On the use of hemagglutination-inhibition for influenza surveillance: Surveillance data are predictive of influenza vaccine effectiveness

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ABSTRACT

The hemagglutination-inhibition (HI) assay is the main tool used by epidemiologists to quantify antigenic differences between circulating influenza virus strains, with the goal of selecting suitable vaccine strains. However, such quantitative measures of antigenic difference were recently shown to have poor predictive accuracy with respect to influenza vaccine effectiveness (VE) in healthy adults. Here, we re-examine those results using a more rigorous criterion for predictive accuracy – considering only cases when the vaccine (V) and dominant (D) circulating strains are antigenically different – and greater numbers of HI titers. We find that the Archetti–Horsfall measure of antigenic difference, which is based on both the normalized HI titer (NHI) of D relative to antisera raised against V and the NHI of V relative to D, predicts VE very well ($R^2 = 0.62$, $p = 4.1 \times 10^{-3}$). In contrast, the predictive accuracies of the NHI of D relative to V alone ($R^2 = 0.01$), and two other measures of antigenic difference based on the amino acid sequence of influenza virus hemagglutinin ($R^2 = 0.03$ for both measures) are relatively poor. Furthermore, while VE in the elderly is generally high in cases when D and V are antigenically identical (VE = 35%, S.E. = 5%), in other cases VE appears to increase with the antigenic difference between D and V ($R^2 = 0.90$, $p = 2.5 \times 10^{-5}$). This paradoxical observation could reflect the confounding effects of prior immunity on estimates of VE in the elderly. Together, our results underscore the need for consistently accurate selection of suitable vaccine strains. We suggest directions for further studies aimed at improving vaccine-strain selection and present a large collection of HI titers that will be useful to such studies.

1. Introduction

For at least a century, influenza A and B viruses have caused regular epidemics and occasional pandemics that have a significant negative impact on public health. On an almost annual basis, antigenically novel "drift" strains of these viruses emerge (primarily by means of mutations to the viral hemagglutinin protein), infecting about 5–15% of the world's population and causing a large number of excess deaths [1,2]. Occasionally, antigenically novel "shift" strains of influenza A viruses capable of efficient human-to-human transmission emerge (via mutation of avian-adapted strains and/or exchange of viral segments among human- and avian-adapted strains), giving rise to pandemics characterized by significantly elevated rates of infection and of death [3]. The most deadly pandemic in recorded history is that of 1918, which killed tens of millions of individuals worldwide. The recent emergence of minimally transmission-competent and highly pathogenic shift

strains of the influenza H5N1 virus subtype has raised awareness concerning the danger of a future pandemic [4].

To help forecast the emergence of, and assist in developing vaccines against, both epidemic and pandemic influenza virus strains, the World Health Organization oversees an extensive influenza surveillance network comprising researchers from over 80 countries [5,6]. The main tool used by these researchers to quantify (antigenic) changes to the hemagglutinin of circulating influenza viruses that could have deleterious consequences for influenza vaccine effectiveness (VE) is the hemagglutination-inhibition (HI) assay [7]. However, measures of antigenic changes based on HI assay results (i.e., HI titers) were recently criticized for having limited predictive accuracy with respect to VE, and alternative measures were proposed [8–10]. This criticism calls for improved ways of interpreting HI titers, especially in light of the limited effectiveness of influenza vaccines administered in recent seasons (e.g., Refs. [11,12]). To shed light on the possible reasons for the reportedly poor predictive accuracy of HI titers, we describe briefly the conceptual basis of the HI assay.

The HI assay takes advantage of two facts about the biology of influenza viruses [13]: (i) the ability of an influenza virus to bind simultaneously to hemagglutinin receptors found on more than one red blood cell, causing the bound cells to become agglutinated, and

(ii) the fact that infection of an individual by a given influenza virus strain induces the production of antibodies with differing degrees of specificity for different strains. The degree to which antisera extracted from individuals infected by one strain (the "infecting" or "homologous" strain) prevent another strain (the "heterologous" strain) from agglutinating red blood cells – the heterologous HI titer – is used to measure the antigenic difference between the two virus strains. However, HI titers depend on several properties of virus strains that are not directly related to antigenic difference, including the capacity of strains to induce the production of antibodies [14]; the avidity of strains for red blood cells; and experimental conditions (i.e., temperature, pH, etc.). In addition, the concentrations of induced antibodies with specificity for heterologous strains are sometimes small, suggesting that fluctuations in those concentrations could be an important source of error in measured HI titers. We further address these issues in the next paper of this series.

