and rural settings to establish population mixing patterns, and to have information of the population density and population structure, which may vary considerably. This may lead to differences

in transmission patterns and, therefore, age-stratified incidence.

Sample size and power calculations

Power calculations need to be undertaken to determine the number of samples required at each time point and in each age group to estimate a change in prevalence according to specified scenarios.

For example, to estimate the incidence of pandemic influenza AH1N1 in England during the first wave of activity, a serosurvey was performed using samples from prior to the pandemic and then at monthly intervals. The sample consisted of 1600 sera taken prior to the pandemic (200 in each of eight age groups < 1, 1–4, 5–14, 15–44, 45–64, 65–74, 75–79, 80+) and then 1000 per month, spread across five age groups, with 200 in each (< 5, 5–14, 15–44, 45–64, 65+) with a final post-first-wave cross-sectional survey of 1600 using samples collected May–June 2010.

Using this sample size, the 95% CIs for the estimation of prevalence within each age group and overall are shown below (Table 23), for various observed prevalences.

TABLE 23

Prevalence (%)	95% CIs	
	n = 200	n = 1000
0	0.0 to 1.8	0.0 to 0.4
5	2.4 to 9.0	3.7 to 6.5
10	6.2 to 15.0	8.2 to 12.0
15	10.4 to 20.7	12.8 to 17.4
20	14.7 to 26.2	17.6 to 22.6
25	19.2 to 31.6	22.3 to 27.8
30	23.7 to 36.9	27.2 to 32.9
35	28.4 to 42.0	32.0 to 38.0
40	33.2 to 47.1	36.9 to 43.1
45	38.0 to 52.2	41.9 to 48.1
50	42.9 to 57.1	46.9 to 53.1

To estimate incidence from prevalence the difference between the prevalence at two time points can be calculated. When combining all age groups this would give reasonable precision for estimating incidence from prevalence as shown in Table 24 below.

TABLE 24

P1	P2	P2–P1	95% CI
5	10	5	2.7 to 7.3
10	15	5	2.1 to 7.9
15	20	5	1.7 to 8.3
20	25	5	1.3 to 8.7
5	15	10	7.4 to 12.6
10	20	10	6.9 to 13.1
15	25	10	6.5 to 13.5

Within each age group the precision would be much lower if incidence were calculated this way. However, if incidence (or force of infection) is modelled as a function of time and age then this sample size will still give good precision within age groups.

Assay methodology

Consideration should be given to the choice of assay used and the decision should include epidemiologist, statistician and microbiology involvement. Assays for diagnostic purposes often have different criteria than those selected for seroepidemiology purposes, which do not necessarily require accurate results at the individual patient level.

The magnitude and the kinetics of antibody detection using the chosen methodology need to be understood in relation to the measurements undertaken.

Choice of assay

The choice of assay [e.g. HI, MN, single radial haemolysis (SRH)] for influenza antibodies will depend on the parameters to be measured.

Ideally, the assay chosen should provide a direct correlate of protection. ELISA is not recommended for influenza antibody detection, as it does not measure protective antibody or provide a good measure of disease incidence.

It is important to understand the serological response to infection with the assay methodology used, i.e. the relationship between symptomatic and asymptomatic infection and the relationship between seroconversion and clinical illness.

Statistical analysis

- Statistical analysis of seroepidemiology data typically provides estimates of age-specific prevalence. If repeat sampling is used this

[17b]

[17a]

[17b]

can be used to provide a direct measure of incidence of infection in population.

- These direct incidence estimates can be compared with those derived from clinical surveillance using direct virus detection tests and modelling to provide an insight into the epidemiology and clinical expression of the infection.
- Linear regression may be used to estimate the effect of age, sex, geographical origin, gender or other available variables on antibody levels.
- Reverse cumulative distribution curves can be constructed to compare proportions above various threshold titres.
- Mixture modelling techniques may be used instead of pre-defined assay cut-off, especially where there is not a clear correlate of protection.

