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Review Article

The first influenza pandemic of the new millennium

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In the spring of 2009, a novel influenza A virus of the H1N1 subtype emerged that transmitted efficiently among humans; by June of 2009, the outbreak reached pandemic status. The pandemic virus possesses six viral RNA segments from so-called triple reassortant swine viruses that emerged in North American pig populations in the late 1990s and two viral RNA segments from Eurasian avian-like swine influenza viruses. Most human infections with the virus have been mild; however, severe and fatal infections occurred among certain risk groups, but also among those without any known risk factors. Here, we summarize the evolutionary, epidemiological, clinical, and molecular findings on the pandemic virus. We also discuss the arsenal of antiviral compounds and vaccines available to prevent and treat infections with the virus.

Keywords Antiviral compounds, H1N1, influenza virus, pandemic, reassortment, vaccines.

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Introduction

Influenza A viruses (family *Orthomyxoviridae*) cause respiratory infections with mild to severe symptoms. Based on the antigenicity of their surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), they are subdivided into 16 HA subtypes (H1–16) and nine NA subtypes (N1–9) (reviewed in Refs.[1,2]). Influenza pandemics (i.e., outbreaks on a global scale with sustained human-to-human transmission) are caused by viruses to which people have little or no immunity. In 1918, an influenza virus of the H1N1 subtype caused the 'Spanish influenza' that killed an estimated 20–50 million people worldwide. Descendants of this virus circulated until 1957, when they were replaced with an avian/human reassortant of the H2N2 subtype that caused a new pandemic ('Asian influenza'). In 1968, another pandemic was caused by an avian/human reassortant virus of the H3N2 subtype ('Hong Kong influenza'); this virus replaced the previously circulating viruses of the H2N2 subtype. In 1977, an H1N1 virus reemerged that closely resembled the viruses isolated in the mid-1950s. The reemergence of this virus did not cause a full-scale pandemic, likely because of pre-existing immunity to viruses of this subtype in individuals born before 1957. Since 1977, viruses of the H3N2 and H1N1 subtypes have cocirculated in humans. In 2009, a novel influenza virus of the H1N1 subtype caused the most recent pandemic (reviewed in Refs.[3–6]). Notably, this most recent pandemic did not involve a change in the HA subtype. Here, we summarize current knowledge about this novel H1N1 virus, referred to as 2009 H1N1 virus.

The emergence of a new pandemic virus

In late March of 2009, two children in Southern California experienced an influenza-like illness,[7,8] and soon after, similar cases were reported in Mexico.[9–12] On April 14, the CDC identified the causative agents as swine influenza H1N1 influenza viruses.[7] Ten days later, the CDC reported that the Mexican and Californian cases were caused by a genetically similar virus.[11,13] In response to the efficient spread of the novel virus, the World Health Organization (WHO) declared a 'public health emergency of international concern' on April 25.[11] Within 2 months, sustained human-to-human transmission in several countries on different continents was reported, prompting the WHO to announce the highest alert level (phase 6, pandemic) on June 12, 2009.[14,15]

The 2009 H1N1 virus swiftly spread around the world and dominated the circulating, seasonal influenza

viruses – from August 30, 2009 through March 27, 2010, 99·4% of subtyped influenza A viruses were novel 2009 H1N1 viruses.[16] The United States experienced a first wave in late spring of 2009, followed by a second wave that peaked in mid-October. As of February 13, 2010, the CDC estimated 42–86 million cases in the United States (mid-level: 59 million cases) (http://www.cdc.gov/H1N1flu/estimates/April_February_13.htm), with 188 000–389 000 hospitalizations (mid-level: 265 000) and an estimated 8520–17 620 deaths (mid-level: 12 000). This number is still less than that of seasonal influenza-related deaths, which is estimated to be 30 000 per year in the United States. The estimated case-fatality rate ranges from 0·2% to 1·23%[17–19]; however, significant differences between age groups were apparent.[17–23] Children experienced a high attack rate, but a low case-fatality rate; by contrast, the elderly experienced a low attack rate but a high case-fatality rate.[17–23] The relatively low attack rates among the elderly suggest some pre-existing immunity in this age group (see 'HA Antigenicity'). In terms of absolute numbers, the age group of 5–59 years was most affected, accounting for close to 90% of deaths and about 70% of cases of severe pneumonia, compared with 17% and 32% for recent outbreaks of seasonal influenza.[19,20]

