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Vaccines for Pandemic Influenza

Springer

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Preface

Recent years have seen unprecedented outbreaks of avian influenza A viruses. In particular, highly pathogenic H5N1 viruses have not only resulted in widespread outbreaks in domestic poultry, but have been transmitted to humans, resulting in numerous fatalities. The rapid expansion in their geographic distribution and the possibility that these viruses could acquire the ability to spread from person to person raises the risk that such a virus could cause a global pandemic with high morbidity and mortality. An effective influenza vaccine represents the best approach to prevent and control such an emerging pandemic. However, current influenza vaccines are directed at existing seasonal influenza viruses, which have little or no antigenic relationship to the highly pathogenic H5N1 strains. Concerns about pandemic preparedness have greatly stimulated research activities to develop effective vaccines for pandemic influenza viruses, and to overcome the limitations inherent in current approaches to vaccine production and distribution. These limitations include the use of embryonated chicken eggs as the substrate for vaccine production, which is time-consuming and could involve potential biohazards in growth of new virus strains. Other limitations include the requirement that the current inactivated influenza vaccines be administered using needles and syringes, requiring trained personnel, which could be a bottleneck when attempting to vaccinate large populations in mass campaigns. In addition, the current inactivated vaccines that are delivered by injection elicit limited protective immunity in the upper respiratory tract where the infection process is initiated. Most of these limitations of the current vaccines are being addressed by research on novel approaches to vaccine development that are described in many of the chapters in this volume.

As an introduction to the topic, H.L. Yen and R.G. Webster describe the reservoir of influenza viruses with pandemic potential present in aquatic birds, particularly focusing on the evolution of highly pathogenic H5N1 viruses in Asia. As these viruses have continued to spread geographically, they also continue to diversify genetically, raising a strain selection problem for vaccine development. However, A.C.M. Boon and R.J. Webby review recent studies that show that substantial levels of antigenic cross reactivity are exhibited among the surface antigens of H5N1 strains, and that they can elicit cross-protective immune responses. A better definition of antigenic epitopes involved in cross-protection will be an important advance in enabling the design of effective vaccines.

To put new approaches into perspective, several chapters are devoted to reviewing current methods of developing and evaluating seasonal and pandemic influenza vaccines. A.E. Fiore, C.B. Bridges, and N.J. Cox review current efforts to produce seasonal vaccines and the impact of these vaccines on preventing influenza and its complications. E. O'Neill and R.O. Donis describe how candidate vaccine strains are detected, processed, and evaluated, bringing together surveillance, genetic and antigenic characterization, production of reassortant vaccine strains, and analysis of their safety and growth. Live attenuated, cold-adapted, temperature-sensitive influenza vaccine strains (LAIV) have proved highly effective, particularly in young children, against seasonal influenza. G.L. Chen and K. Subbarao show how the lessons learned in developing LAIV can be used to develop effective pandemic vaccines.

In addition to human vaccines, there is high interest in developing vaccines to control infection in poultry. D.R. Kapczynski and D.E. Swayne review the production of inactivated vaccines for avian species, many of which are formulated with oil-based adjuvants. In addition to commercial poultry, such vaccines have also been used in exotic and endangered species. Live attenuated vaccines have not been utilized in birds because of their potential to reassort with other avian influenza viruses. The development and the application of avian H5N1 influenza vaccines in China are discussed by H. Chen and Z. Bu. These include inactivated vaccines as well as live-vectored vaccines based on recombinant Newcastle disease virus. These vaccines have been widely used in Southeast Asia as well as Egypt, and have played an important role in control disease outbreaks.

A number of novel approaches for pandemic influenza vaccine development are now being actively pursued. T. Horimoto and Y. Kawaoka review the use of reverse genetics to develop recombinant virus strains for use in vaccine development, and present an overview of alternative strategies that are available for the development of H5N1 influenza vaccines. Genetically modified viruses with alterations in the NS1 gene have been evaluated as attenuated vaccines. This approach is reviewed by J. Richt and A. Garcia-Sastre. These viruses exhibit reduced virulence because these NS1 mutants do not inhibit interferon responses, unlike the native NS1 protein, which enhances viral replication. These genetically altered viruses represent new live vaccine candidates that confer protection in several animal models. The development of DNA plasmids as vaccines is also being pursued for influenza viruses; strategies to improve the potency and efficacy of such vaccines are described in the chapter by J. Kim and J. Jacob. An attractive alternative to egg-based vaccine production is the use of cell culture systems, in which recombinant expression vectors can be used for antigen production. Vaccines consisting of the purified HA protein have been produced using recombinant baculovirus expression in insect cells; J. Treanor reviews clinical trial results which show that these recombinant vaccines are well tolerated and induce functional antibody responses. Although the HA protein is considered the major component of most vaccines, the neuraminidase (NA) protein is also able to elicit protective immunity, probably by inhibiting cell-to-cell spread of the virus. The role of the neuraminidase in influenza vaccines is the subject of the chapter by M. Sylte and D. Suarez. The use of recombinant virus vectors that express influenza antigens represents an attractive