Quality assurance

It is recommended that one or more 'gold standard' laboratories are identified at a European level to ensure that country results are comparable. These laboratories would be responsible for the development and distribution of panels of serum specimens, which would include known negatives, acute and convalescent specimens, and specimens from people who have been vaccinated. The panels would be used to establish the sensitivity and specificity of assays.

Use of antibody standards

International antibody standards, such as those produced by the HPA NIBSC are primarily designed for use in serological assays where either population based seroepidemiology is being performed, or where vaccine immunogenicity studies are being undertaken. The significant advantage of a calibrated standard is that it helps to overcome the methodological differences that give rise to significant variation between laboratories in antibody titres. These standards are not designed to be used as 'run controls' for routine serological assays.⁴

Standard operating procedures

It is recommended that a limited number of laboratories undertake testing, and the number of SOPs is kept to a minimum to limit variability in testing. SOPs are required for sample handling,

result validation and result interpretation. Assay reproducibility should be built into existing quality assurance programmes.

Audit and quality assurance

Good practice is required, especially when dealing with large data sets and complex sample collection protocols. Regular monitoring, audit of processes and procedures and data verification is needed to ensure integrity of the process from end to end.

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Drafted by Elizabeth Miller, Maria Zambon and Nichola Goddard.

Appendix 4

Study protocol, version 1 July 2009: no subsequent amendments

Protocol for the assessment of baseline age-specific antibody prevalence and incidence of infection to novel influenza AH1N1 2009

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Background

Knowledge of the baseline prevalence of antibodies to H1N1 2009 is essential for understanding the current and future epidemiology of H1N1 2009. It will inform vaccine policy, as it will indicate whether the lower attack rates so far documented in the elderly in the UK are indeed the result of pre-existing cross-protective antibody, thus reducing the clinical benefit and cost-effectiveness of delivering vaccine to this group. Seroepidemiological data combined with age-specific morbidity data will greatly assist in understanding the spread of this novel virus, and provide valuable information for real-time modellers who will be predicting the likely burden of illness and impact of the pandemic as it progresses during the coming year. The need for such data has been identified as a high priority by the Scientific Advisory Group for Emergencies (SAGE).

The proposal below is designed to generate information on the baseline prevalence of cross-reactive antibodies to the H1N1 2009 virus in England prior to the start of the epidemic and to

generate monthly age-specific incidence as the disease progresses during the next year.

Documenting baseline prevalence of antibodies to H1N1 2009 virus in England

Sera taken in 2008 and already stored by the Health Protection Agency (HPA) serological surveillance programme (www.hpa.org.uk/webw/HPAweb&Page&HPAwebAutoListName/Page/1158313434390?p=1158313434390) will be used to document age-stratified prevalence of antibodies to H1N1 2009 prior to the arrival of swine flu in the UK. The sera are collected by 16 laboratories in eight regions in England and are residual aliquots from samples submitted to HPA and NHS laboratories for diagnostic testing or screening and despatched to the Seroepidemiology Unit (SEU) at Manchester. The sera collections have been extensively used for policy-related seroepidemiological studies, and for deriving force of infection estimates for disease modelling purposes (Osborne K, Gay N, Hesketh L, Morgan-Capner P, Miller E. Ten years of serological surveillance in England and Wales: methods, results, implications and action. *Int J Epidemiol* 2000;**29**:362–8).

The age groups have been chosen to match those in the Royal College of General Practitioners (RCGP) and Q Flu influenza surveillance data set, with expansion of the 74+ age group to ensure separate groups for 74- to 79-year-olds and those aged 80+ years as follows: < 1, 1–4, 5–14, 15–44, 45–64, 65–74, 75–79 and 80+ years. Two hundred samples from each of these groups will be tested.

Demographic data on each sample will be: age in months if under a year; age in years if over; month/year sample taken, gender, collecting laboratory.