In fact, HI titers measured in independent assays can be quite variable. For example, in three assays that compared the two influenza H3N2 virus strains A/Moscow/10/199 and A/Sydney/5/97, the normalized HI titers (that is, the ratio of the heterologous HI titer to the homologous HI titer) of A/Moscow/10/199 relative to antisera derived from ferrets infected by A/Sydney/5/97 were 1/2, 1, and 2, while the corresponding normalized HI titers for A/Sydney/5/97 were 1/64, 1/4, and 1/2 [15]. Due to the high intrinsic variability of HI titers, estimates of antigenic difference should be based on many replicated measurements of these titers, when possible. Here, we show that an estimate of antigenic difference based on multiple HI titers accurately predicts VE against influenza-like illness (ILI) in healthy adults, much more so than do other measures of antigenic difference that we tested. Furthermore, motivated by the need to improve access to HI titers we constructed a large database of HI titers, which we here make public. We hope that this database will serve as a catalyst for the establishment of other publicly accessible repositories of HI titers, which could dramatically improve the pace of research on the evolution of influenza viruses.

2. Materials and methods

2.1. HI data

The HI data used in this study were collected as part of a larger effort to make data on the antigenic evolution of influenza viruses more widely available. The data were assembled by searching relevant research articles and other documents published by the US Centers for Disease Control and Prevention and other collaborating centers of the World Health Organization's global influenza surveillance network. Presently, the data include 112 HI tables containing a total of 12,500 HI titers obtained using influenza A/H1N1, A/H3N2, A/H5N1, and B virus strains. The data in each HI table are in the form of an m by n matrix H, with m ($\geq n$) the number of strains found in the HI table, and n the number of antisera raised against a subset of those strains. Each entry H^{DV} of H represents the HI titer of strain D relative to antisera raised against strain V. The data in all HI tables were obtained by following the same general experimental guidelines [7]. The data accompany this paper as Supplemental Material.

2.2. Using HI data to quantify antigenic difference

The standard measure of the antigenic difference between virus strains D and V is the reciprocal of the normalized HI titer of D relative to antisera raised against strain V [7]: rNHT = H^{VV}/H^{DV}. Strain D is said to be antigenically drifted relative to V if rNHT is greater than or equal to 4 [7]. An alternative measure of the antigenic difference between D and V is the reciprocal of the Archetti–Horsfall mea-

sure [9,16,17]: rAHM = $\sqrt{H^{DD}H^{VV}/\left(H^{DV}H^{VD}\right)}$. We expect rAHM to be less dependent on non-antigenic factors than rNHT (see the next paper of this series); thus, rAHM may provide a more accurate measure of antigenic difference. Often, we have the four HI titers, H^{DD}, H^{DV}, H^{VD}, and H^{VV}, from several independent HI assays of the antigenic difference between D and V. In such cases, we use the means of rAHM and rNHT computed using data from each assay. Observe that rNHT is an asymmetric measure of antigenic difference, while rAHM is symmetric.

2.3. Using antigenic difference to predict vaccine effectiveness

Vaccine effectiveness was defined in the usual way: VE = $(1 − \text{RR}) \times 100$, where RR is the relative risk of contracting influenza-like illness (e.g., Refs. [11,18]). RR is given by $ad/(bc)$, where b (respectively d) denotes the total number of vaccinated (respectively unvaccinated) individuals taking part in a given VE study, and a (respectively c) denotes the number of vaccinated (respectively unvaccinated) individuals diagnosed with ILI. We quantify the predictive accuracy of the antigenic difference between virus strains D and V with respect to VE by means of weighted linear regression. The weights used in the regression model control for differences in the variability of VE estimates. The weight associated with a particular VE estimate is defined as the reciprocal of the square of the width, δ^{VE}, of the 95% confidence interval (CI) for that VE estimate. We compute CIs under the assumption that the number of vaccinated (respectively unvaccinated) individuals diagnosed with ILI follows a binomial distribution, with parameters $(a+c)$ and $a/(a+c)$ (respectively $(a+c)$ and $c/(a+c)$). The lower and upper 95% confidence limits for VE are computed using the method of Clopper and Pearson (e.g., see Ref. [19]). When VE is calculated as the average of m different VE estimates, the width of its CI is given by $1/m\sqrt{\sum_{i=1}^{m}\left(\delta_{VE_i}\right)^2}$, where δ_{VE_i} denotes the width of the CI for the ith VE estimate.