approach for rapid vaccine production; S.A. Kopecky-Bromberg and P. Palese discuss the advantages and limitations of several recombinant vectors that are currently under investigation as influenza vaccines. Another novel approach to vaccine development is the use of virus-like particles that are assembled through the expression of viral structural proteins, particularly the HA, NA and M1 proteins. These particles closely resemble the influenza virion but lack the viral genome, and thus have a high degree of safety. S.-M. Kang and co-workers describe recent studies that demonstrate the production and characterization of influenza VLPs and their evaluation in animal models. A major limitation of current influenza vaccines is their induction of neutralizing antibodies that are highly strain-specific and are thus not able to protect against newly arising variant strains; it is therefore highly desirable to develop vaccines that would induce immune responses with an enhanced breadth of immunity. L.J. DiMenna and H.C.J. Ertl describe some approaches that are under investigation to develop potential universal vaccines against influenza A viruses. Results of initial human trials of H5N1 vaccines have shown that these antigens elicit relatively low immune responses, and it was observed that two immunizations with high doses of antigen were needed to achieve satisfactory responses. Such studies have stimulated research on the use of adjuvants to enhance responses to such vaccines. R.L. Atmar and W.A. Keitel provide an overview of current research on a number of these candidate adjuvants being evaluated with influenza vaccines.

Inactivated influenza vaccines are now delivered by hypodermic needles and syringes. This is a time-consuming process that complicates the ability to rapidly deploy a new vaccine to immunize a large population. As an alternative approach to vaccine delivery, I. Skountzou and S.-M. Kang review vaccine delivery by transcutaneous immunization (i.e., the direct application of vaccines to the skin). Mild chemical or physical disruption of the stratum corneum allows macromolecules as well as large particulate antigens to penetrate the skin and elicit immune responses. Such topical delivery provides an alternative approach to vaccination that could potentially result in self-administered vaccines. Alternatively, vaccine delivery through the skin can be accomplished by using micron-scale needles, as reviewed by M.R. Prausnitz and colleagues. Microneedles of various designs have been successfully used to deliver a range of vaccine antigens, including proteins, DNA vaccines and recombinant viruses. This approach to vaccine delivery has a number of advantages, including little or no pain compared to hypodermic needles, possible dose sparing, and the potential for the development of a stable patch formulation that could be self-administered.

Animal models are a critical means of evaluating the effectiveness of pandemic influenza vaccines. R.A. Tripp and S.M. Tompkins review a variety of animal models used to study influenza, and their strengths and weaknesses. Current seasonal influenza vaccines have limited immunogenicity in the age group that is most at risk of influenza complications, the elderly. This age group suffered disproportionately during the influenza pandemics of 1957 and 1968. S. Sambhara and J.E. McElhaney describe what is known about the molecular mechanisms that lead to hyporesponse in the elderly as a potential guide to finding ways to strengthen the

response. A variety of vaccines against potential avian influenza pandemic virus candidates have been developed and tested in human clinical trials. W.A. Keitel and R.L. Atmar discuss the results of these candidates in humans, including the effects of dose, number of doses, and both aluminum- and oil-in-water-containing adjuvants.

All potential influenza vaccines that could be used in humans in prepandemic preparedness efforts or in reaction to a pandemic must be approved by regulatory authorities. N.W. Baylor and F. Houn review some of the challenges that the Food and Drug Administration (FDA) faces in evaluating pandemic influenza vaccines for licensure, and describe some of the efforts being made by the FDA to speed up the development of such vaccines, such as accelerated approval and priority review. They comment on guidance documents that help manufacturers ensure that they collect the critical information needed for these reviews.

Pandemics have the potential for massive global impact. Thus, vaccines should ideally be available throughout the world. K.M. Edwards et al. discuss potential global needs and current global production capacity. It is likely that vaccine supply in the early phases of a pandemic will not be adequate to meet the needs of even an industrialized country such as the United States. B. Schwartz and W.A. Orenstein review efforts within the United States to set priorities for mass vaccination, including the criteria used and the public input process that went into establishing the current proposed priorities.

The editors hope that this volume will stimulate research on improved influenza vaccines, including those that will be able to effectively prevent the next pandemic. We thank all of the authors for their contributions. We are extremely indebted to Erin-Joi Collins for all she did to make this volume possible; this included helping to organize the chapters, communicating with the authors, tracking progress, identifying and resolving problems, and much more.

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Part I
Pandemic Influenza Overview

Pandemic Influenza as a Current Threat

Hui-Ling Yen and Robert G. Webster

Contents

Abstract Pandemics of influenza emerge from the aquatic bird reservoir, adapt to humans, modify their severity, and cause seasonal influenza. The catastrophic Spanish H1N1 virus may have obtained all of its eight gene segments from the avian reservoir, whereas the Asian H2N2 and the Hong Kong H3N2 pandemics emerged by reassortment between the circulating human virus and an avian H2 or

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H3 donor. Of the 16 hemagglutinin subtypes, the H2, H5, H6, H7, and H9 viruses are considered to have pandemic potential. While this chapter focuses on the evolution of the Asian highly pathogenic (HP) H5N1 influenza virus, other subtypes are also considered. The unique features of the HP H5N1 viruses that have devastated the domestic poultry of Eurasia are discussed. Although they transmit poorly to humans, they continue to kill more than 60% of infected persons. It is unknown whether HP H5N1 will acquire human pandemic status; if it does not, another subtype eventually will do so, for a future influenza pandemic is inevitable.

1 Influenza Virus as a Noneradicable Zoonosis

1.1 Natural Reservoirs for Influenza A Virus

The established reservoirs of all 16 hemagglutinin (HA) and nine neuraminidase (NA) subtypes of influenza A viruses are the aquatic birds of the world (Fouchier et al. 2005; Webster et al. 1992). In this reservoir, the low pathogenic avian influenza viruses replicate in the respiratory tract and the intestine and live in apparent harmony with their hosts, causing no apparent disease signs (Webster et al. 1978; Kida et al. 1980).