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Measuring incidence during the evolving epidemic

It is planned to test approximately 1000 serum samples across the age range each month to provide incidence estimates. For this, the principal source of sera will be those from chemical pathology laboratories, as these are usually discarded after 48 hours, whereas the samples from microbiology laboratories are generally retained for longer while testing is completed. This would allow more timely provision of sera for testing. Also sera from the chemical pathology laboratories are not submitted from patients with acute infections and are therefore potentially less biased with respect to risk of influenza. Chemical pathology laboratories on the sites of the existing 16 collecting laboratories will be approached to provide these sera so that they can be despatched together with the microbiological sera using existing procedures.

Collection and documentation of sera

The procedure put in place by the SEU for selection, despatch and documentation of sera is attached in Annex 1. The methods ensure that sera are irreversibly unlinked to any possible patient identifying information. Ensuring that testing is carried out by unlinked anonymous methods is a condition of the ethics approval that has been obtained for the HPA seroepidemiology programme [National Research Ethics Service (NRES) reference no. 05/Q0505/45].

Laboratory testing

Samples will be tested in the Centre for Infections (CFI) Virus Reference Unit (VRU). The HPA SEU will provide the VRU with an EXCEL spreadsheet containing the unique identifier for each sample. The VRU will check the identity of all received samples and feed back mismatches where necessary. All samples will be analysed by microneutralisation (MN) and haemagglutination inhibition (HI) with the NIBRG122 virus (rg virus based on A/Engl/195/2009(vH1N1) and A/Puerto Rico/8/34). Results will be entered onto the EXCEL spreadsheet, which will be returned to the SEU for linking with the basic demographic data available for that sample.

Details of the laboratory methods are attached in Annex 2.

Sample size for swine flu seroprevalence

In order to estimate the incidence of H1N1 2009 during the pandemic, serosurveys will be performed using samples from prior to the

pandemic and then monthly through the first wave from August 2009 to May 2010.

The sample size will consist of 1600 sera taken prior to the pandemic (200 in each of eight age groups: <1, 1–4, 5–14, 15–44, 45–64, 65–74, 75–79 and 80+ years) and then 1000 per month, spread across five age groups with 200 in each (<5, 5–14, 15–44, 45–64 and 65+ years) with a final post-first-wave cross-sectional survey of 1600 using samples collected around May–June 2010.

With this sample size the 95% CI for the estimation of prevalence within each age group and overall is shown in *Table 1* below for various observed prevalences.

TABLE 1

Prevalence (%)	95% CIs	
	n = 200	n = 1000
0	0.0 to 1.8	0.0 to 0.4
5	2.4 to 9.0	3.7 to 6.5
10	6.2 to 15.0	8.2 to 12.0
15	10.4 to 20.7	12.8 to 17.4
20	14.7 to 26.2	17.6 to 22.6
25	19.2 to 31.6	22.3 to 27.8
30	23.7 to 36.9	27.2 to 32.9
35	28.4 to 42.0	32.0 to 38.0
40	33.2 to 47.1	36.9 to 43.1
45	38.0 to 52.2	41.9 to 48.1
50	42.9 to 57.1	46.9 to 53.1

To estimate incidence from prevalence the difference between the prevalence at two time points can be calculated. When combining all age groups this would give reasonable precision for estimating incidence from prevalence as shown in *Table 2* below.

TABLE 2

P1	P2	P2–P1	95% CI
5	10	5	2.7 to 7.3
10	15	5	2.1 to 7.9
15	20	5	1.7 to 8.3
20	25	5	1.3 to 8.7
5	15	10	7.4 to 12.6
10	20	10	6.9 to 13.1
15	25	10	6.5 to 13.5

Within each age group the precision would be much lower if incidence were calculated this way. However, if incidence (or force of infection) is modelled as a function of time and age then this sample size will still give good precision within age groups.

Data analysis and reporting

Data will be analysed by the CFI Statistics Unit. For the monthly incidence estimates it is intended

that testing and analysis of each month's samples will be completed within a month of receipt of that month's batch. Tabulations of the proportion positive to H1N1 2009 by test method and age group (< 5, 5–14, 15–44, 45–64 and 65+ years) will be provided regularly to SAGE and the Scientific Pandemic Influenza Sub-Group on Modelling (SPI-M) as appropriate.

