

## 9. Avian Influenza Viruses and Pandemic Influenza

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### 9.1. Introduction

Human influenza viruses can hardly be labeled as reemerging pathogens because they cause annual human epidemics of symptomatic disease, affecting approximately 20% of children and 5% of adults worldwide, and have probably done so since ancient times. Around the year 400 B.C., Hippocrates recorded “epidemic catarrhs associated with seasonal periods,” which may well have been attributable to influenza viruses. Periodically, however, completely novel antigenic subtypes of influenza viruses were introduced in the human population, causing large-scale global outbreaks with high death tolls. These pandemic strains can certainly be regarded as (re)emerging pathogens. The “Athen’s plague” described by Hippocrates’ contemporary Thucydides is believed by some to constitute the first account of such a devastating influenza epidemic. Since the 16th century, many large-scale outbreaks of influenza-like illnesses have been described in Europe. In 1580, one of such outbreaks spread from Europe into Africa and Asia, possibly making it the first recorded influenza pandemic. The most devastating influenza pandemic in modern recorded history, known as the “Spanish flu,” occurred in 1918–1919, killing up to 100 million people worldwide. Other less destructive pandemics during the past century occurred in 1957 and 1968. Avian influenza A viruses are key to the emergence of human influenza pandemics. The virus strains implicated in the 20th century’s influenza pandemics originated directly from avian influenza viruses, either through genetic reassortment between human and avian influenza strains (1957, 1968) or possibly through adaptation of purely avian strains to humans (1918). It was long thought that the host range of avian influenza viruses precluded direct transmission to humans and that the emergence of pandemic strains required genetic reassortment between avian and human strains. However, occurrences of direct bird-to-human transmission of

avian influenza viruses have increasingly been reported in recent years, culminating in the ongoing outbreaks of influenza A (H5N1) among poultry and wild birds in several Asian, European and African countries with continuing instances of human infections. These unprecedented developments have resulted in increasing global concerns about the (re)emergence of pandemic influenza A strains and the role of avian influenza viruses in this.

## 9.2. Virology

### 9.2.1. Biological Properties

Influenza viruses are pleomorphic, enveloped RNA viruses belonging to the family Orthomyxoviridae. Protruding from the lipid envelope are two distinct glycoproteins: the hemagglutinin (HA) and neuraminidase (NA). HA attaches to cell-surface sialic acid receptors, thereby facilitating entry of the virus into host cells. Because it is the most important antigenic determinant to which neutralizing antibodies are directed, HA represents a crucial component of current vaccines. NA is the second major antigenic determinant for neutralizing antibodies. By catalyzing the cleavage of glycosidic linkages to sialic acid on host cell and virion surfaces, this glycoprotein prevents aggregation of virions thus facilitating the release of progeny virus from infected cells. Inhibition of this important function represents the most effective antiviral treatment strategy to date. A third membrane protein, the M2 protein, is present in small quantities in influenza A viruses. By functioning as an ion channel, this protein regulates the internal pH of the virus, which is essential for uncoating of the virus during the early stages of viral replication. This function is blocked by the antiviral drugs amantadine and rimantadine.

The genome of influenza viruses is segmented, consisting of eight single-stranded, negative-sense RNA molecules that encode 10 proteins. The RNA segments are contained within the viral envelope in association with the nucleoprotein (NP) and three subunits of viral polymerase (PA, PB1, and PB2), which together form the ribonucleoprotein (RNP) complex responsible for RNA replication and transcription. Additional proteins contained within the virion include M2 and the viral nuclear export protein (NEP), which function in assembly and budding and in export of RNP from the nucleus, respectively. The only nonstructural protein of influenza A viruses is NS1, which has multiple functions in viral replication and is also thought to counteract interferon activity of the host thereby evading the immune response.

### 9.2.2. Classification

Based on antigenic differences in NP and M proteins, influenza viruses are classified as types A, B, and C. Influenza B and C viruses are not divided into subtypes. All avian influenza viruses are classified as type A. Further subtyping of influenza A viruses is based on antigenic differences between the two surface glycoproteins HA and NA. To date, 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) of influenza A viruses have been identified (Fouchier et al., 2005). The standard nomenclature for influenza viruses include the influenza type, the host of origin (excluding humans), the place of isolation, the strain number, the year of isolation, and finally the influenza A subtype in parentheses (e.g., A/Duck/Vietnam/11/04 [H5N1]).

### 9.2.3. Natural Hosts

The natural reservoir of influenza A viruses are aquatic birds, in which the viruses appear to have achieved an optimal level of host adaptation and do not cause disease (Webster et al., 1992). From this principal reservoir, viruses are occasionally transmitted to other animals, including mammals and domestic poultry, causing transitory infections and outbreaks. Through adaptation by mutation or genetic reassortment, some of these viruses may establish species-specific permanent lineages of influenza A viruses and cause epidemics or epizootics in the new host. In the human population, the establishment of these lineages in the 20th century was preceded by influenza pandemics. Transmission of viruses and transitory infections may also occur among the new hosts (e.g., between humans and pigs or chickens and humans).

Although all HA and NA subtypes are found in aquatic birds, the number of subtypes that have crossed the species barrier and established stable lineages in mammals is limited. Only three HA and two NA subtypes (i.e., H1N1, H1N2, H2N2, and H3N2) have circulated in humans since 1918. In horses, only two influenza A subtypes (H7N7 and H3N8) are found, while, despite susceptibility to all avian subtypes in experimental settings, the only subtypes recovered from pigs in nature are H1, H3, N1, and N2. The molecular, biological, or ecological factors determining the apparent subtype-specific ability of viruses to cross species barriers and spread among a range of hosts remain largely unresolved.

### 9.2.4. Determinants of Host Range

Although interspecies transmission does occur at times, there certainly are host-range restrictions. For example, avian influenza viruses

usually do not replicate efficiently in humans and vice versa (Hinshaw et al., 1983; Beare and Webster 1991). Relatively little is known about the viral and host factors governing the host range of influenza viruses and the mechanisms by which species barriers are crossed. However, in view of their role in entry of the virus, the viral HA glycoproteins and their sialic acid receptors on host cells clearly are important determinants of host-range restrictions. Human influenza strains preferentially bind to sialic acid residues linked to galactose by the  $\alpha$ 2,6 linkage, and avian and equine influenza strains recognize sialic acid linked to galactose by  $\alpha$ 2,3 linkage (Rogers et al., 1983; Rogers and Paulson 1983; Rogers and D'Souza 1989; Connor et al., 1994; Gambaryan et al., 1997; Matrosovich et al., 1997, 2004). Correspondingly, human respiratory epithelial cells predominantly contain  $\alpha$ 2,6 sialic acid–galactose linkages, whereas the host cells in birds and horses mainly contain  $\alpha$ 2,3 linkages (Couceiro et al., 1993; Ito et al., 1998; Matrosovich et al., 2004). Interestingly, in contrast with the human respiratory tract, epithelial cells in the human eye predominantly contain  $\alpha$ 2,3-linked sialic acid receptors, which may explain why conjunctivitis is a common symptom of human infections with avian influenza viruses (Paulsen et al., 1998; Terraciano et al., 1999; Diebold et al., 2003). It has been hypothesized that, by serving as the main port of entry and site of initial replication, the eye may play a role in the adaptation of avian influenza viruses to humans (Olofsson et al., 2005). The presence of  $\alpha$ 2, 3-linked sialic acid receptors has also recently been demonstrated in the lower respiratory tract of humans, i.e. on bronchiolar and alveolar cells, which may explain the propensity of avian H5N1 viruses to cause pneumonia and not upper respiratory illnesses, in humans (Shinya et al., 2006; van Riel et al., 2006).

Respiratory epithelial cells in the pig contain both  $\alpha$ 2,3 and  $\alpha$ 2,6 linkages, which explains why this animal is susceptible to both human and avian influenza viruses (Ito et al., 1998). Because of this trait, the pig is widely regarded as a potential source of new pandemic strains, because it could serve as a nonselective host in which mixed infection of avian and human strains efficiently occurs, potentially resulting in new reassortant viruses, or in which purely avian strains can adapt to human receptor recognition (Figure 9.1).

The receptor specificity of HA for either of the two sialic acid–galactose linkages is determined by the structure of the receptor-binding site of HA. Although several residues have been implicated, the amino acids at positions 226 and 228 particularly seem to determine HA receptor specificity, that is, Glu-226 and Gly-228 are predicted to have affinity for avian and equine receptors, whereas Leu-226 and Ser-228 confer specificity for human receptors (Wilson et al., 1981; Rogers et al.,

1983; Naeve et al., 1984; Weis et al., 1988; Suzuki et al., 1989). Albeit less important, substrate specificity of NA for either  $\alpha$ 2,3- or  $\alpha$ 2,6-linked sialic acid also contributes to the efficiency of viral replication in different hosts (Hinshaw et al., 1983). This is illustrated by the fact that during its evolution in humans, the NA of H2N2 viruses, which were of avian origin and therefore highly specific for hydrolyzation of  $\alpha$ 2,3-linked sialic acids, acquired high affinity for the human  $\alpha$ 2,6-linked sialic acids (Baum and Paulson, 1991). In addition to the surface glycoproteins, laboratory experiments with reassortant viruses suggest that the genes encoding internal proteins, such as M, NP, PB1 and PB2, may also play a role in determining the host range (Almond 1977; Scholtissek et al., 1978a; Snyder et al., 1987; Subbarao et al., 1993). However, because most of these experiments evaluated reassortant viruses with different constellations of gene segments, it remains difficult to interpret whether the proteins themselves contribute to host-range restrictions or whether certain combinations of gene segments from different origins are incompatible. Manipulation of the genome using reverse genetics approaches will undoubtedly provide more definitive insight in the role of other host range determinants.