In addition to aquatic birds, diverse animal species are susceptible to influenza A virus infection in nature or under laboratory conditions. Current information suggests host-specific lineages have been established in birds, pigs, and horses, as well as humans. Phylogenetic analyses suggest that mammalian influenza viruses all are derived from the avian influenza reservoir (Webster et al. 1992). However, the possibility exists that established host-specific influenza viruses may be introduced and further established in other species, as was observed with the equine H3N8 virus in dogs (Crawford et al. 2005).

The clinical outcome of influenza A virus infection depends on the host and the virus. Domestic poultry are susceptible to most subtypes of avian influenza virus infection. Intensive surveillance activities in the United States during 2002–2005 detected avian influenza virus or specific antibodies to H1–H13 subtypes and all nine NA subtypes (Senne 2007). Of the 16 HA subtypes, only two (H5 and H7) subtypes are known to have the capacity to become highly pathogenic (HP) in chickens and other gallinaceous birds. The HP H5 and H7 viruses usually produce asymptomatic to mild clinical infection in ducks or wild birds and are rarely lethal to wild birds, with the exception of the HP H5N1 virus that has emerged in Asia since 1997. The HP phenotype is related, but not restricted, to the presence of multiple basic amino acids at the HA cleavage site (Rott et al. 1995; Horimoto and Kawaoka 2001).

The error-prone viral RNA polymerase, the segmented RNA genome that allows dynamic genetic reassortment within the large gene pool perpetuated in aquatic birds, and the existence of multiple natural reservoirs all point to the influenza A virus as a noneradicable zoonosis.

1.2 Ecology of Influenza A Virus in Asia

Southern China is the hypothetical pandemic epicenter of influenza, as this environment may have provided the conditions for the emergence of 1957 Asian and 1968 Hong Kong pandemic influenza viruses (Shortridge and Stuart-Harris 1982). In tropical and subtropical areas, human influenza can be detected year-round. The warm winter in Southeast Asia attracts migratory birds from northern climes to spend the winter in this region. The high density of human population and prevalence of backyard poultry (ducks, geese, and chickens) and pigs provide the opportunity for close interaction between these influenza reservoir animals and the possibility of interspecies transmission and genetic reassortment. Pigs that possess both receptors for avian (sialic acids with α-2,3-galactose linkage) and human (sialic acids with α-2,6-galactose linkages) influenza viruses were considered "mixing vessels" for generating reassortant viruses (Scholtissek 1995). In addition, the live-poultry market ("wet market") system provides optimal conditions for influenza virus evolution, with transmission between avian species and possible infection of humans (Shortridge et al. 1998; Peiris et al. 2007). Transmission between different host species and serologic evidence of human infection with H4, H5, H6, H7, H10, and H11 subtypes of avian influenza virus were documented in this region prior to the 1997 Hong Kong H5N1 outbreak (Shortridge 1992; Peiris et al. 2007).

2 Human Influenza Epidemics and Pandemics

2.1 Epidemiology of Human Influenza

Humans can be infected with influenza A, B, or C viruses, all of which belong to the *Orthomyxoviridae* family and are distinguished by serologic reactions of conserved viral nucleoprotein or matrix protein (Beard 1970). Influenza in humans may occur in two epidemiologic forms: pandemics and epidemics (Nicholson 1998). An influenza pandemic is a large-scale global outbreak of the disease, while an epidemic is more sporadic and localized, as seen with seasonal influenza outbreaks. Influenza epidemics result from newly immune-selected variant strains that contain accumulated point mutations that result in amino acid changes in the antigenic sites in the HA glycoprotein (predominantly in HA1) as well as NA glycoprotein (antigenic drift) (Fig. 1a). Current epidemics are caused by antigenic variants of influenza A viruses of the H1N1 H3N2 or their reassortant H1N2 subtypes as well as influenza B viruses. Because most of the population possesses cross-reacting antibodies for recent antigenic variants, severe clinical signs and death are observed mostly among young children, the elderly, and people with other underlying diseases.

Pandemic influenza results from the emergence of a new subtype of influenza A virus (antigenic shift) (Fig. 1b). Because the population does not possess immunity to the new subtype of influenza A virus, the new subtype may spread globally with