Clinical manifestations and risk factors

Most cases of 2009 H1N1 infection presented as mild upper respiratory tract illness. The most common symptoms were fever, cough, sore throat, shortness of breath, headache, and rhinorrhea.[8,19,22,24–29] Gastrointestinal symptoms including diarrhea and vomiting (which are unusual with seasonal influenza infections) have been reported and seem to be associated primarily with mild cases. In some instances, the disease progressed to primary and secondary pneumonia that resulted in multi-organ failure, respiratory failure, acute respiratory distress symptoms, and sometimes death.[8,19,22,24–30] Pathological findings included diffuse alveolar damage, hemorrhagic interstitial pneumonitis, and peribronchiolar and perivascular lymphocytic infiltrates,[22,27,31] reminiscent of infections with highly pathogenic avian H5N1 influenza viruses.[32–34] These findings indicated that 2009 H1N1 viruses cause lower respiratory tract infections, in contrast to seasonal influenza viruses, which typically affect the upper respiratory tract. Secondary bacterial infections have been reported in a substantial number of cases[22,27,35–37] and may exacerbate the course of the disease.

Certain factors significantly increased the risk of severe disease.[8,14,19,22,24,25,27,37–41] These factors include chronic conditions such as respiratory diseases (notably asthma), autoimmune diseases, cardiovascular diseases, diabetes, and obesity. In addition, pregnancy, particularly in the last trimester, significantly increased the risk for complications.[8,14,19,27,38–41] Increased risk of severe disease was also reported for indigenous people, probably because of overall lower health status and/or limited access to health care.[39,42] In general, early treatment with antiviral compounds (see 'Antiviral Compounds') appeared to be critical in mitigating the risks associated with 2009 H1N1 infections.

Pigs – a 'mixing vessel' of influenza A viruses?

Sequence and evolutionary analyses of 2009 pandemic isolates revealed an H1N1 virus that resembles viruses isolated from pigs.[7,8,43–45] Influenza A viruses are known to replicate in pigs, where they typically cause mild respiratory symptoms (reviewed in Ref.[46]). While most avian influenza viruses are restricted in their ability to replicate in humans, and *vice versa*, pigs can be infected by both virus types[47] and were therefore proposed as a 'mixing vessel'[48] in which human and avian influenza viruses may reassort, potentially leading to viruses with novel gene combinations against which humans are immunologically naïve. In fact, the pandemic viruses of 1957 and 1968 were human/avian reassortants, but it is not known whether the reassortment occurred in pigs. In 1976, a swine influenza virus of the H1N1 subtype caused an outbreak among soldiers at Fort Dix, New Jersey.[49–51] Since then, multiple human infections with swine influenza viruses have been reported[26,52–56]; however, these infections did not cause widespread outbreaks. The finding of a pandemic influenza virus that likely originated from pigs was therefore of great interest.

In North American pig populations, so-called classical H1N1 influenza viruses (descendants of the 1918 pandemic virus) circulated until 1997/1998, when H3N2 triple human/avian/swine reassortant viruses emerged; these viruses have now spread throughout the swine populations (reviewed in Ref.[46]) (Figure 1). These viruses possess HA, NA, and PB1 polymerase genes of human virus origin, PB2 and PA polymerase genes of avian virus origin, and matrix (M) and non-structural (NS) genes of classical H1N1 swine virus origin. Through further reassortment, the H3N2 triple reassortant viruses acquired HA, or HA and NA genes from classical H1N1 viruses, resulting in triple reassortant H1N2 and H1N1 viruses. The 2009 H1N1 viruses possess six genomic RNA segments from triple reassortant swine viruses and acquired their HA and M segments from a Eurasian avian-like swine virus[7,8,43–45] (Figure 1).