### 9.2.5. Antigenic Variation and the Emergence of Pandemic Influenza Strains

#### 9.2.5.1. Antigenic Drift

Antigenic variation of influenza A viruses can occur gradually by accumulation of point mutations (antigenic drift) or drastically by genetic reassortment (antigenic shift). Antigenic drift, driven by immunological pressure on HA and NA, allows the virus to evade the immune response and is the reason that influenza viruses manage to cause yearly epidemics. It is also because of antigenic drift that periodic replacements of human vaccine strains are needed. In contrast with human and other non-avian influenza strains, antigenic drift in avian viruses is very limited despite similar mutation rates (Austin and Webster, 1986; Kida et al., 1987; Liu et al., 2004). Most likely, this reflects optimal adaptation of these viruses to the host resulting in limited immunological pressure and consequent evolutionary stasis of these viruses in their natural reservoir.

#### 9.2.5.2. Antigenic Shift

Drastic changes in antigenicity can occur through the acquisition of completely new surface proteins by genetic reassortment (Webster et al., 1982). The segmented nature of the influenza virus genome facilitates the

exchange of genes between two viruses (e.g., human and avian strains) that coinfect a host cell. Although such exchange can result in 256 possible combinations of the eight different genomic segments of the virus, antigenic shift only arises when the reassortment at least includes the HA gene. Provided that the reassortant virus is efficiently transmissible from infected to noninfected hosts, such an antigenically novel virus strain has pandemic potential when introduced in a population that completely lacks immunity against the new surface protein (Figure 9.1A). The pig is regarded as the ideal host for reassortment in view of its equal susceptibility for human and avian influenza strains (Ito et al., 1998). However, the increasing reports of bird-to-human transmissions of avian viruses indicate that coinfections, and consequently reassortments, could also take place in humans.

Beside genetic reassortment, antigenic shift is also caused by direct transmission of non-human influenza viruses to humans, as occurred or is still occurring on a relatively large scale in Hong Kong in 1997 (H5N1), in The Netherlands in 2003 (H7N7), and in Asia, the Middle East, Europe and Africa since 2004 (H5N1) (Yuen et al., 1998; Fouchier et al., 2004; Hien et al., 2004a). As is true for reassortant viruses, these viruses are of pandemic potential when acquiring the ability for efficient transmission between humans through adaptation in either humans or an intermediate host (Figure 9.1B).

Finally, antigenic shift can occur when a previously circulating human influenza virus reemerges after an extended period of time. This happened in 1977 when H1N1 virus, which circulated in the 1950s, reappeared in the human population (“Russian flu”), possibly after escaping a laboratory (Nakajima et al., 1978; Scholtissek et al., 1978b). The reemergence of this virus gave rise to a relatively mild pandemic affecting mainly young persons who were still immunologically naive to this subtype. The same could have happened in 2005, when H2N2 virus, which had disappeared from the human population after the emergence of H3N2 viruses in 1968, was inadvertently sent to more than 3000 laboratories worldwide as part of an external quality assurance scheme (Enserink, 2005).

### **9.3. Pathogenesis of Avian Influenza**

#### **9.3.1. Avian Influenza Virus Infections in Natural Hosts**

Avian influenza viruses can infect a wide range of domestic and wild birds, including (but not restricted to) chickens, ducks, turkeys, geese, quail, pheasants, seabirds, shore birds, and migratory birds. In these natural hosts, influenza viruses replicate in the gastrointestinal tract and are secreted in large amounts into the feces (Webster et al., 1978).

Transmission between birds occurs directly or indirectly through fecally contaminated aerosols, water, feed, and other materials.

The spectrum of disease in birds ranges from asymptomatic infection, to mild respiratory illness, to severe and rapidly fatal systemic disease. Most avian influenza viruses isolated from birds are avirulent (i.e., result in asymptomatic infection or only mild disease). Avian influenza viruses capable of causing outbreaks of severe disease (fowl plague) in chickens or turkeys are classified as highly pathogenic and are currently restricted to H5 and H7 subtypes. Typically, these highly pathogenic strains do not cause disease in ducks or geese. Infection of poultry by highly pathogenic avian influenza viruses is characterized by disseminated infection and clinically manifested by decreased egg production, respiratory signs, excessive lacrimation, edema of the head, diarrhea, neurological symptoms, and death.

### 9.3.2. Viral Determinants of Pathogenicity

The knowledge concerning the viral factors that determine the pathogenicity of influenza viruses is limited and is primarily derived from studies of highly pathogenic avian influenza viruses. A broad tissue tropism and the ability to replicate systemically are the hallmarks of these viruses. The most important and well-studied molecular correlate of these properties resides in the cleavability of the HA precursor glycoprotein (Webster and Rott, 1987; Garten and Klenk, 1999; Steinhauer, 1999). In the viral life cycle, post-translational cleavage of the precursor HA molecule into two subunits (HA1 and HA2) by host proteases is essential for infection to proceed. This cleavage generates a fusogenic domain at the amino terminus of HA2 that mediates fusion between the viral envelope and the endosomal membrane. HAs of avirulent avian influenza strains are cleaved only in a limited number of cell types, resulting in localized respiratory or gastrointestinal infections and mild illness. In contrast, HAs of highly pathogenic H5 and H7 strains can be cleaved in several different host cells, resulting in a broad cell tropism and the ability of causing systemic infection (Klenk and Garten, 1994; Senne et al., 1996). This apparent promiscuity of HA for a broad range of cellular proteases is determined by the structure of the HA cleavage site: HAs with high cleavability (i.e., from highly pathogenic strains) have multiple basic amino acid residues immediately upstream of the cleavage site, whereas HAs from avirulent subtypes usually have only a single arginine residue at this site (Bosch et al., 1981; Walker and Kawaoka, 1993; Senne et al., 1996; Chen et al., 1998). Evidence for the correlation between a multibasic cleavage site, susceptibility for proteases and virulence has been provided

by experiments in which viruses were generated with altered cleavage sites in otherwise unchanged genetic backgrounds (Ohuchi et al., 1991; Horimoto and Kawaoka, 1994). The reason why multibasic cleavage sites seem restricted to the HAs of H5 and H7 subtypes is unclear but may suggest that the number of basic residues is limited by structural features of HA. Analyses of nucleotide sequences of H5 and H7 HA genes has shown the occurrence of direct repeats of purine-rich sequences (AAGAAA) at the cleavage site in many cases (Hirst et al., 2004). Such repeats may arise because of pausing of the transcriptase-complex at a region of secondary structure, resulting in slippage of the complex and insertion of a short repeat sequence. Additionally, recombination events between two genes of the same virus (e.g., from M or NP to HA) may result in the insertion at the cleavage site of short sequences that code for multibasic amino acid residues (Orlich et al., 1994; Suarez et al., 2004). In addition to the presence of multiple basic amino acids, susceptibility to ubiquitous proteases is also determined by the loss of a glycosylation site in the vicinity of the cleavage site (Deshpande et al., 1987; Kawaoka and Webster, 1988, 1989).

Although HA clearly is an important determinant of viral pathogenicity, animal studies indicate that virulence in mammals is a polygenic trait involving a constellation of other genes that can vary with the specific virus strain and host (Lipatov et al., 2004). However, besides HA, two genes have specifically been implicated in viral pathogenicity in mammals; that is, PB2 and NS.

By reverse genetics experiments, it has been shown that a lysine residue at position 627 (Lys627) of PB2 seems essential for high virulence and systemic replication in mice of highly pathogenic influenza H5N1 viruses responsible for the outbreak among poultry and humans in Hong Kong in 1997 (H5N1/97) (Hatta et al., 2001). The presence of Lys627 in PB2 of H5N1/97 viruses appears to determine the viral replicative efficiency in mouse cells (and not avian cells) but does not increase the tissue tropism of the virus in mice (Shinya et al., 2004). Lys627 has also been found in PB2s of some, but not all highly pathogenic H7N7 and H5N1 viruses isolated from humans during the outbreaks of these viruses among poultry and humans in 2003 (The Netherlands) and 2004 (Viet Nam, Thailand), respectively (Fouchier et al., 2004; Li et al., 2004). Interestingly, a lysine residue at position 627 of PB2 is also present in all human influenza subtypes (H1, H2, H3) (Webster, 2001). Furthermore, single-gene reassortant viruses carrying a PB2 gene of avian origin and all other genes from a human virus showed efficient replication in avian but not mammalian cells (Subbarao et al., 1993). This host cell restriction could be traced to a glutamic acid residue at position 627 of the avian PB2

instead of a lysine residue at the same position in the human virus (Subbarao et al., 1993). These observations suggest that an amino acid change to Lys627 in PB2 may help avian viruses to adapt to efficient replication in mice and possibly other mammals, thereby increasing the virulence in these hosts. Reverse genetics experiments with highly pathogenic avian influenza H5N1 and H7N7 viruses have indicated that other members of the viral polymerase complex beside PB2, i.e. PA and PBI, likely also play a role in adaptation of avian viruses to the mammalian host, suggesting that host factors are important for viral polymerase activity (Gabriel et al., 2005; Salomon et al., 2006).