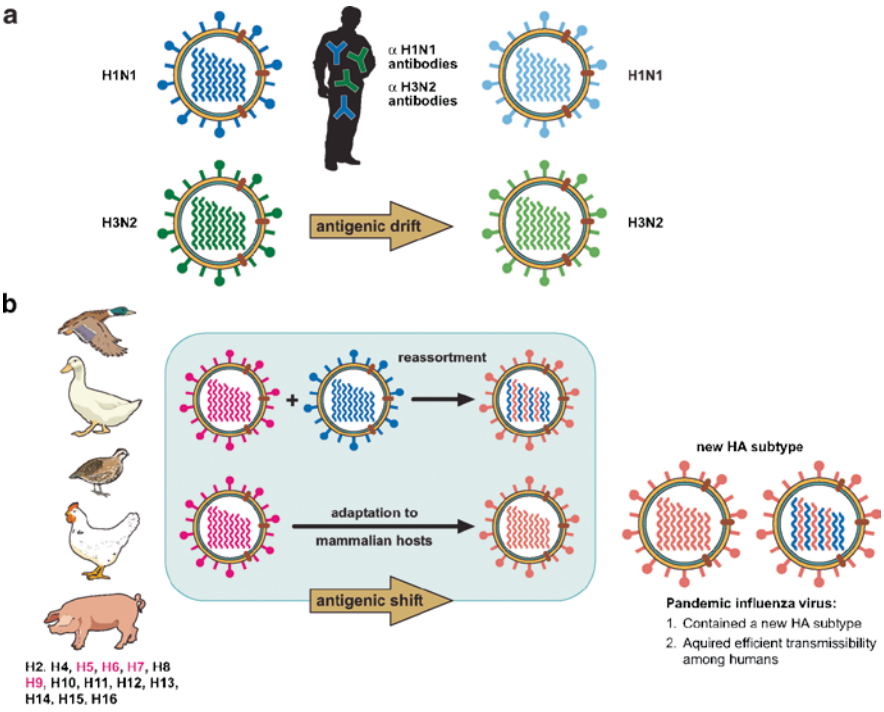

Fig. 1 **a–b** Antigenic drift and antigenic shift of influenza virus. a Pre-existing antibody response against the HA and NA glycoproteins of influenza A virus of H1N1 or H3N2 subtypes or influenza B virus selects antigenic variants with amino acid changes modifying the antigenic structure that allow influenza virus to evade immunity. Antigenic drift is a result of both immune and natural selection. **b** Reassortment between avian and human influenza A virus or continued adaptation of an avian influenza virus may result in a new subtype of influenza A virus with sustained human-to-human transmissibility. Pre-existing antibody response provides little or no cross-protection for this major change in the HA (and NA); however, cytotoxic T lymphocyte responses that target the conserved peptides encoded in viral internal proteins may provide protection

high attack rates and may cause significant morbidity and mortality (Nicholson 1998). However, the severity of a pandemic may be dependent on the composition of the virus, as cytotoxic T lymphocyte responses that target the relatively conserved internal proteins may provide protection (Rimmelzwaan et al. 2007). A mild pandemic is possible when the pandemic virus emerges through genetic reassortment (see below) and by acquiring internal gene segments from previously circulated human influenza virus. During the twentieth century, there were three global pandemics. These pandemics occurred in 1918 (Spanish pandemic, H1N1 subtype), 1957 (Asian pandemic, H2N2 subtype), and 1968 (Hong Kong pandemic, H3N2 subtype). In addition, in 1977 there was a reemergence of the H1N1 subtype (Russian pandemic). With the emergence of a new subtype, the old subtype is usually replaced. The exception was the 1977 H1N1 pandemic virus, which continues to circulate along with the H3N2 subtype.

2.2 Molecular Requirements for a Pandemic Strain: Emergence of 1918, 1957, and 1968 Pandemic Strains

The minimum molecular requirement for a pandemic influenza strain is a new HA subtype derived from the avian reservoir with sustained human-to-human transmissibility. Influenza pandemics that occurred during the last century suggest that such a virus may emerge in two ways: (1) genetic reassortment between avian influenza virus and circulating human influenza A viruses (as seen with the 1957 and 1968 pandemic viruses) and (2) interspecies transmission from an avian reservoir into an intermediate host, followed by continued adaptations (as seen with the 1918 pandemic virus) (Horimoto and Kawaoka 2005; Webby et al. 2004; Belshe 2005) (Fig. 1b).

Genetic analyses showed that the H2N2 1957 Asian pandemic virus acquired three gene segments from an avian reservoir (PB1, HA, and NA) and kept five other gene segments from the H1N1 human strain circulating prior to 1957. Similarly, the 1968 H3N2 Hong Kong pandemic virus acquired two gene segments from an avian reservoir (PB1 and HA) and kept six other gene segments from the H2N2 human strain that circulated in 1957–1968 (Webster et al. 1992). Pig tracheae, which have sialyl receptors for avian and human influenza viruses, have been proposed as the site for genetic reassortment (Ito et al. 2000; Scholtissek 1995). Additionally, the intermediate host may not be restricted to pigs. A report also demonstrated the presence of sialyl receptors with α-2,3- and α-2,6-galactose linkages in chicken and quail intestines (Guo et al. 2007). Unlike the 1957 or 1968 pandemic viruses, genetic analysis of the 1918 pandemic virus suggests that all of its eight gene segments originated from the avian reservoir without genetic reassortment (Belshe 2005; Taubenberger et al. 2005). However, it is not clear how long it took for an avian-originated influenza virus to become adapted in mammals or in which mammalian reservoirs the adaptations occurred.

The HA glycoprotein of avian and human influenza viruses preferentially recognizes sialic acids with α-2,3- or α-2,6-galactose linkages, respectively. As the HA segments of the 1918, 1957, and 1968 pandemic strains were derived from the avian reservoir, one common feature between the pandemic strains is the acquisition of amino acid changes in the receptor binding site of the HA glycoprotein that alter the virus' receptor binding specificity from the α-2,3 to the α-2,6 linkage between sialic acid and galactose (Matrosovich et al. 2000; Stevens et al. 2006). The switch to a predominantly α-2,6-linked sialyl receptor specificity facilitated transmission of 1918 pandemic virus (Tumpey et al. 2007) and likely the 1957 and 1968 pandemic viruses. The effect of the switch in receptor specificity on viral pathogenicity is less understood. Theoretically, the changes in receptor specificity may result in a change in target cells from the lung epithelial cells (exhibit α-2,3-linked sialyl receptor) to the epithelial cells lining the upper respiratory tract (exhibit α-2,6-linked sialyl receptor), thereby reducing the occurrence of pneumonia.