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Annex I: Seroepidemiology Unit user guide



**SEROEPIDEMIOLOGY
UNIT**

USER GUIDE

April 2009

Introduction.

The Health Protection Agency (HPA) Seroepidemiology Programme.

The basis for the HPA Seroepidemiology Programme is a large collection of sera approximating the general population of England and Wales, forming a unique and valuable public health resource. The serum collection is now stored and maintained by the seroepidemiology unit (SEU) at the HPA (North West) Manchester laboratory having previously been stored at the Lancashire Teaching Hospitals NHS Trust (formerly Preston Public Health Laboratory). Sera used are residues of specimens submitted for diagnostic testing, they represent the entire age range of the population and are anonymised (retaining age, sex, date of collection and source laboratory only). Collection of sera is continuing through collaboration with the HPA Regional Microbiology Network and laboratories throughout England and Wales, and has occurred annually since 1986, with over 150,000 sera now stored and catalogued. The collection is available for testing to anyone wishing to use it for public health purposes.

The programme is focused on cross-sectional antibody prevalence studies to help in the understanding of the epidemiology and burden of infectious diseases of public health importance, and how this may be changing. This provides key evidence to assist with making informed decisions regarding health policy where intervention is possible.

Cross-sectional antibody prevalence studies.

Cross-sectional prevalence studies involve collecting biological samples at a point in time that represents a population of interest and subsequently screening them for a marker that relates to a disease. They are relatively simple to conduct, take only a short time and are relatively inexpensive. Serum is often the sample of choice with specific IgG the marker chosen, as the presence of this antibody indicates previous exposure (or vaccination) to the disease for which it is specific. If samples collected at several different time points are able to be used it is possible to estimate changes in the epidemiology of a particular disease.

Regular cross-sectional IgG prevalence studies form the basis of serological surveillance, an important technique for continually monitoring the behaviour of a disease within a population.

The collection is run on a voluntary basis with over 150,000 sera now available. If you would like to contribute, please contact us. Some financial assistance may be available.

Further information:

<http://www.hpa.org.uk/webw/HPAweb&Page&HPAwebAutoListName/Page/1158313434390?p=1158313434390>

SEU Staff and Contact Details:

SEU at HPA Centre for Infections (CfI):

For general enquiries and requests to use the archive:

Address: HPA CfI,
Immunisation Department,
61, Colindale Avenue,
London,
NW9 5HT.

Staff: Dr Richard Pebody richard.pebody@hpa.org.uk 0208 327 7423

SEU at HPA North West, Manchester Laboratory:

For contributions of sera to the archive, general enquiries and the return of tested samples and results:

Delivery Address: Elaine Stanford,
HPA Seroepidemiology Unit,
Vaccine Evaluation Unit,
Manchester Medical Microbiology Partnership,
2nd Floor, Clinical Sciences Building 2,
Manchester Royal Infirmary,
Oxford Road,
Manchester
M13 9WL,
UK.

Hays: HPA North West, Manchester Lab, DX 6962410, Manchester 90 M

Fax: 0161 276 6792

Staff: Ms Elaine Stanford elaine.stanford@hpa.org.uk 0161 276 6791

Dr Jamie Findlow jamie.findlow@hpa.org.uk 0161 276 5697

Dr Ray Borrow ray.borrow@hpa.org.uk 0161 276 6793

The laboratory is open to receive samples on Mondays to Fridays from 8.00am to 4.00pm (excluding bank holidays).