In addition to PB2, the NS gene seems to play a role in the pathogenesis of avian and human influenza virus infections. This gene encodes two proteins: the nuclear export protein (NEP) and the only non-structural protein of influenza viruses, NS1. The NS1 gene or its product contribute to viral pathogenesis by allowing the virus to evade the interferon response of the host (Garcia-Sastre, 2001, 2002; Krug et al., 2003). This evasion may occur through multiple mechanisms, including interference with the activation of cell-signaling pathways and protein kinases involved in interferon induction or interference with the maturation of cellular pre-mRNA at the post-transcriptional level. The NS gene has also been implicated in determining the high pathogenicity of influenza H5N1/97 viruses in mammals. Experiments in pigs using recombinant viruses showed that the presence of the NS gene of H5N1/97 viruses greatly increased the pathogenicity of an H1N1 virus, possibly by escaping the antiviral effects of interferons and tumor necrosis factor alpha (TNF- $\alpha$ ) (Seo and Webster 2002; Seo et al., 2002, 2004). This enhanced virulence in pigs required the presence of glutamate instead of aspartate at position 92 (Glu-92) of the H5N1 NS gene, but this amino acid change has not been found in all highly pathogenic H5N1 viruses isolated from humans or animals (Seo et al., 2002).

Beside the apparent cytokine resistance of H5N1/97 viruses, *in vitro* studies in human macrophages and respiratory cells showed that these viruses also seem to induce the transcription of proinflammatory cytokines, in particular TNF- $\alpha$  and interferon- $\beta$ , and that the NS gene contributes to this induction (Cheung et al., 2002; Chan et al., 2005). Similar results were obtained in mice, in which infection with a recombinant H1N1 virus containing the H5N1/97 NS gene caused a cytokine imbalance in mouse lungs, characterized by increased concentrations of proinflammatory cytokines and decreased levels of anti-inflammatory cytokines (Lipatov et al., 2005a). Cytokine dysregulation by H5N1/97 viruses is also suggested by observations in human infections.

Pathological examination of patients who died of influenza H5N1 infection during the 1997 outbreak in Hong Kong showed reactive hemophagocytic syndrome, which is believed to be a cytokine-driven condition, as the most prominent feature (To et al., 2001). In addition, exceptionally high levels of certain chemokines were observed in the serum of human cases with avian influenza H5N1 (Peiris et al., 2004). Together, these observations may suggest that a combination of increased resistance against, and high induction of cytokines by the virus synergistically lead to a profound cytokine dysregulation that may play a role in explaining the severity of illness in mammals, including humans. Although the NS gene seems to play a crucial role in this, it is likely that other particular gene constellations involving different internal genes also contribute.

### 9.3.3. Host Factors

The virulence of highly pathogenic avian influenza viruses is clearly influenced by the specific host. Two variants of the H5N1/97 virus, one of which was isolated from a human patient with mild respiratory illness and the other from a fatal human case, displayed similar differential pathogenicity in mice (Zitzow et al., 2002). However, in ferrets, both variants caused indistinguishable severe systemic disease (Zitzow et al., 2002). Conversely, experimental infection with H5N1/97 viruses exhibiting high virulence in mice caused localized respiratory illness without systemic spread in primates and only viral replication in the respiratory tract without clinical illness in pigs (Shortridge et al., 1998; Rimmelzwaan et al., 2001). The host factors determining the clinical outcome in animals are unclear.

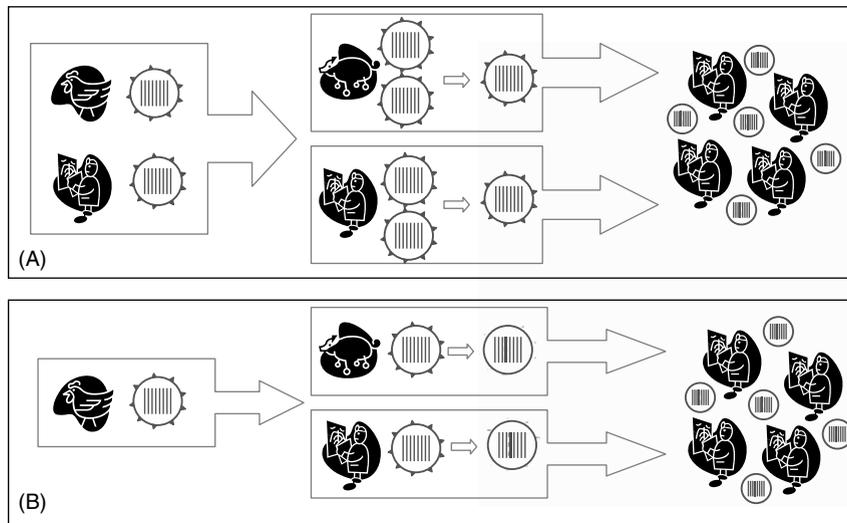
The clinical outcome of human influenza is influenced by factors such as the patient's age, the level of preexisting immunity, immunosuppression, comorbidities, pregnancy, and smoking habits, indicating that host-related factors certainly contribute to pathogenesis in humans. Most of the above factors may be explained by differences in local, innate, or specific immunity at different stages of life or under specific circumstances, but other factors likely also play a role. For example, the observation that influenza-related encephalopathy seems well-recognized in Japan but less so in other countries may suggest that there are differences in proneness for certain disease manifestations among populations, possibly related to genetic differences (Morishima et al., 2002; Sugaya, 2002). Although evidence is lacking at present, it is not unlikely that host factors also play a role in the susceptibility and pathogenesis of human infections with avian influenza viruses.

## 9.4. Avian Influenza Viruses Infecting Humans

### 9.4.1. Pandemics of the 20th Century

Introduction of an influenza A virus with a novel HA gene in a population that lacks immunity to this HA has the potential to cause a pandemic when the virus possesses the ability to spread efficiently among humans (Figure 9.1). During the 20th century, this has happened three times, in 1918, 1957, and 1968, killing millions of people worldwide. In all three pandemics, the viruses originated from avian influenza viruses.

The virus strains responsible for the influenza pandemics of 1957 and 1968 both first emerged in southeastern Asia, and both arose through reassortment of genes between avian viruses and the prevailing human influenza strain (Scholtissek et al., 1978c). The “Asian influenza” pandemic of 1957 was caused by an H2N2 virus that had acquired three genes (H2, N2, and PB1) from avian viruses infecting wild ducks, in a backbone of the circulating H1N1 human influenza strain. As the Asian flu strain emerged and established a permanent lineage, the H1N1 strains soon disappeared from the human population for unclear reasons. Similarly, the H3N2 virus causing the “Hong Kong influenza” pandemic of 1968



**Figure 9.1.** Mechanisms for generation of a pandemic influenza A strain. Pandemic influenza A strains could result from genetic reassortment involving the hemagglutinin gene between avian and human strains in coinfecting pigs or humans, followed by adaptation to human receptors in either host and human-to-human transmission (A); or through adaptation to humans of a purely avian influenza strain, either in humans or in an intermediate host such as the pig (B). Mechanism A was implicated in the “Asian” (1957) and “Hong Kong” (1968) influenza pandemics. The H1N1 virus that caused the “Spanish flu” influenza pandemic of 1918 likely resulted from mechanism B.

consisted of two genes from a duck virus (H3 and PB1) in a background of the human H2N2 strain circulating at that time. The latter virus disappeared with the emergence of the H3N2 virus and since then has not been detected in humans. Sequence analysis of the hypothetical precursor strain that immediately preceded the pandemic H3N2 virus suggested that fewer than six amino acids in HA had changed during the avian-to-human transition (Bean et al., 1992). Interestingly, a number of these changes may reflect adaptation to the new host because they modified the area surrounding the receptor-binding pocket of HA, including a Glu to Leu change at position 226, which is particularly implicated in determining specificity for human receptors (see Section 9.2.4). The fact that beside one or two novel surface glycoproteins, both pandemic strains also possessed a PB1 gene of avian origin is intriguing and may suggest a role of this gene in interspecies transmission (Kawaoka et al., 1989).

Although millions of people died during the 1957 and 1968 pandemics, the viruses involved did not appear particularly virulent, suggesting that lack of immunity was the main reason for the excess mortality. This was different during the “Spanish flu” pandemic of 1918, in which lack of immunity in the human population was combined with an apparent extremely high virulence of the virus, resulting in the demise of up to 100 million people worldwide. Because the 1918 pandemic occurred before viruses were identified as the causative agents, no intact virus has been available for analysis. This and the similar lack of available human and animal influenza strains circulating before 1918 has made it difficult to determine the exact origin of the pandemic H1N1 virus and the reason for its extreme virulence. However, valuable insight has been provided by the recovery of fragments of viral RNA isolated from archived autopsy specimens and tissue from Alaskan flu victims buried in the permafrost (Taubenberger et al., 1997). This enabled sequence analysis of all eight genes of the virus (Reid et al., 2004; Taubenberger et al., 2005). Phylogenetic analyses of these genes suggest that the 1918 H1N1 virus may not have arisen by the same mechanism as the 1957 and 1968 pandemic viruses (i.e., by reassortment of avian and human influenza viruses) but perhaps by direct transmission from an avian source after adaptation in humans or another permissive mammalian host, such as the pig (Reid et al., 2004; Taubenberger et al., 2005). This is supported by the observation that human H1N1 strains, including the 1918 pandemic strain, have retained the amino acid residues at positions 226 and 228 of HA predictive for binding to avian receptors (see Section 9.2.4) (Taubenberger et al., 1997). Recent crystallographic studies showed that structural changes in the H1 HA allowed the virus to recognize human receptors despite the presence of these avian-like residues, which may explain why