It is important to note that in addition to the antigenic difference between D and V, VE depends on a range of other factors, including the age and immune status of vaccinated individuals and the presence in the population under study of other causative agents of ILI [20]. These factors are often not controlled for (e.g., Ref. [11]). Therefore, the correlation between antigenic difference and VE is not expected to be 100%. Conversely, a high correlation between antigenic difference and VE, although highly suggestive of a causal relationship, should be interpreted with caution. In addition, note that using estimates of VE against virologically confirmed influenza (rather than ILI) could lead to improvement in the calculated predictive accuracy of antigenic difference with respect to VE. However, we only have access to a limited number of such VE estimates (see Supplemental Material).

3. Results

We quantified the predictive accuracies (with respect to VE against ILI) of two HI-based measures of the antigenic difference between virus strains D and V, namely (i) the reciprocal of the normalized HI titer of D relative to antisera raised against V (denoted by rNHT), and (ii) the reciprocal of the geometric mean of both the normalized HI titer of D relative to V and the normalized HI titer of V relative to D (denoted by rAHM). For comparison, we also quantified the predictive accuracies of two measures of antigenic difference based on the amino acid sequence of influenza virus hemagglutinin (HA); one of these measures (denoted by p_{sequence}) is the Hamming distance between the HA amino acid sequences of D and V normalized by the length of HA, while the other measure (denoted by p_{epitope}) is the maximum of the Hamming distances

between corresponding HA epitopes normalized by the total number of amino acids found in each epitope [9,10]. The former measure of antigenic difference is essentially a lower bound on the normalized phylogenetic-tree distance between the HA amino acid sequences of D and V, while the latter measure is thought [9,10] to quantify changes to the "dominant" epitope of HA.

We focused on predicting VE in healthy adult vaccines, since VE in these individuals is more likely to be indicative of the antigenic difference between D and V than VE in the elderly, for whom there is a preponderance of confounding factors (e.g., Ref. [21]). We compiled a total of 22 VE estimates and the corresponding antigenic difference between D and V, for all previous influenza seasons in which (i) virus strains belonging to the H3N2 influenza virus subtype were dominant, and (ii) the relevant data are publicly available (see Table 1).[2] Eleven of the 22 estimates correspond to cases when D and V are antigenically identical (i.e., both rAHM and rNHT equal 1, by definition). In these 11 cases, VE is consistently high (43%, S.E. = 5%; see Table 1). Note that the predictive accuracy of a given measure of antigenic difference can only be tested rigorously in cases when D and V are actually antigenically different. Considering only such cases, we find, using a weighted linear regression of VE against antigenic difference (see Section 2), that rAHM has good predictive accuracy with respect to VE ($R^2 = 0.62$, $p = 4.1 \times 10^{-3}$), while rNHT ($R^2 = 0.01$), $p_{epitope}$ ($R^2 = 0.03$), and $p_{sequence}$ ($R^2 = 0.03$) all have relatively poor predictive accuracies (see Table 1).

The results obtained using rAHM suggest the existence of a maximum antigenic difference between D and V (about 5) above which vaccines are likely to be ineffective: vaccines were effective (defined as a positive mean value of VE) in 16 out of 17 cases when the computed antigenic difference between D and V was less than 5, but were ineffective in 4 out of 5 cases in which the computed antigenic difference was greater than or equal to 5. This prediction is consistent with the antigenic difference threshold of 4, which is currently in use by epidemiologists [7]. Note that in the elderly, VE is generally high (35%, S.E. = 5%) when D and V are antigenically identical (see Table S1), although it is not as high as in healthy adults. In contrast to the situation in healthy adults, there is a statistically significant *positive* correlation between VE in the elderly and the antigenic difference between D and V, as measured using both rNHT ($R^2 = 0.53$, $p = 0.02$) and rAHM ($R^2 = 0.90$, $p = 2.5 \times 10^{-5}$) (see Table 2). This unexpected positive correlation could be due to, among other things, the confounding effects of chronic immunization of the elderly on estimates of VE (e.g., Ref. [21]).

In particular, previous empirical and theoretical results suggest that the immunogenicity (and hence the effectiveness) of influenza vaccines could decrease as the antigenic difference between V and the vaccine strain used in the previous influenza season decreases [21,29]. Consistent with those results, we find that there is a statistically significant ($p = 0.02$) interaction between the rAHM of the antigenic difference between D and V and the rAHM of the antigenic difference between V and the H3N2 vaccine strain used in the previous influenza season (see Table 2); VE is predicted to increase with the product of both antigenic differences. In addition, the antigenic difference between D and V is predicted to have a negative effect on VE, but this effect is not statistically significant (see Table 2). Importantly, the regression model that accounts for the abovementioned interaction effect explains a greater amount of the variation in VE than the model that only accounts for the antigenic difference between D and V ($R^2 = 0.98$ versus 0.90), and it

also possesses a smaller Akaike information measure (88.27 versus 97.67), suggesting that it is indeed a better model (see Table 2).