Contributions of sera to the SEU archive:

Serum requirements:

The current yearly approximate target age ranges for collection per HPA region, distributed evenly between male and female patients, are as follows:

- 1 - 24 years: 25 sera in each 1 year age group
- 25 - 44 years: 50 sera in each 5 year age group
- 45 - 84 years: 50 sera in each 10 year age group

A minimum volume of 200 μ L is requested for each sample
 Recent repeat sera from the same individual should be excluded
 Sera from individuals known to be immuno-compromised should be excluded
 Sera submitted for genitourinary investigations (GU) and/or antibody testing to Human Immunodeficiency Virus (HIV) and Hepatitis B Virus (HBV) or Hepatitis C can be included but should be flagged appropriately by annotating the GU column of Appendix I or an electronic copy thereof.

All contributions of serum samples to the archive are to be sent frozen in secure, appropriately-labelled packaging including freezer packs or dry ice. Serum vials should be rigid polypropylene with a screw-cap with O-ring or phlange seal, with a capacity of no more than 2 mL. **Shipping costs to the SEU archive laboratory are the responsibility of the contributing laboratory.**

It is requested that contributing laboratories supply the following data for all samples submitted to the archive:

Gender

Unique sample identifier

Date of birth (or age, if DOB not available)

Date of sample

Whether or not sample collected for GU investigation and/or testing for HIV/HBV.

The data must be sent to the SEU laboratory in electronic form by email to Elaine Stanford (elaine.stanford@hpa.org.uk) as a Microsoft Office Excel spreadsheet with column headings as above (an electronic template will be provided on request) or as a text file, as well as a printed list of samples or box plan to accompany all sample shipments. **It is important that no patient identifying data (names) are included.** Upon receipt of a shipment of samples into the SEU laboratory, the samples are checked against the sample information provided, and confirmation and/or notes of discrepancies are notified to the contributing laboratory by email or telephone. **Please include full contact details of the person(s) to whom queries can be directed.**

Payments to Contributing Laboratories.

Payment may be available for contributing laboratories, but can vary each year depending on budgetary allowances. Information will be sent to current contributing laboratories at the start of each financial year. New contributors can contact the SEU for up to date information.

Using the SEU archive

The SEU archive is freely available to be used for public health research and requests to use the archive should be submitted to the SEU at Cfl stating required sample numbers and demographic criteria. All requests are considered by Cfl, who then use the SEU database to select a subset of samples within the demographic criteria requested, if appropriate. A list of samples is then sent to the SEU laboratory and the samples are retrieved from the archive and sent to the organisation who is conducting the research.

The samples are packed securely in labelled boxes, kept frozen on dry ice and sent with a list and box plan, using a courier service. Samples must be stored at -65°C to -95°C by the testing laboratories, and freeze-thaw cycles must be kept to a minimum. Testing laboratories are requested to confirm receipt of samples by email or fax. Sample packaging is re-usable and should be retained for shipping samples back to the SEU laboratory when testing is complete.

Arrangement of shipping from and to the archive laboratory and associated costs are the responsibility of the requesting laboratory.

Returning samples to the SEU archive

When testing is complete, all SEU samples must be returned to the archive (even if insufficient volume to test). Samples must be returned frozen on dry ice with complete lists/box plans, preferably in the same packaging used to send them for testing. Confirmation of receipt of samples by the SEU laboratory will be faxed or emailed.

Arrangement of shipping from and to the archive laboratory and associated costs are the responsibility of the requesting laboratory.

Reporting test results

It is requested that the following basic results of tests on the serum archive are reported back to the SEU laboratory in electronic format, preferably a Microsoft Office Excel spreadsheet (an electronic template will be provided on request) or text file with the following headings:

SEU sample number/barcode
Analyte
Result
Units (i.e., \square g/mL)

Results will be entered into the SEU results database held on the shared network drive at Cfl.

Annex 2: laboratory methods

Microneutralisation

The MN will be performed in a 96-well format according to previously described protocols (Nicholson KG, Colegate AE, Podda A, Stephenson I, Wood J, Ypma E, *et al.* Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. *Lancet* 2001;**357**:1937–43) and standard operating procedures (SOPs) developed at the Respiratory Virus Unit (RVU).