Another molecular characteristic observed in the 1918 and 1957 pandemic influenza viruses is the loss of the secondary sialic acid binding site with hemadsorbing activity in NA (Matrosovich 2008), which is a molecular signature for NA derived

from avian influenza viruses (Hausmann et al. 1995; Varghese et al. 1997). In addition to the surface glycoproteins, genetic analyses of influenza viruses isolated from different hosts have identified 32 residues from PB2, PA, NP, M1, and NS1 proteins as host-specific markers differentiating human and avian influenza viruses (Finkelstein et al. 2007). Among these 32 residues, 13 were conserved among the 1918, 1957, and 1968 pandemic influenza viruses (Finkelstein et al. 2007). The clear genetic difference between avian and human influenza viruses in these gene segments may be functionally related to the differences in cooperation with avian and human cellular machinery. It is likely that a pandemic strain should contain some of the human-specific markers that allow efficient replication and transmission.

3 H5N1 Virus as a Pandemic Threat

3.1 Emergence and Spread of H5N1 Virus

Before 1996, low-pathogenic H5 avian influenza viruses had been isolated from domestic ducks and geese in Southeastern China but not from chickens (Shortridge et al. 1998), and neutralizing antibodies to H5 virus were detected in pig sera from Southeastern China collected in 1977–1982 (2 of 127 samples) and 1998 (10 of 101 samples) (Peiris et al. 2007).

Genetic evidence showed that the precursor (A/Goose/Guangdong/1/96) of the currently circulating HP H5N1 virus was first detected in domestic geese in Guangdong, China, in 1996 (Peiris et al. 2007). To date, the precursor of this virus is unknown, although eight gene segments are closely related to those from low-pathogenic H5 viruses isolated from migratory birds and wild ducks in Hokkaido, Japan (Okazaki et al. 2000; Duan et al. 2007).

The index human case of H5N1 influenza occurred in May 1997, and the causative virus was identified in August 1997 (de Jong et al. 1997) as the first HP avian influenza virus known to cause lethal infection in humans. During the remainder of 1997, 17 additional human cases were detected, and six patients succumbed to H5N1 infection. Surveillance and epidemiologic studies established that poultry markets were the source of human H5N1 infection, as H5N1 virus was isolated from approximately 20% of fecal samples from chickens and from approximately 2% of fecal samples from ducks and geese in the market (Shortridge et al. 1998). Subsequent genetic analysis of the index human virus revealed that six internal genes were closely related to those in A/Quail/Hong Kong/G1/97 (H9N2) and that the NA gene was genetically similar to that of A/Teal/Hong Kong/W312/97 (H6N1), raising the possibility that reassortment between these viruses was involved in the genesis of the HP H5N1 virus (Peiris et al. 2007).

The culling of all poultry in Hong Kong effectively eradicated that particular genotype of HP H5N1 influenza virus. There were no more human cases in Hong Kong, but H5N1 viruses continued to circulate among apparently healthy domestic ducks in the coastal provinces of China between 1999 and 2002 (Chen et al. 2004). HP H5N1 viruses were also detected in geese in a live-poultry market in Vietnam

in 2001 and from duck meat exported from China to Korea and Japan in 2001 and 2003 (Peiris et al. 2007). During 2001 and 2002, multiple H5N1 genotypes were detected in poultry in Southern China (Li et al. 2004). These viruses had HA typical of the A/Goose/Guangdong/1/96-like lineage but with a plethora of different internal genes. In addition, the NA genes of these variant H5N1 viruses were typical of that of A/Goose/Guangdong/1/96 but frequently had deletions of amino acids in the stalk region (Li et al. 2004). In 2002, H5N1 outbreaks of lethal disease in waterfowl occurred in Penfold Park and Kowloon Park in Hong Kong; many aquatic species as well as tree sparrows and pigeons were killed (Ellis et al. 2004).

The next key event in the development of H5N1 viruses was its re-emergence in humans in 2003. The daughter of a Hong Kong family died while visiting the Fujian province of China in February 2003. On their return to Hong Kong, H5N1 infection was diagnosed in her father and brother (Peiris et al. 2004); the father subsequently died, but the brother recovered.

In late 2003 to early 2004, outbreaks of HP H5N1 viruses in domestic poultry were reported in South Korea, Japan, Vietnam, Laos, Cambodia, and Indonesia. During this period, avian-to-human transmission resulted in lethal H5N1 human infection in Vietnam and Thailand. Serologic evidence suggests that limited human infections occurred in Japan and South Korea during the 2003–2004 H5N1 outbreaks. Genetic analysis showed that the viruses that spread to Japan and South Korea (genotype V) differed in the PA gene from the viruses that became dominant in Vietnam, Thailand, Cambodia, Indonesia, and Southern China (genotype Z) (Li et al. 2004).