The reassortment event(s) that led to the generation of the novel viruses likely occurred several months to years before the pandemic outbreak, and the novel virus may have circulated in humans for several months before its detection.[17,45,57–60] This would explain why no (major) influenza virus activity was reported in Mexican pigs at the

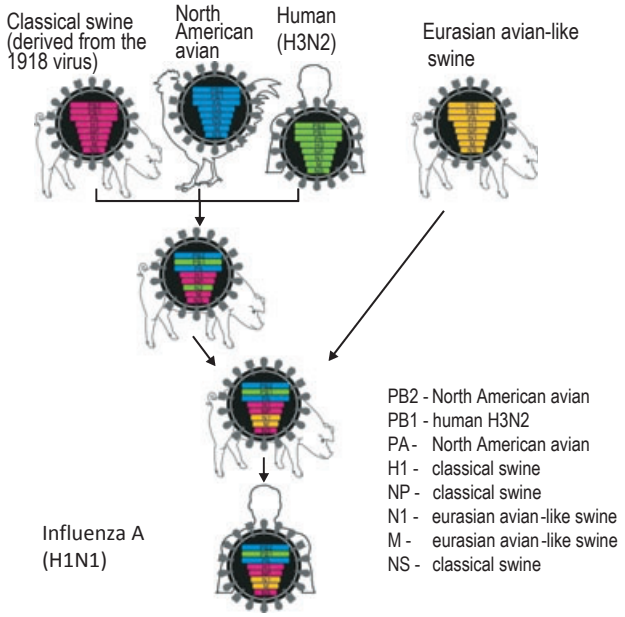

Figure 1. Genesis of pandemic 2009 H1N1 viruses. The NA and M genes were derived from a Eurasian avian-like swine virus (yellow). The remaining six genes were derived from triple resssortant swine viruses that possessed genes originating from classical H1N1 swine (red), North American avian (blue) and human H3N2 (green) viruses. Reprinted by permission from Macmillan Publishers Ltd: Nature, advance online publication 14 June 2009 (doi:10.1038/nature08157).

time the outbreak was recognized in humans. However, pigs can be experimentally[61–65] and naturally[66–68] infected with 2009 H1N1 viruses, resulting in mild respiratory infection, and contact transmission can be demonstrated in an experimental setting.[61,62,65] The extent of 2009 H1N1 virus circulation in pigs is not known. In contrast to efficient replication in humans and pigs, avian species such as chickens, ducks, and turkeys are resistant to experimental infection with 2009 H1N1 viruses,[69–72] although infection of two turkey flocks was reported in Chile.[73] These findings suggest that 2009 H1N1 viruses do not replicate efficiently in avian species, possibly with the exception of turkeys.

Determinants of virulence and pathogenicity

Soon after their recognition, 2009 H1N1 viruses were characterized in mice, ferrets, and non-human primates.[74–79] Although the new pandemic viruses are not uniformly lethal in these animal models, they cause more severe lung pathology than seasonal influenza viruses. In addition, 2009 H1N1 viruses replicate efficiently in the upper and lower respiratory tract of infected animals, unlike seasonal influenza viruses, which are restricted in their growth to the lower respiratory tract. The ability of 2009 H1N1 viruses to replicate efficiently in the lower respiratory tract may explain, at least in part, the viral pneumonia observed in severe human cases of 2009 H1N1 virus infection. In addition, these studies demonstrated the transmission of 2009 H1N1 viruses in ferrets.[74–77]

Sequence analysis revealed that 2009 H1N1 viruses do not possess recognized markers of high virulence (discussed below),[8,17,43,45] leaving in question the mechanisms for the increased virulence of 2009 H1N1 viruses compared to seasonal H1N1 viruses. With the availability of thousands of complete genomic influenza virus sequences, efforts are now underway to identify amino acid changes that may have facilitated virus transmission to and among humans.[80–82] Others studies are focused on identifying host factors critical for efficient viral replication.[83–85] Together, these studies have identified a number of viral mutations and cellular factors that may play a role in the viral life cycle; however, for most of these candidates, experimental confirmation has yet to be obtained.