4. Discussion

In this paper, we showed that vaccine effectiveness against influenza-like illness in healthy adults is high in seasons when vaccine (V) and dominant (D) circulating influenza virus strains are antigenically identical. In other seasons, VE is predicted accurately by the reciprocal Archetti–Horsfall measure (rAHM) of the antigenic difference between D and V [9,16,17], which is given by the reciprocal of the geometric mean of both the normalized hemagglutination-inhibition (HI) titer of D relative to antisera raised against V and the normalized HI titer of V relative to D. In contrast, measures of antigenic difference based on (i) the normalized HI titer of D relative to V alone, (ii) amino acid differences between the entire hemagglutinin sequences of D and V, and (iii) amino acid differences between "dominant" antibody-binding sites of the hemagglutinin sequences of D and V [9,10] were found to have relatively poor predictive accuracies with respect to VE. The superior predictive accuracy of rAHM could be explained by the fact that it has less dependence on non-antigenic factors than the other considered measures of antigenic difference (see the next paper of this series). Also, the considered sequence-based measures of antigenic difference provide only indirect information about the antigenic properties of influenza virus strains that determine the outcome of their interactions with vaccine-induced antibodies.[3] The above results address previous criticisms of HI titers [8–10], and they suggest that rAHM should be used more often when selecting suitable vaccine strains.

Furthermore, we found that in seasons when D and V are antigenically identical, vaccines are generally effective in preventing influenza-like illness in the elderly, but in other seasons there is a significant *positive* correlation between VE and the rAHM of the antigenic difference between the two strains. This unexpected observation could be explained by a host of factors known to confound estimates of VE in the elderly, including impaired immune function and chronic immunization [20,21]. Indeed, we found that the correlation between VE and the antigenic difference between D and V becomes negative when the antigenic difference between D and the previous season's vaccine strain was accounted for, although it was not statistically significant. Importantly, we found a statistically significant interaction between the antigenic difference between D and V and the antigenic difference between V and the previous season's vaccine strain; VE was predicted to increase with the product of both antigenic differences. These results are consistent with the antigenic distance hypothesis [21,29], which postulates that prior immunization with a vaccine strain that has low antigenic difference from V could lead to low immunogenicity and, hence, effectiveness of vaccines containing V. Further studies of the mechanistic basis of these results could be of value. In addition, it would be helpful to investigate the relationship between antigenic difference and (more accurate) estimates of VE against virologically confirmed influenza, once a sufficient amount of those VE estimates becomes available. Overall, our results highlight the importance of consistently selecting the most suitable influenza vaccine strains.

Influenza vaccine-strain selection is currently very challenging, as evinced by the antigenic mismatches between vaccine and dominant strains observed recently [54,57–59]. These mismatches

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[2] We found another VE estimate from the 1968–1969 influenza season [63,64], but the rNHT of the corresponding dominant strain (Hong Kong/8/68) relative to the vaccine strain (Aichi/2/68) was 0.4 [65], which is lower than the expected value for two strains that are antigenically identical. This data point was therefore considered to be an outlier, and it was not included in our calculations.

[3] The much lower predictive accuracies of the considered sequence-based measures of antigenic difference compared to their previously reported accuracies could be explained, in part, by the fact that we used a more rigorous criterion for predictive accuracy than was used in previous studies [9,10].

Table 1
Estimates of antigenic difference and of VE in healthy adults.