Qinghai Lake in Western China is a leading breeding site of migratory waterfowl. In May 2005, a lethal outbreak of HP H5N1 influenza occurred at Qinghai Lake that affected bar-headed geese (*Anser indicas*), great black-headed gulls (*Larus ichthyaetus*), brown-headed gulls (*Larus brunnicephalus*), ruddy shelducks (*Tadorna ferruginea*), and great cormorants (*Phalacrocorax carbo*) and killed more than 6,000 migratory waterfowl (Chen et al. 2006; Peiris et al. 2007). Other wild birds that have been affected by H5N1 include whooper swans (*Cygnus cygnus*), black-necked cranes (*Grus nigricollis*), and pochards (diving ducks that belong to the subfamily *Aythyinae*) (Peiris et al. 2007). This event was the first major outbreak of H5N1 influenza virus in wild migratory birds. The precursors of the dominant Qinghai H5N1 virus were detected in mallard ducks at Poyang Lake, China, in March 2005 (Chen et al. 2006) and may have came from domestic poultry. During the outbreak at Qinghai Lake, at least four genotypes of H5N1 virus were detected in the waterfowl, but one genotype became dominant and rapidly spread to wild and domestic birds in Siberia (July 2005), Mongolia and Kazakhstan (August 2005), Croatia, Romania, and Turkey (October 2005), Middle Eastern and European countries (2006), and Nigeria and India (February 2006) (Chen et al. 2006; Peiris et al. 2007). Although the Qinghai-like H5N1 virus can transiently infect migratory waterfowl, available surveillance evidence does not indicate the perpetuation of this virus in this natural influenza reservoir.

In 2005, two major clades with no overlapping geographic distributions were identified on the basis of HA sequence analysis (World Health Organization 2005). Viruses isolated from Thailand, Cambodia, and Vietnam during the 2004–2005 outbreaks were clustered into clade 1, whereas viruses isolated from China, Indonesia,

Korea, and Japan during the 2003–2004 outbreaks were clustered into clade 2. In 2005, human infection with H5N1 viruses continued to be reported in Vietnam and Thailand, and new cases were reported in Cambodia, China, and Indonesia. Effective control measures taken by Vietnam (vaccination of poultry) and Thailand (stamping out) since 2006 have significantly reduced the number of outbreaks in these two countries as well as the circulation of clade 1 virus. On the other hand, clade 2 viruses continued to evolve into three major subclades that differ in geographic distribution. Indonesian H5N1 viruses isolated since 2003 continue to cluster into one sublineage (subclade 2.1, which can be further grouped into subclades 2.1.1, 2.1.2, and 2.1.3), suggesting the possibility of a single introduction of the virus into Indonesia and its continued evolution within the region since 2003 (World Health Organization 2005, 2006; Peiris et al. 2007; Smith et al. 2006b). Subclade 2.2 contains the H5N1 virus that caused the large-scale lethal outbreak in wild birds at Qinghai Lake during summer 2005 and the H5N1 viruses that subsequently spread to the Middle East, Europe, and Africa, suggesting a potential role for migratory birds in spreading the virus (World Health Organization 2005, 2006). Surveillance in Southern China from July 2005 to June 2006 identified a dominant sublineage that had replaced most of the previously established sublineages. These Fujian-like viruses formed a separate subclade 2.3 (which can be further grouped into subclades 2.3.1, 2.3.2, 2.3.3, and 2.3.4) and further spread to Hong Kong, Malaysia, Laos, Vietnam, and Thailand, causing outbreaks in wild birds and domestic poultry in 2006, 2007, and 2008 (World Health Organization 2005; Smith et al. 2006a).

3.2 Unique Features of H5N1 Viruses: Changing Patterns

As the H5N1 viruses continued to spread and evolve during the past decade, we have learned of and observed several unique features about the virus. The first feature noted was the ability of the H5N1 virus to cause lethal infection in wild birds, including waterfowl, after the outbreak in Hong Kong in winter 2002 (Ellis et al. 2004). These H5N1 isolates were highly lethal to mallard ducks and caused neurologic symptoms (Sturm-Ramirez et al. 2004). Although HP H5 viruses are highly lethal in chickens and other gallinaceous birds, they had rarely been reported to be pathogenic in wild birds. The only recorded incident prior to the Hong Kong H5N1 event was reported in 1961, when an H5N3 virus (A/Tern/South Africa/61) caused deaths in terns. We have further learned that, although some of the H5N1 viruses isolated since 2002 were initially highly lethal to mallard ducks, antigenic variants with decreased pathogenicity can be selected rapidly in this natural influenza reservoir (Hulse-Post et al. 2005). In addition, waterfowl (including domestic ducks) have exhibited higher resistance than chickens and other gallinaceous birds to H5N1 infection and thus can serve as hidden sources (“Trojan horses”) for the maintenance and spread of the virus (Hulse-Post et al. 2005).

Unique features were also noted among the clade 2.2 H5N1 viruses, which spread widely to the Middle East, Europe, and Africa. The spread of this lineage

of H5N1 virus is considered to have occurred partly due to the migration of the birds. Experimental infection with six wild duck species (*Anas* and *Aythya* species) revealed differences in susceptibility to H5N1 virus (Keawcharoen et al. 2008). In addition, it was noted among these wild duck species that virus shedding from the throat was higher and of longer duration than from the cloaca (Keawcharoen et al. 2008). This property of respiratory shedding must be considered when studying the ecology of this H5N1 virus in migratory birds. The collection of both oral and cloacal samples from birds is therefore critical for surveillance purposes. Another notable feature of the dominant Qinghai H5N1 virus is that it had a mutation of the PB2 gene (E→K at residue 627) that is one of the conserved host markers (E627 for avian and K627 for human influenza viruses) and is associated with increased viral virulence in mice (Chen et al. 2006; Hatta et al. 2001).