Role of HA in viral pathogenicity

The HA protein mediates two critical functions in the viral life cycle – binding of the virus to host cells and the fusion of the viral and endosomal membranes for the release of viral ribonucleoprotein complexes into the cytoplasm. HA also plays a critical role in virulence and host range restriction.

Receptor-binding specificity

Human influenza viruses preferentially bind to receptors that possess sialic acid linked to galactose by an α2,6-linkage (SAα2,6Gal), whereas avian influenza viruses preferentially recognize SAα2,3Gal.[86,87] This receptor preference is matched by SAα2,3Gal on epithelial cells in the intestinal tract of waterfowl (the main replication site of avian influenza viruses) and by SAα2,6Gal on epithelial cells in the human trachea. We now know, however, that SAα2,3Gal (i.e., avian-type receptors) are also expressed in the lower respiratory tract of humans.[88,89] Interestingly, pigs express both receptor types on their respiratory epithelial cells,[87,90,91] although avian-type receptors may only be expressed in bronchioli and alveoli.[90,91]

Differences in receptor-binding specificity are determined by specific amino acids in HA. For H1 HA, Asp at positions 190 and 225 (H3 numbering) confers binding to SAα2,6Gal, whereas Glu-190 and Gly-225 confer binding to SAα2,3Gal.[92] Hence, human and avian influenza viruses typically possess Asp-190/Asp-225 or Glu-190/Gly-225, respectively. The pandemic 2009 H1N1 viruses possess SAα2,6Gal receptor-binding specificity,[75,93] although one study reported binding of a 2009 H1N1 to both SAα2,3Gal and SAα2,6Gal.[94] Recently, a change in HA (Asp-to-Gly at position 225) was reported that appears to correlate with more severe disease outcomes in humans.[95–102] This variant

does not appear to transmit efficiently among humans, as only a few transmission events have been reported to date.[103] Following experimental inoculation of pigs with mixed virus populations encoding Glu-225 and Gly-225, the Glu-225 variant was found in nasal secretions, while the Gly-225 variant was found in the lower respiratory tract.[65]

Hemagglutinin cleavage

The HA protein is synthesized as a precursor protein (HA0) that is post-translationally cleaved into two disulfide-linked subunits, HA1 and HA2. This cleavage event exposes the N-terminus of HA2 (the so-called fusion peptide), which mediates the fusion between the viral envelope and the endosomal membrane. HA cleavage is therefore essential for viral infectivity. The 2009 H1N1 viruses, like other human and swine influenza viruses, possess a single basic amino acid at the HA cleavage site, which is cleaved by proteases in the respiratory and/or intestinal organs, hence restricting systemic spread. By contrast, highly pathogenic avian influenza viruses (including H5N1 and H7N7) possess multiple basic amino acids at the HA cleavage site. This cleavage motif is recognized by ubiquitous proteases and leads to systemic infection.

Hemagglutinin antigenicity

Sequence analysis, serology data, and experimental studies in animal models demonstrated that the 2009 H1N1 HA proteins more closely resemble the 1918 HA protein than the HA proteins of recent seasonal H1N1 viruses.[43,45,74,93,104–109] These findings offer an explanation for the serum cross-reactivity with 2009 H1N1 viruses observed for some elderly people, but not younger individuals.[20,22,74,110–112] For H1 HA proteins, four antigenic sites (Ca, Cb, Sa, and Sb) have been identified.[113,114] The Sa, Sb, and Cb sites of 2009 H1N1 HA proteins differ by one, two, and one amino acid, respectively, from the 1918 HA protein. By contrast, the respective antigenic sites of a recent seasonal H1N1 HA protein differ by five, eight, and four amino acids from the 1918 HA protein. The Ca site is more divergent.