the virus could nevertheless efficiently infect and spread among humans (Gamblin et al., 2004; Stevens et al., 2004). The possibility that the 1918 strain had retained the structure and biological properties of its avian ancestors while acquiring the ability to recognize and efficiently infect human cells may explain the high virulence of this virus. Mathematical modeling studies have suggested that the transmissibility of the 1918 virus was not remarkably different than regular human influenza strains, indicating that extremely efficient spread did not account for the high morbidity and mortality (Mills et al., 2004). Although part of the high mortality of the 1918 pandemic could be explained by the lack of antibiotics to treat secondary bacterial pneumonia and poor living conditions, the extremely rapid and severe clinical course implies high pathogenicity of the virus as the major cause. The molecular basis for this high virulence remains unclear. The 1918 H1 HA lacks the multibasic cleavage site characteristic of highly pathogenic avian influenza viruses (see Section 9.3.2) (Taubenberger et al., 1997; Reid et al., 1999). There are conflicting observations concerning the role of the NS gene in the 1918 pandemic strain. In mice, the presence of the complete NS or only the NS1 segment seemed to confer decreased, rather than enhanced pathogenicity of reassortant H1N1 viruses (Basler et al., 2001). In contrast, *in vitro* experiments in human lung cells suggested more efficient inhibition of interferon-regulated genes by H1N1 virus in the presence of the 1918 NS gene (Geiss et al., 2002). The most convincing evidence implicates HA as an important determinant of the high virulence. The presence of HA of the 1918 virus conferred high pathogenicity in mice to human strains that were otherwise non-pathogenic in this host (Kobasa et al., 2004). Furthermore, these infections were associated with severe hemorrhagic pneumonia and the induction of high levels of macrophage-derived cytokines and chemokines, strikingly reminiscent of clinical observations in humans during the Spanish flu pandemic, as well as of recent *in vitro* and *in vivo* observations of infections with highly pathogenic avian influenza H5N1 viruses (Oxford 2000; To et al., 2001; Cheung et al., 2002; Peiris et al., 2004).

#### 9.4.2. H7N7 Viruses

Before the year 2003, a few isolated cases of human infections with highly pathogenic H7N7 viruses were reported. These infections concerned laboratory accidents involving exposure to viral cultures or infected animals, and in one case presumed exposure to infected waterfowl in the absence of an overt outbreak (DeLay et al., 1967; Campbell et al., 1970; Taylor and Turner, 1977; Webster et al., 1981; Kurtz et al.,

1996). All reported cases, except one, were clinically characterized by self-limiting conjunctivitis. Influenza H7N7 virus was isolated from blood of one patient with hepatitis, but the clinical relevance of this finding was unclear as was the source of this infection (DeLay et al., 1967; Campbell et al., 1970).

In 2003, a large-scale outbreak of H7N7 viruses decimated the poultry industry in The Netherlands and was associated with several human infections (Fouchier et al., 2004; Koopmans et al., 2004). After diagnosing the first case of human infection with H7N7 virus, active case finding among exposed persons and their close contacts identified a total of 89 laboratory-confirmed infections in humans (Koopmans et al., 2004). This amounted to approximately 2% of the estimated number of people potentially exposed to the virus. Highest infection rates were observed in veterinarians and chicken cullers.

Of the 89 H7N7 cases, 83 persons presented with conjunctivitis, of whom 5 also complained of influenza-like symptoms (Fouchier et al., 2004; Koopmans et al., 2004). It cannot be excluded that the remaining six patients also had conjunctivitis. Although two individuals reported an influenza-like illness only, one had suffered a previous eye injury that precluded evaluation of conjunctivitis, while the other had chronic blepharitis. Four individuals did not fit any case definition, either because of missing data or because they complained of red eyes only and therefore did not meet the criteria for a diagnosis of conjunctivitis. The prominence of conjunctivitis as the presenting syndrome in these and other reported human cases of influenza H7N7 is striking and may be explained by the presence of  $\alpha$ 2,3-linked sialic acid receptors in the eye, which are preferentially recognized by HA of avian influenza viruses, including H7N7 subtypes (see Section 9.2.4) (Olofsson et al., 2005). The observation that, in contrast with human influenza strains, viral loads in conjunctival specimens of the Dutch H7N7-infected individuals seemed higher than in respiratory specimens, supports the notion that H7N7 virus may replicate efficiently in cells in or near the eye and not in the respiratory tract (Fouchier et al., 2004).

Six of the seven cases of influenza-like illnesses were mild. One patient, a previously healthy 57-year-old veterinarian, complained of fever and headache 2 days after visiting an infected farm (Fouchier et al., 2004). He subsequently developed pneumonia complicated by acute respiratory distress syndrome and multiorgan failure, of which he died 13 days after the onset of illness. Autopsy revealed pathologic changes in the lungs similar to those found in influenza H5N1-infected humans and no significant abnormalities in other organs. Direct human-to-human transmission of H7N7 virus during the Dutch outbreak is suggested by the fact

that three individuals with confirmed infections had not been in direct contact with infected poultry but were family members of poultry workers with H7N7 conjunctivitis (Koopmans et al., 2004).

The H7N7 virus causing the outbreak in The Netherlands most likely evolved from a low pathogenic virus from wild ducks after the introduction of this virus into the poultry population (Fouchier et al., 2004). In agreement with its classification as a highly pathogenic avian influenza virus, the HA contained multiple basic amino acids at the cleavage site. Sequence comparison of virus isolates from chickens and humans, including those implicated in human-to-human transmission, revealed virtually no differences, indicating no significant accumulation of mutations on bird-to-human or human-to-human transmission. The only exception was the virus isolated from the fatal case, which showed a total of 14 amino acid substitutions not seen in the other isolates. Most of these substitutions involved the HA, NA, and PB2 genes, which have all been implicated as determinants of host range and pathogenicity. Intriguingly, the mutations in PB2 included a glutamine to lysine change at positions 627, associated with high virulence of H5N1 viruses in mice (see Section 9.3.2) (Fouchier et al., 2004).

The H7N7 outbreak in poultry was effectively contained by the culling of approximately 30 million chickens, which amounts to about 28% of the total chicken population in The Netherlands (Koopmans et al., 2004). After the first human infections were identified, individuals exposed to potentially infected chickens were vaccinated with the available human vaccine to prevent possible dual infection with human and avian strains and the resulting risk of reassortment. As the outbreak progressed, the recommendation for vaccination was extended to all poultry farmers in a 3-km radius of infected farms and to persons suspected of H7N7 infection. In addition, a prophylactic regimen of the neuraminidase-inhibitor oseltamivir was started for all people handling potentially infected poultry to prevent bird-to-human transmission and human-to-human transmission of avian viruses. Prophylactic treatment was to be continued for 2 days after the last exposure. These control measures may serve as a model for the control of emerging influenza viruses because they, at least theoretically, minimize the possibility that the virus spreads among the human population.

#### 9.4.3. H7N3 Viruses

In early 2004, an outbreak of highly pathogenic H7N3 viruses occurred in poultry farms in British Columbia, Canada (Tweed et al., 2004). The causative virus probably had evolved by homologous recombination

between the HA and M genes in a low pathogenic H7N3 virus (Hirst et al., 2004). This recombination event resulted in the introduction of a multi-basic sequence at the cleavage site of HA.

Surveillance among potentially exposed people identified two laboratory-confirmed cases of H7N3 infection. In these cases, conjunctivitis and mild influenza-like symptoms (coryza, headache) developed 1–3 days after exposure (Tweed et al., 2004). Both were treated with oseltamivir and recovered fully. No secondary cases were identified. The outbreak among poultry was contained by extensive culling. Control measures in potentially exposed people were similar to those during the Dutch H7N7 outbreak.

#### 9.4.4. H9N2 Viruses

In 1999, human infections with H9N2 viruses were reported in two unrelated children from Hong Kong, aged 1 and 4 years (Peiris et al., 1999). Both children had a mild influenza-like syndrome, associated with mild lymphopenia in one, and slightly raised transaminase levels in the other child. Neither child developed pneumonia and both recovered uneventfully within 3–7 days. There was a history of probable contact with live chickens in one of the patients, but otherwise the source of transmission was unclear. No serological evidence of H9N2 infection was found in the children's family members or health care workers. Three serum samples from 150 volunteer blood donors in Hong Kong showed the presence of neutralizing antibodies against H9N2 virus, suggesting that additional infections had occurred in Hong Kong (Peiris et al., 1999). Around the same time as the infections in Hong Kong, five additional, similarly mild cases of human H9N2 in humans were reported from mainland China (Guo et al., 1999).

The human infections in Hong Kong and mainland China were caused by non-highly pathogenic H9N2 viruses of two distinct lineages. The Hong Kong virus was related to a quail H9N2 virus (A/quail/HK/G1/97 [H9N2]) and possessed internal genes similar to the H5N1 virus that caused the outbreak in poultry and humans in 1997 (Guan et al., 1999, 2000; Lin et al., 2000). This may suggest that the quail H9N2 virus has been the donor of all internal genes to the H5N1 outbreak strain (Guan et al., 1999). The strains isolated from humans in mainland China were related to a different lineage of H9N2 viruses found in ducks and chickens (A/duck/HK/Y280/97 [H9N2] and A/Chicken/HK/G9/97 [H9N2]) (Guo et al., 2000). Most H9N2 strains isolated since 1999 seem to be related antigenically to the latter virus but possess a variety of internal gene constellations, including those of H5N1/97-like origin (Choi et al., 2004; Lipatov et al., 2004).

Interestingly and perhaps concerning, H9N2 viruses isolated from poultry have acquired a preference for binding to the human-like  $\alpha 2,6$  sialic acid–galactose linkages, which may suggest that certain species of poultry could act as an intermediate host in the zoonotic transmission of influenza viruses from their natural reservoir in aquatic birds to mammals, including humans (Matrosovich et al., 2001). Indeed, H9N2 viruses have also been isolated from pigs in Southeastern China, indicating widening of the host range (Peiris et al., 2001). In addition, contemporary human H3N2 strains are cocirculating in southeastern Chinese pigs, providing ideal circumstances for potential genetic reassortment leading to the emergence of viruses with pandemic potential (Peiris et al., 2001).