Influenza season	Vaccine strain	Dominant strain	Antigenic difference			VE data	
			NHT/AHM	$p_{epitope}$	$p_{sequence}$	a/b/c/d[*]	% VE (width)[**]
1968	Hong Kong/8/68	Hong Kong/8/68	1		0	52/465/33/118; 92/479/33/118 [22]	45.6 (35.6)
–1969	Aichi/2/68	Aichi/2/68	1	0	0	11/195/10/49;14/190/10/49 [23]	68.2 (49.0)
1972–1973	Hong Kong/1/68[***] (AF201874)	England/42/72 (ABQ58930)	4.76/3.85 [24–27]	.263	.055	590/58370/713/59926 [28]	15.1 (18.9)
1973–1974	England/42/72	PortChalmers/1/73	16.67/2.78 [27,29–31]	.143	.018	532/59784/604/60349 [28]	11.1 (21.1)
1975–1976	Portchalmers/1/73	Victoria/3/75	7.14/7.14 [27,30,31]	.190	.055	680/61811/653/61049 [28]	−2.9 (22.5)
1980–1981	Bangkok/1/79	Bangkok/1/79	1	0	0	1/37/1/20 [32]	46.0 (4242.4)
1984–1985	Philippines/2/82	Mississippi/1/85	4.76/5.00 [33–35]	.190	.033	110/456/55/241 [36]	−5.7 (73.1)
1985–1986	Philippines/2/82	Mississippi/1/85	4.76/5.00 [33–35]	.190	.033	80/577/36/253; 21/91/19/88 [37,38]	−2.2 (161.0)
1987–1988	Leningrad/360/86	Shanghai/11/87	2.00/2.00 [34]	.143	.024	106/789/28/145; 251/1060/262/1064 [37,39]	17.1 (46.9)
1989–1990	Shanghai/11/87	England/427/88	3.33/2.38 [40,41]	.105	.021	154/1016/146/1016 [39]	−5.5 (49.6)
1992–1993	Beijing/32/92	Beijing/32/92	1	0	0	26/131/64/131 [42]	59.4 (40.3)
1993–1994	Beijing/32/92	Beijing/32/92	1	0	0	4/26/2/8 [43]	38.5 (671.5)
1994–1995	Shangdong/9/93	Shangdong/9/93	1	0	0	105/100/140/100 [44]	25.0 (39.6)
1995–1996	Johannesburg/33/94	Johannesburg/33/94	1	0	0	31/297/67/384; 26/387/32/268 [45]	42.0 (42.7)
1996	Wuhan/359/95	Nanchang/933/95	1.33/1.47 [46–48]	.095	.006	19/273/286/2978 [49]	27.1 (72.6)
–1997	Nanchang/933/95	Nanchang/933/95	1	0	0	86/294/98/299 [50]	10.8 (54.5)
1997–1998	Nanchang/933/95 (AF008725)	Sydney/5/97 (AJ311466)	14.29/14.29 [47,48]	.227	.040	161/576/132/554 [11]	−17.3 (56.2)
1998–1999	Sydney/5/97	Sydney/5/97	1	0	0	82/582/128/596 [11]	34.4 (38.1)
1999–2000	Sydney/5/97	Sydney/5/97	1	0	0	22/341/37/325 [51]	43.3 (66.8)
2001–2002	Panama/2007/99	Panama/2007/99	1	0	0	8/968/18/982 [52]	54.9 (92.0)
2003–2004	Panama/2007/99	Fujian/411/2002	2.63/7.14 [53,54]	.143	.040	149/1000/59/393 [55]	.8 (63.7)
2004–2005	A/Wyoming/3/2003 (AY531033)	A/California/07/2004 (EF473342)	7.69/4.76 [56]	.273	.030	50/367/6/40 [12]	9.2 (220.3)

Shown are VE estimates from influenza seasons in which H3N2 virus strains were dominant. When VE estimates where obtained from multiple VE studies, the average of those estimates was used. Antigenic difference was quantified using two measures based on hemagglutination-inhibition titers (rAHM and rNHT; see Section 2); a measure based on the entire amino acid sequence of hemagglutinin ($p_{sequence}$); and a measure based on hemagglutinin epitopes ($p_{epitope}$) [9,10]. See Table S3 for details on the amino acid positions found in these epitopes. Except when noted otherwise, estimates of $p_{epitope}$ and $p_{sequence}$ were taken from [9]. In cases when $p_{epitope}$ and $p_{sequence}$ were computed by us the Genbank IDs of the sequences used are given. For 2003–2004 the more accurate [55] VE estimate, which does not take into account individuals vaccinated <2 weeks prior to developing symptomatic influenza, was used. The vaccine and dominant strains for a given season were taken from publications reporting VE estimates for that season, if the strains and/or their antigenic similarity were indicated. Otherwise, the vaccine and dominant strains reported by the World Health Organization were used.

[*] a – number of vaccinated individuals diagnosed with ILI; b – total number of vaccinated individuals; c – number of unvaccinated individuals diagnosed with ILI; d – total number of unvaccinated individuals.

[**] The width of the confidence interval associated with each VE estimate was calculated as described in Section 2.

[***] Antigenic data for the correct vaccine strain (A/Hong Kong/x-31/68) were not available, so the vaccine strain (A/Hong Kong/1/68) recommended by the World Health Organization was used instead.

Table 2
Fits of four linear models to estimates of VE in the elderly.