Antigenic sites can be masked by glycans: the 1918 and 2009 H1N1 HA proteins do not possess glycosylation sites near the Sa antigenic site; by contrast, seasonal H1N1 HA proteins possess several oligosaccharides that block the Sa site. Together, these data suggest the following scenario: the 1918 HA remained relatively unchanged in pigs; people infected with the 1918 pandemic virus, or a close descendant thereof, are therefore partially protected against swine-origin 2009 H1N1 viruses. In humans, however, the pandemic 1918 HA faced significant immune pressure that has led to the accumulation of mutations in the antigenic sites and the circulation of seasonal H1N1 viruses up to 2009. Younger people, who have not been exposed to 1918-like viruses, are therefore not similarly protected.

Role of PB2 in viral pathogenicity

The polymerase subunit PB2 is critical for influenza virus replication and transcription, and is a determinant of host range restriction in mammalian species[115,116]: human influenza viruses (with the exception of the 2009 H1N1 viruses) possess Lys at position 627, which confers efficient replication in mammalian cells. By contrast, most avian influenza viruses possess Glu-627, which restricts replication in mammals, particularly in the upper respiratory tract. The PB2 gene of 2009 H1N1 viruses originated from an avian influenza virus and possesses Glu (i.e., the avian-type amino acid) at PB2-627[43,45]; substitution of Glu with Lys does not increase viral growth rates in cell culture or virulence in mice.[117,118] Two recent studies now offer an explanation for the efficient replication of 2009 H1N1 influenza viruses in mammals despite the lack of lysine at position 627.[119,120] A Lys at position 591 of PB2 (which replaces the Glu typically found at this position) confers efficient replication in mammalian cells in the background of Glu-627.[119,120] X-ray crystallography of a 2009 H1N1 PB2 protein demonstrated that Lys-591 alters the surface charge and structure of PB2,[120] which may then prevent the binding of an inhibitory factor that suppresses the activity of avian-type polymerases (possessing PB2-627-Glu) in mammalian cells, as suggested by Mehle and Doudna.[121]

Another study found that the amino acid at position 701 of PB2 affects the replicative ability of an H5N1 virus in mammals.[122] 2009 H1N1 viruses possess the 'low-pathogenic' amino acid (Asp) at this position, and its replacement with the 'high-pathogenic' amino acid (Asn) did not increase virus replication in mammalian cells or virulence in mice and ferrets.[117] Recent data also suggest a role for the amino acid at position 271 of PB2 in adaptation to mammals.[123] The novel pandemic viruses possess the human-type amino at this position, which contributes to the high replicative ability of the 2009 H1N1 virus replication complex.[123]

Role of NS1 in viral pathogenicity

The NS1 protein is an interferon antagonist[124] that executes its role through several mechanisms (reviewed in Refs.[125,126]); it counteracts virus-stimulated RIG-I signaling, binds to double-stranded RNA to prevent the activation of 2′-5′ oligo(A)synthetase and RNaseL, and interferes with the activation of transcription factors and IFN-β-stimulated gene products. The amino acid at position 92 may play a role in regulating interferon responses, with Glu (found in highly pathogenic avian influenza viruses) resulting in high virus pathogenicity, and Asn resulting in low virus

pathogenicity.[127] 2009 H1N1 viruses possess the 'low-pathogenic' amino acid at this position. The four C-terminal amino acids of NS1 form a PDZ ligand domain motif[128] that may affect virulence.[129] The NS1 protein of the novel pandemic viruses lacks the 11 C-terminal amino acids,[43,45] yet restoration of the PDZ ligand domain motif does not increase the virulence of 2009 H1N1 viruses,[130] suggesting that this motif is not a major determinant of virulence in the genetic background of the 2009 pandemic viruses.