#### 9.4.5. H5N1 Viruses

##### 9.4.5.1. Outbreaks of Influenza H5N1 in Poultry and Humans

In recent years it has become clear that, in contrast with the usually mild illnesses caused by H7 and H9N2 viruses, human infections with highly pathogenic influenza H5N1 viruses are associated with severe, often fatal disease. In May 1997, after outbreaks of influenza H5N1 among poultry on three farms in the New Territories of Hong Kong, an influenza H5N1 virus was isolated from a 3-year-old boy in Hong Kong, who died of severe pneumonia complicated by acute respiratory distress syndrome and Reye syndrome (Subbarao et al., 1998). In November and December of the same year, concomitant with outbreaks of influenza H5N1 among chickens in poultry markets and on farms in Hong Kong, 17 additional cases of human H5N1 infections were identified, five of which were fatal (Yuen et al., 1998; Chan 2002). The outbreak was contained after the slaughtering of all 1.5 million chickens in Hong Kong. In response to the outbreak, influenza surveillance in poultry was intensified permitting early recognition of other outbreaks of avian influenza in 2001 and 2002. No further human H5N1 infections were reported until February 2003, when two laboratory-confirmed cases and one probable case were identified in one family from Hong Kong (Peiris et al., 2004). The daughter died of an undiagnosed respiratory infection while visiting Fujian Province in mainland China. Upon their return to Hong Kong, the father and son developed severe respiratory illnesses of which the father died. H5N1 virus was isolated from both patients.

In December 2003, an outbreak of highly pathogenic H5N1 virus was identified among poultry in the Republic of Korea (Lee et al., 2005). Subsequently, outbreaks by antigenically related viruses were reported among poultry in Thailand, Viet Nam, Japan, China,

Cambodia, Laos, Malaysia, and Indonesia. The reason for this apparent simultaneous occurrence of H5N1 outbreaks in many Asian countries remains unclear. However, H5N1 viruses have also been found in dead migratory birds, which may suggest a role of wild birds in the spread of H5N1 viruses in the region (Li et al., 2004). Since 2005, migratory birds indeed have also been responsible of spreading the virus to regions outside Asia, including several countries in the Middle East, Europe and Africa.

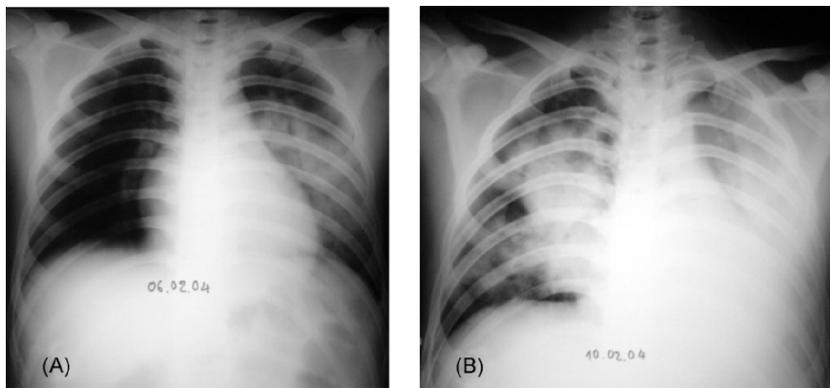
Human infections during the Southeast Asian outbreaks were first reported in early 2004 from Viet Nam and Thailand, (Hien et al., 2004a; Chotpitayasunondh et al., 2005). Since then, concurrent with the spread of the virus by migrating birds and consequent poultry outbreaks elsewhere, human H5N1 infections have been reported in several other countries in Asia (China, Cambodia, Indonesia), Eurasia and Europe (Azerbaijan, Iraq, Turkey), and Africa (Egypt, Djibouti). At the time of this writing (May 2006) more than 200 human infections have been reported worldwide of which more than half were fatal (WHO, 2005). It cannot be excluded and may even be likely that additional cases have gone unnoticed in affected countries due to a lack of clinical awareness, active surveillance, or diagnostic facilities (Hien et al., 2004b).

Although many countries initially affected by poultry outbreaks in 2004 have been declared free of the virus, H5N1 virus seems to have reached endemic levels in poultry and aquatic birds in several Asian countries, despite attempts to contain the outbreak by extensive culling of poultry. This is also suggested by the establishment of multiple geographically distinct sublineages of H5N1 influenza viruses in Asia (Chen et al., 2006). Continuing occurrences of bird-to-human transmissions increase the opportunity of the virus to adapt to humans and acquire the ability to spread between humans. In addition, continuing cocirculation of avian and human viruses in countries, where humans live in close proximity with poultry and pigs, increases the risk of reassortment between both in coinfecting humans or other mammalian hosts, such as the pig. The isolation of H5N1 viruses from pigs in China and Indonesia is concerning in this respect (Chen et al., 2004). For all these reasons, the current developments in Asia and other regions in the world seem to justify the global concern that, similar to 1957 and 1968, a new pandemic influenza strain may emerge in the near future.

#### 9.4.5.2. *The Clinical Spectrum of Human H5N1 Infections*

At presentation, most cases of human H5N1 infections were characterized by a severe influenza syndrome, clinically indistinguishable

from severe human influenza, with symptoms of fever, cough, and shortness of breath, and radiological evidence of pneumonia (Yuen et al., 1998; Hien et al., 2004a; Chotpitayasunondh et al., 2005). Abnormalities on chest radiographs at presentation included extensive, usually bilateral infiltration, lobar collapse, focal consolidation, and air bronchograms (Figure 9.2). Radiological evidence of pulmonary damage could still be observed in surviving patients several months after the illness (T.T. Hien, personal communication). Beside respiratory symptoms, a large proportion of patients also complained of gastrointestinal symptoms such as diarrhea, vomiting, and abdominal pain, which are common in children with human influenza, but not in adults. In some cases, diarrhea was the only presenting symptom, preceding other clinical manifestations (Apisarnthanarak et al., 2004; de Jong et al., 2005). Unlike human infections with H7 or H9 viruses, conjunctivitis was not prominent in H5N1-infected patients. The clinical course of the illness in severe cases was characterized by rapid development of severe bilateral pneumonia necessitating ventilatory support within days after onset. Complications included acute respiratory distress syndrome, renal failure, and multiorgan failure. Evidence that the clinical spectrum of human H5N1 infections is not restricted to pulmonary symptoms was provided by a reported case of possible central nervous system involvement in a Vietnamese boy who presented with diarrhea, followed by coma and death. Influenza H5N1 virus was isolated from throat, rectal, blood, and cerebrospinal fluid specimens, suggesting widely disseminated viral replication (de Jong et al., 2005). His sister had died of a similar illness 2 weeks earlier, but no diagnostic specimens were obtained. Although highly virulent H5N1 viruses have shown neurotropism in mammals such as mice and



**Figure 9.2.** Influenza H5N1 pneumonia. Chest radiographs of an influenza H5N1 patient obtained at admission (A) and 4 days later (B).

cats (Lipatov et al., 2003; Tanaka et al., 2003; Keawcharoen et al., 2004), these cases may be similarly rare as central nervous system manifestations associated with human influenza (Morishima et al., 2002; Sugaya, 2002). Genetic predisposition of the host to such manifestations may play a role.

Striking routine laboratory results in H5N1-infected patients, especially in severe cases, were an early onset of lymphopenia, with a pronounced inversion of the CD4+/CD8+ ratio, thrombocytopenia, and increased levels of serum transaminases (Yuen et al., 1998; Hien et al., 2004a; Chotpitayasunondh et al., 2005). High levels of cytokines and chemokines have been observed in several H5N1-infected patients, suggesting a role of immune-mediated pathology in the pathogenesis of H5N1 infections (see Section 9.3.2) (To et al., 2001; Peiris et al., 2004). This was supported by pathological examination in two patients who died during the outbreak in Hong Kong, which showed reactive hemophagocytosis as the most prominent feature (To et al., 2001). Other findings included diffuse alveolar damage with interstitial fibrosis, hepatic central lobular necrosis, acute renal tubular necrosis, and lymphoid depletion. Although the gastrointestinal, hepatic, renal, and hematologic manifestations could suggest wider tissue tropism, there was no evidence of viral replication in organs outside the respiratory tract (To et al., 2001).