The re-emergence of human H5N1 infections in 2004 was accompanied by several unique characteristics of the virus, including an increased host range and increased pathogenicity in mammalian species. Although cats can be infected with influenza virus experimentally, the first report of natural influenza virus infection in felids was caused by the HP H5N1 virus in a zoo in Thailand: tigers and leopards that were fed H5N1-infected poultry carcasses showed severe pneumonia and succumbed to infection (Keawcharoen et al. 2004). Further laboratory study confirmed the susceptibility of domestic cats to HP H5N1 infection as well as experimental transmission among cats (Kuiken et al. 2005; Rimmelzwaan et al. 2006). In addition to cats, the fatal infection of a dog fed H5N1-infected duck carcasses in Thailand was reported (Songserm et al. 2006). Stone martens, a wild mammalian species that, like ferrets, belong to the *Mustelidae* family, were also infected during an H5N1 outbreak in wild birds in Germany, and H5N1 infection in Owston's palm civet (*Chrotagale owstoni*) was reported in Vietnam (Peiris et al. 2007). These cases highlight the potential threat of H5N1 in wild mammalian species.

Additionally, increased viral pathogenicity in mammalian species is associated with H5N1 viruses isolated from human infection (Govorkova et al. 2005). Characterization of an HP H5N1 virus isolated from a fatal human case in Vietnam showed that viral polymerase activity is a key factor for increased pathogenicity in mammals (Salomon et al. 2006). Other factors that may determine the host range and the pathogenicity of H5N1 viruses include viral surface glycoproteins, the presence of K at residue 627 in the PB2 protein, and the ability to evade the host innate immune response through viral NS1 protein (Neumann and Kawaoka 2006).

Overall, the widespread HP H5N1 virus has several unique characteristics that should be taken into account in any attempts to control the virus. First are the virus's abilities to replicate in both the respiratory and gastrointestinal tracts and cause lethal infection in waterfowl reservoirs. Second is that, in domestic duck and waterfowl reservoirs, selection of antigenic variants with decreased pathogenicity to these species may occur. Domestic ducks and waterfowl that harbor the selected variants without apparent symptoms may transmit the virus to chickens and other wild birds (such as geese and swans) that are highly susceptible to infection, thus causing outbreaks (Fig. 2). Third, HP H5N1 virus with an increased host range to

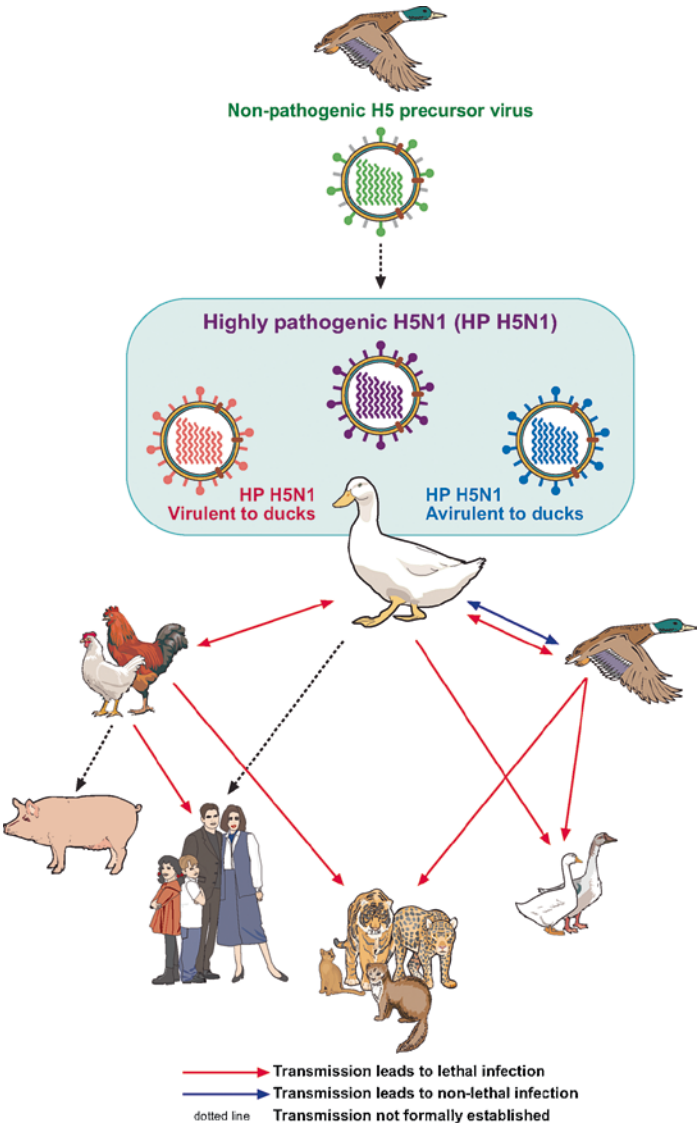

Fig. 2 Drivers of diversity for H5N1 virus. Highly pathogenic (HP) H5N1 influenza viruses (*shown in purple*) evolved from nonpathogenic H5 precursors (*shown in green*) preserved in wild aquatic-bird reservoirs, with eight gene segments derived from the Eurasia influenza gene pool. While universally highly lethal to chickens, the HP H5N1 isolates demonstrate variable pathogenicity in mammals and ducks. In domestic ducks and mallards, inoculation of HP H5N1 viruses that are virulent to the duck species (*shown in red*) may lead to selection of antigenic variants with decreased pathogenicity in ducks (*shown in blue*). Domestic ducks or waterfowl that harbor the selected variants of HP H5N1 virus without apparent symptoms may transmit the virus to chickens or other wild birds (geese or swans) that are highly susceptible to infection, thus causing outbreaks. The proximity of multiple influenza reservoirs and the endemicity of the H5N1 avian influenza virus in Southeast Asia since 1996 have provided numerous opportunities for the viruses to interact with various avian and mammalian species. Because the selection pressure on H5N1 viruses varies with the host, interspecies transmission events may have driven both the antigenic and the host range diversity of the virus

felids or ferret species may provide the virus with opportunities to further adapt in mammals, including humans (Fig. 2).