Several studies have assessed the cytokine/chemokine levels induced by 2009 H1N1 viruses; while some reported similar induction of cytokines/chemokines by seasonal and pandemic H1N1 viruses,[131,132] one study found higher levels of cytokine/chemokine induction upon infection with 2009 H1N1 viruses[74]; these differences likely reflect differences in the test systems used. Comparisons of mild and severe cases of 2009 virus infection demonstrate higher levels of proinflammatory cytokine/chemokines in the latter group.[133–135]

Role of PB1-F2 in viral pathogenicity

The PB1-F2 protein, expressed from the +1 reading frame of the polymerase PB1 gene, induces apoptosis, retains PB1 protein in the nucleus for efficient replication, and increases the frequency and severity of secondary bacterial infections (reviewed in Ref.[136]). 2009 H1N1 viruses encode a PB1-F2 peptide of only 11 amino acids,[43,45] because of stop codons in the open reading frame. Reverse genetics allowed the generation of pandemic viruses with full-length PB1-F2 proteins.[137] In mice and ferrets, these variants were comparable in their virulence to wild-type virus encoding a PB1-F2 peptide of 11 amino acids,[137] suggesting that PB1-F2 is not a major factor in the virulence and pathogenicity of 2009 H1N1 viruses.

Antiviral compounds

As vaccine development and production typically takes more than 3 months, compounds with antiviral activity are the first line of defense to newly emerging viruses. Two classes of antiviral compounds are available to treat influenza virus infections: (a) amino adamantanes, such as amantadine hydrochloride and rimantadine, that block the ion channel formed by the viral M2 protein and (b) inhibitors of the viral NA activity, such as oseltamivir and zanamivir. The new pandemic viruses are resistant to ion channel inhibitors,[7,8,138] because of a Ser-to-Ala mutation at position 31 of the M2 protein,[8,43,45] which is known to confer resistance to amino adamantanes. However, 2009 H1N1 viruses are sensitive to NA inhibitors,[8,138] although oseltamivir-resistant viruses have now been reported, both in oseltamivir-treated and oseltamivir-untreated individuals.[139–144] These viruses possess a histidine-to-tyrosine mutation at position 275 of their NA protein (N1 numbering), a known source of oseltamivir resistance. Currently, oseltamivir-resistant 2009 H1N1 viruses do not appear to transmit efficiently among humans, although isolated cases of transmission have been reported.[143,144] In contrast to 2009 H1N1 viruses, almost all seasonal H1N1 viruses are now resistant to oseltamivir. Concern therefore exists that the pandemic viruses may acquire this trait through mutation. Zanamivir[145] and peramivir (a NA inhibitor in Phase III clinical trials that recently received 'Emergency Use Authorization')[146] would remain as treatment options.