Regression coefficient	Model			
	rNHT	rAHM		
	Diff1	Diff1	Diff1 + Diff2	Diff1 + Diff2 + Diff1 × Diff2
Diff1	3.1 (.02)	2.4 (2.5e − 5)	2.6 (2.0e − 5)	−5.8 (.08)
Diff2	–	–	9.4 (.10)	−42.6 (.05)
Diff1 × Diff2	–	–	–	8.4 (.02)
R^2	.53	.90	.94	.98
Akaike information criterion	113.5	97.7	95.4	88.3

The models were fit using a weighted linear regression (see Section 2), with VE the response variable and both the antigenic difference between vaccine and dominant strains (Diff1) and the antigenic difference between the current vaccine strain and the vaccine strain used in the preceding influenza season (Diff2) serving as predictor variables. The regression coefficients associated with Diff1, Diff2, and the interaction between Diff1 and Diff2 are given. Also given (in brackets) are the *p*-values indicating whether a given predictor variable explains a significantly greater amount of variation in VE than the intercept term of the corresponding regression model. Estimates of VE, Diff1, and the width of the 95% confidence interval for VE (used as weights in the regression model) are given in Table S1, while estimates of Diff2 are given in Table S2. Antigenic differences were computed using both the reciprocal normalized HI titer (rNHT) and the reciprocal Archetti–Horsfall measure (rAHM) (see Section 2).

can have deleterious consequences for VE, especially in the elderly. Also, in the unfortunate event that avian influenza virus strains (e.g., H5N1 viruses) acquire the ability to spread efficiently among humans, an antigenic mismatch between those strains and the recommended "pre-pandemic" vaccine strain [60] could have very serious consequences. When selecting vaccine strains, researchers rely on epidemiologic, genetic and, most importantly, antigenic data (primarily from HI assays) collected by sampling circulating strains [5]. The development and application of accurate sequence-based measures of antigenic difference to data from ongoing influenza virus sequencing projects (e.g., Ref. [61]) could allow sequence data to be used to complement antigenic data. This could help to increase the diversity of sampled strains that are phenotyped antigenically, as well as the probability of identifying suitable vaccine strains. In addition, the collection of better-quality antigenic data (e.g., corresponding to the means of replicate HI titers) combined with the development of quantitative methods for increasing the signal-to-noise ratio of these data (e.g., Ref. [62]) could improve the selection of suitable vaccine strains and allow greater understanding of influenza virus evolution. In order to help spur the development of such quantitative methods and encourage studies on other aspects of influenza virus evolution, we presented a large collection of HI titers culled from various sources.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2009.02.047.