3.3 Human Infection with H5N1

Although transmission of the H5N1 viruses among avian species is highly efficient, interspecies transmission from avian species to mammalians remains infrequent. After a decade of continued circulation, H5N1 viruses have resulted in more than 380 human infections with an approximately 60% case fatality rate. The highest fatality is observed among patients 10–19 years old, and the lowest rate is among patients 50 years or older (bdel-Ghafar et al. 2008). The typical clinical manifestation of human H5N1 infection is severe pneumonia that may progress to acute respiratory distress syndrome, although mild upper respiratory illness without pneumonia has been reported (Beigel et al. 2005; bdel-Ghafar et al. 2008). Depending on the clade of H5N1 virus, gastrointestinal symptoms have been reported among 5–52% of patients (bdel-Ghafar et al. 2008). Encephalopathy has been reported in one human case (de Jong et al. 2005a). High viral load, lymphopenia, increased levels of lactate dehydrogenase, and certain chemokine and cytokine levels correlate with fatal outcome after infection (de Jong et al. 2006; bdel-Ghafar et al. 2008). Seroepidemiology results among high-risk groups with close contact to infected poultry or patients suggest that asymptomatic infection is rare (Beigel et al. 2005; bdel-Ghafar et al. 2008).

Direct avian-to-human transmission as a result of close contact with H5N1-infected poultry, a contaminated environment, or consumption of undercooked poultry products is the predominant cause of human infection (Beigel et al. 2005; bdel-Ghafar et al. 2008). Vertical viral transmission from infected mother to fetus has been reported (Gu et al. 2007). Limited and nonsustained human-to-human infections have been reported from family members attending H5N1 patients (Ungchusak et al. 2005; Kandun et al. 2006). The observation that 90% of case clusters occur among blood-related family members also suggests the possibility of genetic susceptibility (bdel-Ghafar et al. 2008).

4 Other Subtypes with Pandemic Potential

4.1 H9N2 Viruses

Surveillance studies revealed that H9N2 avian influenza virus has become established in chickens and quails and has been detected in pigs in Southern China since the mid-1990s (Guan et al. 1999, 2000; Xu et al. 2007; Peiris et al. 2001). Genetic analysis of the circulating H9N2 avian influenza viruses in China suggested two major lineages in terrestrial poultry: A/Duck/Hong Kong/Y280/97-like and A/Quail/Hong Kong/G1/97-like (Guan et al. 2000; Xu et al. 2007).

The A/Quail/Hong Kong/G1/97 virus shared the six internal genes with H5N1 human isolates in Hong Kong in 1997. The continued circulation of H9N2 viruses as well as H5N1 viruses in Southern China has resulted in multiple reassortment genotypes in recent years (Xu et al. 2007). In Korea, the Middle East, and Europe, H9N2 outbreaks in poultry have also been reported since late 1990 (Alexander 2003, 2007; Cameron et al. 2000). The H9N2 viruses that circulated in the Middle East were genetically related to the A/Quail/Hong Kong/G1/97-like viruses (Aamir et al. 2007; Cameron et al. 2000).

In 1999, human infection with H9N2 avian influenza virus was first documented in two children with mild upper respiratory symptoms in Hong Kong (Peiris et al. 1999), followed by subsequent reports of human infections in mainland China (Guo et al. 2001). The H9N2 human isolates from Hong Kong were genetically related to the A/Quail/Hong Kong/G1/97-like lineage. In 2003, human infection with H9N2 virus was identified again in Hong Kong, and the human H9N2 isolate was genetically more related to the A/Duck/Hong Kong/Y280/97-like lineage (Butt et al. 2005). In December 2008 an H9N2 infection was reported from a two-month old in Hong Kong. To date, there has been little evidence of human-to-human transmission of H9N2 virus. However, H9N2 virus with dual or human-like receptor specificity (Matrosovich et al. 2001) is now prevalent in many Eurasian countries, and the probability of the H9N2 subtype continuing to evolve into a pandemic strain is high.

4.2 H7 Viruses

Self-limited human infections with H7 subtype of avian influenza viruses have been documented since 1970 (Campbell et al. 1970; Kurtz et al. 1996; Webster et al. 1981). Between February and May 2003, outbreaks of the HP H7N7 subtype were reported in the Netherlands, Germany, and Belgium (Alexander 2007). More than 25 million birds were slaughtered during the outbreaks, and H7 virus was detected in at least 86 human infections. Infection with the H7N7 viruses resulted in conjunctivitis in 83 of 89 confirmed cases and one fatal case with pneumonia in combination with acute respiratory distress syndrome (Fouchier et al. 2004). In 2004, HP H7N3 outbreaks were reported in British Columbia, Canada, resulting in the slaughter of more than 19 million domestic poultry and causing two human infections with conjunctivitis (Hirst et al. 2004). Genetic evidence showed that the HP H7N3 virus evolved from a low-pathogenic H7N3 virus by obtaining a 21-nucleotide insertion (derived from the M gene) at the HA cleavage site (Hirst et al. 2004). The continued incidence of human infection with the H7 subtype and the high frequency of human cases associated with conjunctivitis showed that the H7 virus could infect humans without prior adaptation.

4.3 H6 Viruses

Outbreaks of the H6 subtype in domestic poultry have been reported in many Eurasian countries in recent years (Alexander 2007). Surveillance studies in