Serum pretreatment

Elimination of complement (e.g. from fetal calf serum in culture medium) by incubation of the sera and appropriate quality control sera (provided and chosen according to test virus by RVU – usually serum of ferret, sheep or human, with/without neutralisation activity) at +56°C/30 minutes. This step will be performed simultaneously for all study samples and control sera.

Microneutralisation test

The analysis with the NIBRG122 virus will be performed. In the early stage of the outbreak the assumption can be made that most sera will show no evidence of antibody to the H1N1 virus. Therefore, sera could be screened at a limited dilution range or one dilution only (1:10). Only sera that inhibit virus growth at this dilution will be titrated further: A twofold dilution series will be set up for each of the samples and control sera. After addition of a pre-titrated virus (usually around $100 \times \text{TCID}_{50}$ per well or 0.1–1 virus particle per cell – input might vary according to the virus used in the assay) neutralisation will be performed by incubation of the virus–serum mixture at room temperature for 1 hour.

As discussed earlier, the dilution range for the study samples might vary depending on the development of the prevalence of antibody in the population over time. We will routinely perform a six-step dilution (covering titres 20–640), but will determine end point titres for each sample by further titrating those specimen that show titres of > 640.

After neutralisation, a suspension of Madin–Darby Canine Kidney (MDCK) cells will be added and the plates will be incubated for 16 hours at 37°C in a CO₂ incubator. The remaining infectivity of virus after neutralisation is determined in an enzyme immunoassay (EIA) format using a monoclonal antibody to detect expression of viral nucleoprotein. The amount of nucleoprotein

expression is determined photometrically [optical density (OD) 450] using a plate reader.

Reading

An OD reading for each dilution step for each sample will be used to calculate the titre. The titre will be reported as the reciprocal dilution at which 50% of the virus is neutralised (e.g. titre of 100). The microneutralisation analysis will be performed in duplicate (in separate runs on 2 days) for each sample.

The two titres for each sample must not differ by more than a twofold serial dilution. In cases, where samples do not fall within this limit, a third analysis is performed and the two closest titres (which must be within a twofold serial dilution) will be reported.

Haemagglutination inhibition

The principle of the HI test is based on the ability of specific anti-influenza antibodies to inhibit haemagglutination of red blood cells (RBCs) by influenza virus haemagglutinin (HA). The sera to be tested have to be previously treated to eliminate the non-specific inhibitors and the anti-species HAs. The experiment will be performed in accordance to protocols and SOPs established by the RVU.

Serum pretreatment

Elimination of non-specific inhibitors by incubation of the unknown serum samples and quality control sera (serum of ferret or human immunised with influenza virus) with neuraminidase [receptor-destroying enzyme (RDE) II: 18 hours/+36°C followed by heat inactivation 1 hour/+56°C]. Preparation will be performed simultaneously for serum obtained pre-vaccination and post vaccination.

Haemagglutination test

Similar to the microneutralisation we will screen sera in the early phase of the study in a limited dilution range (e.g. 8- to 32-fold dilution) using the NIBRG122 virus. Samples which show activity in this range will be further titrated: eight twofold dilutions, starting at a 1:8 dilution of serum sample [or quality control sera) are performed and incubated with the HA antigen suspension (previously titrated to adjust the dilution at eight haemagglutination units (HAUs)/25 µl]. The HA antigen is not added to the well dedicated to the RDE quality control.

The mixture is incubated for 1 hour at room temperature and 25 µl of the 0.5% RBC suspension

(turkey blood) are added. The reaction is left for 1 hour at room temperature before reading.

Reading

The serum titre is equal to the highest reciprocal dilution, which induces a complete inhibition of haemagglutination. The titre of each quality control serum is close to the previously assigned value (within one serial twofold dilution limits).

The RBC controls (RBC suspension without antigen) and the RDE controls do not produce any agglutination.

Each serum sample is titrated in duplicate and individual titres will be reported (two for each sample). These must not differ by more than a twofold serial dilution. In cases, where samples do not fall within this limit, a third analysis is performed and the two closest titres (which must be within a twofold serial dilution) will be reported.

UNCORRECTED PROOFS

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