Vaccines

The antigenic differences and the resulting lack of serum cross-reactivity between seasonal and pandemic H1N1 2009 viruses in younger people[43,45,74,93,104–109] (see also 'HA Antigenicity') necessitated the development of a new vaccine to the pandemic viruses. Based on antigenic, genetic, and phylogenetic characterization, the WHO recommended on May 26, 2009, that vaccines contain an A/California/07/2009-like virus (http://www.who.int/csr/resources/publications/swineflu/H1N1Vaccinevirusrecommendation26May2009.pdf). Several candidate vaccines were soon developed in which (at least) the HA and NA genes of A/California/07/2009 virus were combined with the remaining genes of A/Puerto Rico/8/34 (H1N1) virus, the virus commonly used for human influenza vaccine production[147] (http://www.who.int/csr/resources/publications/swineflu/summary_candidate_vaccine.pdf). Reassortants were generated by classic reassortment techniques or reverse genetics. Early findings of poor growth of the vaccine candidates (http://www.thelancet.com/H1N1-flu/egmn/0c03c805) and the potential need for two doses[148,149] sparked concerns over a vaccine shortage. Antigen-sparing strategies, such as the use of adjuvants, were therefore considered. Clinical trials tested one- and two-dose regimens of different amounts of adjuvanted or non-adjuvanted split-virion vaccines, or adjuvanted whole virion vaccine.[147,150–157] These studies demonstrated high seroconversion rates with hemagglutination inhibition titers of 1:40 or more [which are considered protective (http://www.fda.gov/cber/gdlns/panfluvac.htm)] in healthy adults after a single dose of vaccine. The strong immune responses induced in adults by a single dose of 2009 H1N1 vaccine suggested a certain level of pre-existing immunity that cross-reacts with the 2009 pandemic virus. By contrast, seroconversion rates were generally lower in children,[155–158] suggesting that two doses may be necessary. A clinical study also demonstrated that the vaccines to pandemic and seasonal influenza viruses can be administered simultaneously without negatively affecting the efficacy of either.[154]

The first vaccines against 2009 H1N1 viruses were approved in September of 2009[159–161] (http://www.tga.gov.au/alerts/medicines/h1n1vaccine.htm) (http://www.ema.europa.eu/influenza/vaccines/home.htm) (http://www.fda.gov/newsevents/newsroom/pressannouncements/ucm182399.htm). In the United States, non-adjuvanted, inactivated split-virus or subunit vaccines for intramuscular injection, or a live attenuated vaccine for intranasal administration was approved with one dose for adults or two doses for children between the age of 6 months and 9 years. In Europe, only inactivated vaccines were approved and two doses are recommended with one exception, these vaccines are adjuvanted[161] (http://www.ema.europa.eu/influenza/vaccines/home.htm). In Australia, an inactivated split-virus vaccine was approved for use in adults and children 10 years of age and older (http://www.tga.gov.au/alerts/medicines/h1n1vaccine.htm).

Vaccine safety has been monitored closely through the Vaccine Adverse Event Reporting System (VAERS). As of April 30, 2010, the percentage of serious adverse reactions was similar to that observed with seasonal influenza vaccines (http://vaers.hhs.gov/resources/2010H1N1Summary_May07.pdf). Particular focus has been on cases of Guillain–Barré syndrome (GBS), a neurologic disease that occurred at a higher incidence with the A/New Jersey/1/76 (H1N1) vaccine and contributed to the cessation of that vaccination program. Currently, there is no indication that the 2009 H1N1 vaccines elevated the number of GBS cases when compared to seasonal influenza vaccines (http://vaers.hhs.gov/resources/2010H1N1Summary_May07.pdf).

Outlook

Since their appearance in the spring of 2009, the 2009 H1N1 viruses have largely replaced seasonal H1N1 viruses. This fact was anticipated by WHO and has been recognized with the recent recommendation by the WHO to replace seasonal H1N1 viruses with pandemic H1N1 viruses in the 2010 (Southern Hemisphere; http://www.who.int/csr/disease/influenza/vaccinerecommendations1/en/index.html) and 2010/2011 (Northern Hemisphere) vaccine formulations.[16] The pandemic of 2009 tested our ability to react to outbreaks of novel influenza viruses that spread efficiently in human populations. Fortunately, the 2009 H1N1 virus was not as pathogenic as originally feared, and low levels of immunity primed by seasonal H1N1 infection provided protection with a single dose of vaccine. The need for two doses, together with the fact that almost all influenza vaccines are still produced in embryonated chicken eggs (a production system that is difficult to scale up rapidly), would have resulted in severe vaccine shortages. Future efforts should therefore be directed toward further evaluation of antigen-sparing strategies and alternative methods of influenza vaccine production such as cell culture.

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