References

[1] Dushoff J, Plotkin JB, Viboud C, Earn DJD, Simonsen L. Mortality due to influenza in the United States – an annualized regression approach using multiple-cause mortality data. Am J Epidemiol 2006;163:181–7.
[2] World Health Organization. Influenza Factsheet. Available at: http://www.who.int/mediacentre/factsheets/fs211/en/ [cited September 15, 2007].
[3] Subbarao K, Swayne DE, Olsen CW. Epidemiology and control of human and animal influenza. In: Kawaoka Y, editor. Influenza virology: current topics. Caister Academic; 2006.
[4] Taubenberger JK, Morens DM, Fauci AS. The next influenza pandemic – can it be predicted? JAMA 2007;297:2025–7.
[5] Cox NJ. Expanding the worldwide influenza surveillance system and improving the selection of strains for vaccines. In: Layne SP, editor. Firepower in the lab: automation in the fight against infectious diseases and bioterrorism. Washington: Joseph Henry Press; 2001. p. 47–53.
[6] Layne SR. Human influenza surveillance: the demand to expand. Emerg Infect Dis 2006;12:562–8.
[7] Webster R, Cox N, Stohr K. WHO Manual on Animal Influenza Diagnosis and Surveillance. WHO/CDS/CSR/NCS/2002.5 Rev.1, 2002.
[8] Deem MW. Entropy disease and new opportunities for chemical engineering research. AIChE J 2005;51:3086–90.
[9] Gupta V, Earl DJ, Deem MW. Quantifying influenza vaccine efficacy and antigenic distance. Vaccine 2006;24:3881–8.
[10] Munoz ET, Deem MW. Epitope analysis for influenza vaccine design. Vaccine 2005;23:1144–8.
[11] Bridges CB, Thompson WW, Meltzer MI, Reeve GR, Talamonti WJ, Cox NJ, et al. Effectiveness and cost-benefit of influenza vaccination of healthy working adults – a randomized controlled trial. JAMA 2000;284:1655–63.
[12] Chan AL, Shie HJ, Lee YJ, Lin SJ. The evaluation of free influenza vaccination in health care workers in a medical center in Taiwan. Pharm World Sci 2008;30:39–43.
[13] Hirst GK. The agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. Science 1941;94:22–3.
[14] Salk JE. A critique of serologic methods for the study of influenza viruses. Arch Virol 1951;4:476–84.
[15] Centers for Disease Control and Prevention. Information for the vaccines and related biological products advisory committee, CBER FDA WHO Collaborating Center for surveillance epidemiology and control of influenza; 2003.
[16] Lee MS, Chen JS. Predicting antigenic variants of influenza A/H3N2 viruses. Emerg Infect Dis 2004;10:1385–90.
[17] Archetti I, Horsfall FL. Persistent antigenic variation of influenza A viruses after incomplete neutralization *in ovo* with heterologous immune serum. J Exp Med 1950;92:441–62.
[18] Halloran ME, Longini IM, Struchiner CJ. Design and interpretation of vaccine field studies. Epidemiol Rev 1999;21:73–88.
[19] Prince RM, Bonett DG. Estimating the ratio of two Poisson rates. Comp Stat Dat Anal 2000;34:345–56.
[20] Centers for Disease Control and Prevention. Flu vaccine effectiveness: questions and answers for health professionals. Available at: http://www.cdc.gov/flu/professionals/vaccination/effectivenessqa.htm 2006 [cited September 15, 2007].
[21] Goronzy JJ, Fulbright JW, Crowson CS, Poland GA, O'Fallon WM, Weyand CM. Value of immunological markers in predicting responsiveness to influenza vaccination in elderly individuals. J Virol 2001;75:12182–7.
[22] Waldman RH, Bond LP, Levitt EC, Hartwig EC, Prather RL, Baratta JS, et al. An evaluation of influenza immunization: influence of route of administration and vaccine strain. Bull World Health Organ 1969;41:543–8.
[23] Waldman RH, Coggins WJ. Influenza immunization: field trial on a university campus. J Infect Dis 1972;126:242–8.
[24] World Health Organization Weekly Epidemiological Record 1975;50:45–52.
[25] World Health Organization Weekly Epidemiological Record 1974;49:41–8.
[26] World Health Organization Weekly Epidemiological Record 1972;47:73–84.
[27] de Jong JC, Smith DJ, Lapedes AS, Donatelli I, Campitelli L, Barigazzi G, et al. Antigenic and genetic evolution of swine influenza A (H3N2) viruses in Europe. J Virol 2007;81:4315–22.
[28] Smith JW, Pollard R. Vaccination against influenza: a five-year study in the post office. J Hyg 1979;83:157–70.
[29] Smith DJ, Forrest S, Ackley DH, Perelson AS. Variable efficacy of repeated annual influenza vaccination. Proc Natl Acad Sci USA 1999;96:14001–6.
[30] Both GW, Sleigh MJ, Cox NJ, Kendal AP. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. J Virol 1983;48:52–60.
[31] Underwood PA. Serology and cross-reactions among the H3 antigens of influenza viruses. Infect Immun 1980;27:397–404.

[32] Tannock GA, Bryce DA, Hensley DJ, Saunders NA, Gillett RS, Kennedy WS. Responses to one or two doses of a deoxycholate subunit influenza vaccine in a primed population. Vaccine 1984;2:100–6.

[33] Hay AJ, Gregory V, Douglas AR, Lin YP. The evolution of human influenza viruses. Phil Trans R Soc Lond 2001;356:1861–70.

[34] World Health Organization Weekly Epidemiological Record 1988;63:57–9.

[35] World Health Organization Weekly Epidemiological Record 1986;61:61–8.

[36] Keitel WA, Cate TR, Couch RB. Efficacy of sequential annual vaccination with inactivated influenza virus vaccine. Am J Epidemiol 1988;127:353–64.

[37] Keitel WA, Cate TR, Couch RB, Huggins LL, Hess KR. Efficacy of repeated annual immunization with inactivated influenza virus vaccines over a five year period. Vaccine 1997;15:1114–22.

[38] Weingarten S, Staniloff H, Ault M, Miles P, Bamberger M, Meyer RD. Do hospital employees benefit from the influenza vaccine? J Gen Intern Med 1988;3:32–7.

[39] Edwards KM, Dupont WD, Westrich MK, Plummer Jr WD, Palmer PS, Wright PF. A randomized controlled trial of cold-adapted and inactivated vaccines for the prevention of influenza A disease. J Infect Dis 1994;169:68–76.