Although many laboratory-confirmed H5N1 infections were associated with severe, often fatal disease, milder cases have also been reported, especially during the outbreak in Hong Kong (Yuen et al., 1998; Chan 2002). An increasing number of milder cases also seemed to occur in Viet Nam, as the outbreak progressed in 2005 (WHO, 2005). Although increased clinical awareness and surveillance may account for such observations, progressive adaptation of the virus to humans is the dreaded alternative explanation. Detailed monitoring of virus evolution during outbreaks is obviously important and may help to distinguish between both possibilities. The occurrence of mildly symptomatic and asymptomatic infections have also been suggested during the outbreak in Hong Kong by seroepidemiological studies in household members of H5N1-infected patients and health care workers. In these studies, 8 of 217 exposed and 2 of 309 nonexposed health care workers were seropositive for H5N1-specific antibodies (Bridges et al., 2002). Seroconversion was documented in two exposed nurses, one of whom reported a respiratory illness 2 days after exposure to an H5N1-infected patient. More importantly than showing the occurrence of asymptomatic infections, these data indicated that nosocomial person-to-person transmission had occurred, albeit limited to a few cases. An additional case of possible human-to-human transmission during the Hong Kong outbreak was suggested by

H5N1-seropositivity in a household contact of a patient, who had no history of poultry exposure (Katz et al., 1999). Seroepidemiological studies in health care workers involved in the care of H5N1-infected patients in Thailand and Viet Nam in 2004 have not shown evidence of person-to-person transmission, despite the absence of adequate infection control measures in the Vietnamese cohort at the time of study (Apisarnthanarak et al., 2005; Liem and Lim, 2005; Schultsz et al., 2005). During the outbreak in Thailand in 2004, extensive epidemiological investigations have suggested person-to-person transmission from a child, who died of presumed H5N1 infection, to her mother who had no history of exposure to poultry and had provided prolonged unprotected nursing care to her daughter (Ungchusak et al., 2005). An aunt of the child may have been infected by the same route because her last exposure to poultry before infection had been 17 days, considerably longer than the estimated incubation period of 2–10 days. There have been several similar family clusters of H5N1 cases in other affected countries, which have all ignited concerns about the possibility of human-to-human transmission, but most of which could be explained by common exposure to poultry. Although there has been no evidence of efficient transmission of influenza H5N1 virus between humans to date, caution and detailed investigations obviously remain warranted in case of any cluster of infections, especially in view of the relatively rapid evolution H5N1 viruses have exhibited in recent years.

#### 9.4.5.3. *The Evolution of H5N1 Viruses, 1997–2004*

In 1996, an H5N1 virus was isolated from geese during an outbreak in Guangdong Province in China (influenza A/Goose/Guangdong/1/96 [A/G/Gd/96]) (Xu et al., 1999). This virus proved to be the donor of the HA gene of the reassortant H5N1 viruses causing the outbreak among poultry and humans in Hong Kong in 1997. The internal genes of the Hong Kong H5N1 viruses were closely related to those of an H9N2 virus isolated from quail (see Section 9.4.3) (Guan et al., 1999). The origin of the NA gene remains unclear but was notable for a 19-amino-acid deletion in the stalk region (Subbarao et al., 1998). Such deletions may be associated with adaptation of influenza viruses to land-based poultry (Matrosovich et al., 1999). The HA gene contained multibasic sequences at the cleavage site, in accordance with its classification as a highly pathogenic strain (Claas et al., 1998; Matrosovich et al., 1999). The role of other genes potentially involved in its pathogenicity is reviewed in Section 9.3.2.

After the eradication of the 1997 Hong Kong strain, the goose precursor viruses continued to circulate in geese in southeastern China

(Cauthen et al., 2000; Webster et al., 2002). Through reassortment between this virus and other avian viruses, multiple antigenically similar genotypes, which were highly pathogenic in chickens but not in ducks, emerged and again were eradicated in Hong Kong in 2001 and 2002 (Guan et al., 2002). Then, in late 2002, H5N1 strains isolated from wild migratory birds and resident waterfowl in two Hong Kong parks showed marked antigenic drift and exhibited high pathogenicity in ducks (Guan et al., 2004; Sturm-Ramirez et al., 2004). The latter property is rarely found in nature and had not been observed in strains isolated during previous years. An antigenically and molecularly similar virus caused the two confirmed human infections in early 2003 in a family from Hong Kong (see Section 9.4.5.1) (Guan et al., 2004; Peiris et al., 2004).

H5N1 influenza viruses isolated from healthy ducks in southern China between 1999 and 2002 were all antigenically similar to the precursor influenza A/G/Gd/96 virus (Chen et al., 2004). It is thought that these ducks played a central role in the generation of the virus responsible for the outbreaks in Southeast Asia since 2003. Detailed genetic analyses of H5N1 strains isolated during the period 2000–2004 from poultry and humans in China, Hong Kong, Indonesia, Thailand, and Viet Nam demonstrated that a series of genetic reassortment events, all traceable to the A/G/Gd/96-precursor virus, ultimately gave rise to a dominant H5N1 genotype (genotype Z) in chickens and ducks (Li et al., 2004). This genotype is implicated in the human cases in Hong Kong in 2003 and the outbreaks among poultry and humans since 2004.

The evolution of H5N1 viruses in recent years has been associated with increasing virulence and an expanding host range, which beside terrestrial poultry and wild birds also includes mammals. Although all H5N1 viruses isolated from ducks in China between 1999 and 2002 were highly pathogenic in chickens, an increasing level of pathogenicity was observed in mice with the progression of time: virus isolated in 1999 and 2000 were less pathogenic than those isolated in 2001 and 2002 (Chen et al., 2004). It has been suggested that the increasing ability to replicate in mammals has resulted from transmission between ducks and pigs. The expanding host range is also illustrated by successful experimental infection of domestic cats and natural infections of cats, tigers and leopards with recent H5N1 strains (Keawcharoen et al., 2004; Kuiken et al., 2004; Songserm et al., 2006).

In summary, continued evolution of H5N1 viruses since 1997, involving multiple genetic reassortment events between A/G/Gd/96-like viruses and other avian viruses and perhaps transmission between birds and pigs or other mammalian hosts, has resulted in a highly virulent genotype with an expanded host range that is causing widespread outbreaks among wild birds,

poultry and humans affecting several regions in the world. Although transmission between birds and humans at present still seems inefficient, as does transmission between humans, this may change when the virus is allowed to continue its evolution through adaptation and reassortment.

### 9.5. Laboratory Diagnosis of Avian Influenza

In clinical trials, human influenza has been clinically diagnosed correctly in approximately two-thirds of adults with influenza-like symptoms, despite the lack of pathognomonic features (Monto et al., 2000). Although virus isolation remains the gold standard of diagnosis and indispensable for virus characterization, rapid laboratory confirmation of suspected human influenza in routine diagnostic laboratories is usually performed by immunochromatographic or immunofluorescent detection of influenza virus antigens or by reverse transcriptase (RT)-PCR detection of viral nucleic acids in respiratory specimens. In addition, serological evidence of human influenza A virus infection can be obtained by commercially available ELISA kits that detect antibodies to conserved viral antigens, such as the nucleoprotein. In the absence of cocirculating avian influenza strains in the human population, further subtyping of influenza viruses or detection of subtype-specific antibodies are usually not done by routine diagnostic laboratories but are restricted to reference laboratories involved in epidemiological analyses and planning of vaccine strains. However, in case of an outbreak of avian influenza, efforts to further subtype the virus (e.g., by subtype-specific RT-PCR methods) should be made by routine laboratories because immediate knowledge about the infecting influenza subtype is essential for infection control and timely epidemiological investigations. Dependence on reference laboratories, which in the case of many Southeast Asian countries affected by avian influenza outbreaks are situated abroad, potentially results in unacceptable delays and hampers timely recognition of outbreaks and institution of adequate control measures (Hien et al., 2004b). However, the reality is that diagnostic facilities in many affected countries are scarce and often not sufficiently equipped for virological diagnostics, let alone subtyping of influenza viruses. Global efforts to improve diagnostic capacity in resource-poor countries may prove an important step toward the prevention and control of pandemic influenza (Hien et al., 2004b).

#### 9.5.1. Virus Isolation

Similar to human influenza viruses, avian viruses can be isolated in embryonated eggs or in cell culture, using permissive cells such as Madin

Darby canine kidney (MDCK) cells or rhesus monkey kidney (LLC-MK2) cells. Unlike human strains and avirulent avian strains and in accordance with their promiscuity for cellular proteases, highly pathogenic avian viruses do not require the addition of exogenous trypsin for efficient replication in cell culture. For safety purposes, the isolation of highly pathogenic avian influenza virus requires biosafety level 3 laboratory facilities or higher. Cytopathic effects in cell culture are nonspecific. Initial identification of influenza A virus can be performed by immunofluorescence staining with monoclonal antibodies against the nucleoprotein. Further HA and NA subtyping is performed by subtype-specific RT-PCRs of culture supernatant or hemagglutination inhibition and neuraminidase inhibition assays using a panel of reference antisera against various subtypes. In human infections, avian influenza viruses have mostly been isolated from conjunctival swabs and respiratory specimens such as throat or nasal secretions or washings (Yuen et al., 1998; Fouchier et al., 2004; Hien et al., 2004a). In one case of H5N1 infection, virus was also isolated from serum, cerebrospinal fluid, and a rectal swab (de Jong et al., 2005).

### 9.5.2. Antigen Detection

Detection of influenza A viral antigens in clinical specimens by direct immunofluorescence or by rapid immunochromatographic assays is widely used for diagnosis of human influenza because of their ability for rapid diagnosis. However, in patients with avian influenza, the usefulness of these assays seems limited due to low sensitivity, possibly because of lower viral loads than during human influenza (Yuen et al., 1998; Peiris et al., 2004). In addition, some rapid antigen detection kits do not distinguish between influenza types A and B, and none of the currently available immunofluorescence and immunochromatographic assays distinguishes between influenza A subtypes. However, developments of H5N1-specific rapid antigen detection tests are ongoing (Xu et al., 2005).

### 9.5.3. RT-PCR

RT-PCR methods allow for sensitive and specific detection of viral nucleic acids and have shown to increase the diagnostic sensitivity for many viral pathogens when compared with culture or antigen detection methods. During the H5N1 outbreaks in Hong Kong and Southeast Asia, RT-PCR methods for specific detection of H5N1 viral nucleic acids proved valuable and seem to be the diagnostic methods of choice in case of an outbreak of avian influenza (Yuen et al., 1998; Hien et al., 2004a; Chotpitayasonondh et al., 2005). Especially when using real-time PCR

technology, a reliable subtype-specific diagnostic result can be generated within a few hours after specimen collection. A disadvantage of RT-PCR methods is its proneness for contamination and the consequent risk of false-positive results, which should be minimized by proper precautions, including physical separation of laboratories for PCR preparation and amplification. In addition, the inclusion of an internal control in RT-PCR assays is highly desirable to monitor for false-negative results due to inefficient nucleic acid extraction, cDNA synthesis, or amplification.

#### 9.5.4. Serology

During outbreaks of avian influenza, the detection of subtype-specific antibodies is particularly important for epidemiological investigations. Hemagglutination inhibition (HI) assays are the gold standard for detection of antibodies against human influenza viruses. However, their usefulness for detection of antibodies against avian viruses in mammalian species, including humans, seems limited (Hinshaw et al., 1981; Beare and Webster, 1991; Kida et al., 1994). Several studies have shown a failure to detect HI antibodies against avian viruses in mammals, even in cases where infection was confirmed by virus isolation. Possible reasons for this failure include poor immunogenicity of some avian viruses and lack of sensitivity to detect low-titered or less avid antibodies induced by avian viruses (Hinshaw et al., 1981; Lu et al., 1982; Kida et al., 1994; Rowe et al., 1999). It has been demonstrated that HI testing with subunit HA, but not with intact virus, could detect antibodies against an avian H2N2 virus (Lu et al., 1982). However, neutralizing antibodies against this virus could readily be detected with intact virus. A direct comparison of HI testing with a microneutralization assay in H5N1-infected persons from the 1997 Hong Kong outbreak indeed showed the latter to be more sensitive (Rowe et al., 1999). Although an indirect ELISA assay using recombinant HA from H5N1/97 showed at least equal sensitivity as the microneutralization assay, the specificity in adult sera was inferior, most likely due to the presence of cross-reactive epitopes common to all HAs (Rowe et al., 1999). Based on these observations, neutralization assays are the methods of choice for detection of antibodies against avian viruses in humans.

Using these assays, it has been shown that the kinetics of the antibody response against H5N1 virus in patients infected during the Hong Kong outbreak are similar to the primary response to human influenza viruses (Katz et al., 1999). Neutralizing antibodies were generally detected 14 or more days after the onset of symptoms, and titers equal to or higher than 1:640 were observed 20 or more days after onset. Using

neutralization assays, antibodies against H9N2 could be detected in a small number of blood donors from Hong Kong (Peiris et al., 1999). However, in two laboratory-confirmed H7N3-infected patients with conjunctivitis, no neutralizing antibodies could be detected in sera obtained more than 20 days after onset of the illness (Tweed et al., 2004). Similarly, no HI antibodies could be detected in an H7N7-infected patient with conjunctivitis (Webster et al., 1981). Although the reason for this apparent failure to mount an antibody response remains unclear, it has been suggested that this could be secondary to the highly localized nature of the infection in these cases (Tweed et al., 2004).

## 9.6. Treatment and Prevention

### 9.6.1. Antiviral Treatment

Currently, two classes of drugs are available with antiviral activity against influenza viruses: inhibitors of the ion channel activity of the M2 membrane protein, amantadine and rimantadine; and inhibitors of the neuraminidase, oseltamivir and zanamivir. The therapeutic efficacy of amantadine in human influenza is unclear due to a paucity of reliable clinical studies, but reductions of fever or illness by 1 day have been observed in adults and children (Nicholson et al., 2003). Major disadvantages of amantadine include neurotoxicity and a rapid development of drug resistance during treatment. Resistance is conferred by single nucleotide changes resulting in amino acid substitutions at positions 26, 27, 30, 31, or 34 of the M2 protein. Rates of resistance against amantadine in human influenza viruses has increased from less than 0.5% in 1994-1995 to more than 12% in 2003-2004. Particularly high resistance frequencies of up to 61% were observed in viruses isolated in Asia (Bright et al., 2005). Rimantadine causes less neurological side effects but is not available in most parts of the world. Although several H5N1-infected patients have been treated with amantadine during the 1997 H5N1 outbreak in Hong Kong, the numbers were too small to draw any meaningful conclusions concerning its activity against this virus (Yuen et al., 1998). *In vitro* sensitivity testing of virus isolated from the first patient during this outbreak showed normal susceptibility to amantadine (Subbarao et al., 1998). Strikingly, the sublineage of genotype Z H5N1 viruses prevalent in Thailand, Viet Nam, Cambodia and Malaysia in 2004 invariably showed an amantadine-resistance conferring amino acid substitution at position 31 of the M2 protein, while this mutation was mostly not present in sublineages of H5N1 viruses isolated in other geographic regions (Li et al., 2004; Puthavathana et al., 2005; Cheung et al., 2006).

Both oseltamivir and zanamivir have proven efficacy in the treatment of human influenza when started early during the course of illness and are particularly effective as seasonal or postexposure prophylaxis (Nicholson et al., 2003). Zanamivir has poor oral availability and is therefore administered by inhalation, which has limited its use in the elderly and may induce bronchospasm. Oseltamivir can be given orally. The development of drug resistance during treatment has been reported for both drugs and is associated with mutations in the active site of neuraminidase or in the hemagglutinin. The latter mutations decrease the affinity of HA for the cellular receptor, thereby obviating the need for neuraminidase to escape the cells.

Data on the efficacy of neuraminidase inhibitors in avian influenza virus infections are scarce. The H5N1 strains implicated in the 1997 Hong Kong outbreak were susceptible *in vitro* to oseltamivir and zanamivir (Leneva et al., 2000; Govorkova et al., 2001). Oral oseltamivir and topical zanamivir also showed therapeutic and protective activities against Hong Kong H5N1 isolates in murine animal models (Gubareva et al., 1998; Leneva et al., 2001). Recent murine studies suggest that, perhaps due to higher virulence, higher doses of oseltamivir and longer durations of treatment are necessary to achieve antiviral effects in mice against H5N1 strains causing the Southeast Asian outbreak since 2004, when compared with the 1997 Hong Kong H5N1 strain (Yen et al., 2005). *In vitro* sensitivity testing of H7N7 isolates during the 2003 outbreak of this virus in The Netherlands showed normal susceptibility to zanamivir and oseltamivir (Koopmans et al., 2004). H7N7 infection was detected in 1 of 90 persons who reportedly received prophylactic treatment with oseltamivir during that outbreak, compared with 5 of 52 persons who had not taken oseltamivir prophylaxis (Koopmans et al., 2004).

Oseltamivir treatment has been given to several patients infected with avian influenza viruses, including H7N7, H7N3, and H5N1 subtypes, but no conclusions can be made concerning its efficacy. However, the timing of antiviral treatment may not have been optimal in many human cases of avian influenza so far. Beneficial effects of antiviral treatment in human influenza are optimal when started within 48 h after onset of the illness. During the H5N1 outbreak in Viet Nam in 2004, H5N1-infected patients were admitted 5 days or later after onset of symptoms (Hien et al., 2004a). Earlier recognition of avian influenza in humans may improve the efficacy of antiviral treatment. Nevertheless, favourable virological responses associated with a beneficial clinical outcome have been reported in H5N1-injected patients despite late initiation of treatment (de Jong et al., 2005). The emergence of drug-resistant H5N1 variants during prophylaxis or treatment with oseltamivir has also been reported, and may

be associated with clinical failure of treatment (de Jong et al., 2005; Le et al., 2005). Treatment strategies which minimize the risk of resistance development, such as antiviral combination treatment, deserve attention. In addition, parenteral formulations of antiviral drugs may be desirable to guarantee systemic drug levels in H5N1 patients with severe disease. A novel intravenously administered neuraminidase inhibitor, peramivir, is currently in clinical development.

Although several H5N1-infected patients have received steroids in addition to oseltamivir, the potential benefits of this need formal evaluation in clinical studies (Hien et al., 2004a). Considering the observed cytokine dysregulation in H5N1-infected animals and humans, a beneficial effect of immunomodulating agents could be hypothesized and perhaps requires further study. Finally, neutralizing monoclonal antibodies have been shown effective in treating established influenza A virus infection in mice with severe combined immunodeficiency (Palladino et al., 1995). Although mice are not men, this strategy deserves attention in the treatment of a severe illness such as influenza H5N1.

### 9.6.2. Infection Control and Prophylaxis

Birds infected with avian influenza excrete large amounts of virus in feces and other secretions, which contaminate the direct environment, such as dust, soil, water, cages, tools, and other fomites. Avian influenza virus may remain infectious in soil, water, or contaminated equipment for weeks to months, depending on the temperature and humidity (i.e., longer in colder climates). Illness in birds caused by highly pathogenic avian influenza viruses results in systemic replication and the presence of infectious virus in their eggs and many tissues and organs. Transmission of avian influenza viruses between birds occurs directly or indirectly through contact with fecally contaminated aerosols, water, feed, and other materials. Bird-to-human transmission likely occurs via the same route (i.e., direct contact with birds or contaminated fomites).

Most, but not all human infections with avian influenza viruses involved handling of affected poultry or direct exposure to live poultry in the week before onset of the illness (Mounts et al., 1999; Hien et al., 2004a; Koopmans et al., 2004). Case-control studies during the 1997 H5N1 outbreak in Hong Kong identified visiting a stall or market selling live poultry during the week before the illness as a risk factor, whereas eating or preparing poultry products were not risk factors (Mounts et al., 1999). In cases in which no apparent direct exposure to poultry could be identified, contact with contaminated environment, such as water, has been suggested (de Jong et al., 2005). Of note, it has been shown that ducks infected by the

currently circulating H5N1 strain in Southeast Asia remain healthy but excrete large amounts of virus for prolonged periods of time (Hulse-Post et al., 2005). Because water in ponds and canals in which large flocks of ducks reside is widely used for bathing and drinking in rural areas of many Southeast Asian countries, it may not be unlikely that such water represents a source of transmission when contaminated by infected ducks. In fact, contact with contaminated water is regarded as the most important mode of transmission between aquatic birds.