[40] Morbidity and Mortality Weekly Report 1990;39:293–96.

[41] World Health Organization Weekly Epidemiological Record 1990;65:53–60.

[42] Campbell DS, Rumley MH. Cost-effectiveness of the influenza vaccine in a healthy, working-age population. J Occup Environ Med 1997;39:408–14.

[43] Powers DC, Smith GE, Anderson EL, Kennedy DJ, Hackett CS, Wilkinson BE, et al. Influenza A virus vaccine containing purified recombinant H3 hemagglutinin are well tolerated and induce protective immune responses in healthy adults. J Infect Dis 1995;171:1595–9.

[44] Nichol KL, Lind A, Margolis KL, Murdoch M, McFadden R, Hauge M, et al. The effectiveness of vaccination against influenza in healthy, working adults. N Engl J Med 1995;333:889–93.

[45] Grotto I, Mandel Y, Green MS, Varsano N, Gdalevich M, Ashkenazi I, et al. Influenza vaccine efficacy in young, healthy adults. Clin Infect Dis 1998;26:913–7.

[46] Morbidity and Mortality Weekly Report 1998;47:196–200.

[47] World Health Organization Weekly Epidemiological Record 1998;73:56–63.

[48] Coiras MT, Aguilar JC, Galiano M, Carlos S, Gregory V, Lin YP, et al. Rapid molecular analysis of the haemagglutinin gene of human influenza A H3N2 viruses isolated in Spain from 1996 to 2000. Arch Virol 2001;146:2133–47.

[49] Millot JL, Aymard M, Bardol A. Reduced efficiency of influenza vaccine in prevention of influenza-like illness in working adults: a 7 month prospective survey in EDF Gaz de France employees, in Rhone-Alpes 1996–1997. Occup Med Oxford 2002;52:281–92.

[50] Mixeu M, Vespa CNR, Forleo-Neto E, Toniolo-Neto J, Alves PM. Impact of influenza vaccination on civilian aircrew illness and absenteeism. Aviat Space Environ Med 2002;73:876–80.

[51] Lester RT, McGeer A, Tomlinson G, Detsky AS. Use of, effectiveness of, attitudes regarding influenza vaccine among house staff. Infect Control Hosp Epidemiol 2003;24:839–44.

[52] Kawai N, Ikematsu H, Iwaki N, Satoh I, Kawashima T, Tsuchimoto T, et al. A prospective, internet-based study of the effectiveness and safety of influenza vaccination in the 2002 influenza season. Vaccine 2003;21:4507–13.

[53] World Health Organization Weekly Epidemiological Record 2003;78:57–64.

[54] Morbidity and Mortality Weekly Report 2004;53:8–11.

[55] Morbidity and Mortality Weekly Report 2004;53:547–52.

[56] Centers for Disease Control and Prevention. Information for the vaccines and related biological products advisory committee, CBER FDA WHO Collaborating Center for surveillance epidemiology and control of influenza; 2005.

[57] Morbidity and Mortality Weekly Report 2005;54:631–34.

[58] Morbidity and Mortality Weekly Report 2007;56:1001–4.

[59] Centers for Disease Control and Prevention: fluview: 2007–2008 Influenza Season Week 9 ending March 1 2008. Available at: http://www.cdc.gov/flu/weekly/.

[60] World Health Organization. Antigenic and genetic characteristics of H5N1 viruses and candidate H5N1 vaccine viruses developed for potential use in human vaccines. Available at: http://www.who.int/csr/disease/avian_influenza/guidelines/200809_H5VaccineVirusUpdate.pdf [cited September 2008].

[61] The Influenza Genome Sequencing Project, http://www3.niaid.nih.gov/research/resources/mscs/Influenza/.

[62] Smith DJ, Lapedes AS, de Jong JC, Bestebroer TM, Rimmelzwaan GF, Osterhaus ADME, et al. Mapping the antigenic and genetic evolution of influenza virus. Science 2004;305:371–6.

[63] Eddy TS, Davies NA. The effect of vaccine on a closed epidemic of Hong Kong influenza. S Afr Med J 1970;44:214–6.

[64] Mogabgab WJ, Leiderma E. Immunogenicity of 1967 polyvalent and 1968 Hong Kong influenza vaccines. JAMA 1970;211:1672–6.

[65] Dowdle WR, Coleman MT, Hall EC, Knez V. Properties of the Hong Kong influenza virus 2 Antigenic relationship of the Hong Kong virus haemagglutinin to that of other human influenza A viruses. Bull World Health Organ 1969;41:419–24.