A limited number of possible human-to-human transmissions have been reported, which involved prolonged, close, and unprotected contact with infected patients (Katz et al., 1999; Koopmans et al., 2004; Ungchusak et al., 2005). Similar to human influenza, droplet and contact transmission are probably the most effective means of transmission of avian influenza virus between humans, should the virus acquire the ability for efficient spread, but airborne transmission remains a possibility. The occurrence of diarrhea in H5N1-infected patients, which may contain infectious virus, represents a potential nonrespiratory route of transmission that needs to be considered in infection control practices (Apisarnthanarak et al., 2004; Hien et al., 2004a; de Jong et al., 2005). Data concerning excretion patterns and periods of potential infectivity are lacking for human infections with avian influenza viruses. Based on exposure histories, the incubation time for human H5N1-infections has been estimated at 2–10 days, but it is not known whether excretion of virus occurs during this time (Yuen et al., 1998; Hien et al., 2004a). Based on the current (lack of) knowledge, infection control measures during contact with potentially infected birds or environment or with patients with suspected or confirmed infection should prevent contact, droplet, and airborne transmission. These measures include mask (preferably high-efficiency masks, with surgical masks as a second alternative), gown, face shield or goggles, and gloves.

The efficacy of neuraminidase inhibitors as seasonal or postexposure prophylaxis against human influenza is high (Nicholson et al., 2003). Offering prophylactic treatment to potentially exposed people in the setting of a poultry outbreak of avian influenza, as has been done during H7-outbreaks in The Netherlands and Canada (Koopmans et al., 2004; Tweed et al., 2004), is rational but hardly feasible during the ongoing outbreak in Asia and Africa for logistical and financial reasons. Postexposure prophylaxis to unprotected health care workers and close contacts of infected patients needs serious consideration. The potential use of specific monoclonal antibodies for prophylaxis warrants further investigation.

Eliminating the source of infection (i.e., infected birds) remains the most effective infection control measure. Culling of all infected poultry

has proved successful during avian influenza outbreaks in Hong Kong, The Netherlands, and Canada (Chan, 2002; Koopmans et al., 2004; Tweed et al., 2004). However, considering the geographic extensiveness of the outbreak, the different farming practices in affected regions, and the occurrence of infection in migratory birds, it is doubtful whether culling of poultry will be able to contain the outbreaks in the various regions.

### 9.6.3. Vaccination

The bulk of human influenza vaccines are produced from inactivated viruses grown in embryonated eggs. Vaccine production against highly pathogenic avian influenza viruses is complicated because of the requirement for high biosafety containment facilities and the difficulty, in some cases, to obtain high virus yields in embryonated eggs because of the virus' pathogenicity (Stephenson et al., 2004; Wood and Robertson, 2004). Several other approaches have been used in an attempt to overcome these obstacles, including the use of reverse genetics techniques, generation of recombinant hemagglutinin, DNA vaccination, and the use of related apathogenic H5 viruses with and without different adjuvants (Nicholson et al., 2003; Stephenson et al., 2004; Webby et al., 2004; Wood and Robertson, 2004). Experimental H5N1 vaccines in which important virulence determinants were altered using plasmid-based reverse genetics have shown protective efficacy to homologous and heterologous H5 strains in animal models and may prove an attractive approach (Li et al., 1999; Takada et al., 1999; Lipatov et al., 2005b). Studies in humans using an H5N3 vaccine developed from a 1997 apathogenic avian virus showed high rates of seroconversions to the vaccine strain and heterologous H5N1 strains after 3 doses, but only when the vaccine was given with the adjuvant MF59 (Stephenson et al., 2005). In animal models, baculovirus-derived recombinant H5 vaccines were immunogenic and protective, but results in humans were disappointing even when using high doses (Crawford et al., 1999; Treanor et al., 2001). In a study using a subvirion influenza H5N1 vaccine, neutralizing antibody responses were observed in approximately half of the subjects receiving the highest dose of the vaccine (two intramuscular injections of 90 micrograms) (Treanor et al., 2006). H5 DNA vaccines protected mice from infection by homologous, but not by heterologous H5N1 viruses (Kodihalli et al., 1999; Epstein et al., 2002).

## 9.7. Pandemic Preparedness and Future Directives

The increasing frequency of outbreaks with highly pathogenic avian influenza viruses among poultry and wild birds, and direct transmission

of these viruses to humans, has ignited grave concerns about an imminent influenza pandemic. Indeed, two of three prerequisites for a human pandemic have been met in the H5N1 outbreaks since 1997: the emergence of an antigenically novel strain to which the population has no immunity, and the transmission of this strain to humans in whom it can cause severe disease. To date, there fortunately is no evidence of efficient spread of H5N1 virus between humans, but continued circulation of this strain, which now has reached levels of endemicity among poultry in several Asian countries, increases the opportunity to adapt to humans through mutation or genetic reassortment in humans or intermediate mammalian hosts. As suggested by the “Spanish flu” pandemic of 1918, extremely high transmissibility is no prerequisite for a severe pandemic killing tens of millions of people, and as shown by the severe acute respiratory syndrome (SARS) virus epidemic in 2003, viruses can rapidly spread across the globe in the current age of intense global travel.

As a consequence of all this, pandemic preparedness has become an increasingly important issue, and pandemic plans are being developed by an increasing number of countries worldwide. Control measures based on case identification (e.g., contact tracing and quarantine) were essential for the control of SARS. However, during an influenza epidemic, such measures may not be as effective because of short incubation periods and the potential infectivity before the onset of case-defining symptoms. Much of the preparedness therefore will rely on clinical management and vaccination. Mathematical modelling studies have suggested the possibility of containing an influenza pandemic at the source by antiviral prophylaxis and other preventive measures (Ferguson et al., 2005; Longini et al., 2005). Many developed countries are now stockpiling antiviral drugs for initial management of illness or prophylaxis during the first months of a pandemic, when vaccines are in development and not yet available. In case of an influenza pandemic, there will be limitations on the timeliness and availability of vaccines (Stohr and Esveld, 2004). It has been estimated that it could take at least 6 months for the first vaccine doses to be produced after identifying a pandemic strain. Currently, global production capacity for influenza vaccines is insufficient for worldwide coverage in case of a pandemic, especially because vaccination for the novel influenza strain likely requires two doses, and interruption of annual production of the human influenza vaccine is undesirable (Schwartz and Gellin, 2005). In response to the pandemic threat by the H5N1 outbreak in Southeast Asia, plans have been made to stockpile candidate H5N1 vaccines based on the currently circulating strain. However, there is no certainty whether the next pandemic will indeed be caused by H5N1 virus, and if so, whether antigenic drift will not have rendered the stockpiled vaccine less effective by

the time pandemic spread occurs. In the latter event, such vaccines may still mitigate the illness, which is beneficial for vaccinated people but may also carry a risk of prolonged excretion and increased spread of the virus. Similar worries exist when poultry would be vaccinated with a suboptimal vaccines.

In case of an influenza pandemic, all possibilities for rapid production of vaccines, as well as potential methods to reduce doses without affecting immunogenicity should be considered. This would require the use of alternative, currently not officially approved methods for vaccine production, such as reverse genetics techniques and cell culture-based vaccine production, and the use of alternative adjuvants that may enable dose reduction (Webby and Webster, 2003; Stephenson et al., 2004; Wood and Robertson, 2004; Schwartz and Gellin, 2005). In addition, vaccine doses may be spared by alternative administration routes. It has been shown that intradermal, instead of intramuscular vaccination for human influenza may require less antigen by recruiting efficient antigen-presenting cells present in the dermis (Belshe et al., 2004; Kenney et al., 2004).

Notwithstanding the importance of current efforts to prepare for a possible H5N1 pandemic, more structural and longer term global efforts are needed to allow for early recognition of emerging novel influenza viruses in the future. In 2002, a WHO Global Agenda for Influenza Surveillance and Control has been adopted, of which the main objectives are to strengthen surveillance, improve knowledge of the disease burden, increase vaccine use, and accelerate pandemic preparedness (Stohr, 2003). It is essential that these objectives are increasingly focused on the Southeast Asian region, which has been the source of previous pandemics and is the epicenter of the current pandemic threat. However, many Southeast Asian countries currently lack the expertise, financial means, and infrastructure for human and animal surveillance. Global investments to improve public health care infrastructures and laboratory facilities and to transfer clinical, epidemiological, and technical knowledge to these countries are much needed (Hien et al., 2004b). The window of opportunity in the era of global travel is narrow. Local capacity, and less dependence on foreign laboratories and expertise, will allow for earlier recognition and quicker responses to epidemics. In addition, local availability of clinical, scientific, and laboratory capacity facilitates and expedites clinical, virological, and epidemiological analyses needed to optimize outbreak control, infection control, and clinical management and guarantees the timely availability of virus strains for monitoring virus evolution and planning of vaccines by reference laboratories.

In response to the 1997 H5N1 outbreak in Hong Kong, influenza surveillance in poultry was intensified, which permitted early recognition

of outbreaks by other avian influenza strains and timely interventions and has helped to keep Hong Kong free of H5N1 influenza despite the outbreak of this virus in many other countries in the region. The Hong Kong response may serve as a model, but wider implementation of this approach will require global efforts.

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