CHAPTER 2
CURRENT STATE OF KNOWLEDGE ON HIGHLY PATHOGENIC AVIAN INFLUENZA

This chapter provides background information on avian influenza viruses and the factors that have led to the emergence, spread and persistence of HPAI viruses. It uses experiences before and after the emergence of Asian-lineage H5N1 HPAI viruses as a guide.

2.1 AVIAN INFLUENZA VIRUSES

Aquatic birds are the natural hosts of type A influenza viruses. Influenza viruses are categorized by the glycoprotein spikes on their surface – the 'HA' or haemagglutinin protein (16 different subtypes identified so far) and the 'NA' or neuraminidase protein (nine subtypes). All 16 HA and nine NA subtypes (see box on Terminology) have been detected in various combinations in aquatic wild birds, and for the most part these viruses live harmoniously with their natural hosts, establishing a short-lived subclinical enteric infection (Webster and Hulse, 2004). Occasionally, viruses cross from aquatic wild birds to poultry or mammals, and new genotypes of virus may become established in these non-natural or spillover hosts. Once adapted to non-avian hosts, these viruses may lose the capacity to multiply in the gut of aquatic birds (Kobasa *et al*, 2001)

Of the influenza viruses that cross to terrestrial poultry, the most important are those of the H5 and H7 subtypes. These have the capacity to mutate into HPAI viruses that multiply systemically in chickens, often causing very high mortality in infected flocks (Alexander, 2000).

Other influenza virus subtypes have also crossed into terrestrial poultry and have established in these birds. Among these, the H9N2 subtype is the most frequently detected, and has spread across Asia and into the Middle East over the past 15 years (Alexander, 2000). This subtype is a low pathogenicity virus but, in combination with other pathogens, can cause severe respiratory disease in poultry (Kishida *et al*, 2004). H9N2 viruses are now circulating in pig populations in China (Chen *et al*, 2006b) and have been associated with cases of clinical disease in this species (Peiris *et al*, 2001). They have also caused non-fatal infections in humans (Butt *et al*, 2005).

Terminology

Throughout this document, the term 'Asian-lineage H5N1 HPAI' is used to describe highly pathogenic avian influenza viruses of the H5N1 subtype linked to the line of viruses first detected in geese in Guangdong province in China in 1996. 'H5N1 HPAI' is used to describe the disease caused by these viruses. Although multiple genetic variants of these viruses have emerged, they still form a lineage distinct from other H5N1 viruses. All HPAI viruses of the H5N1 subtype detected in the past 11 years belong to this lineage.

The term HPAI has a very specific meaning, and relates to the ability of the virus to cause disease in experimentally inoculated chickens (i.e. its virulence). It does not reflect the capacity of these viruses to produce disease in other species.

The term 'H5N1 HPAI' is used rather than just 'H5N1' to avoid confusion with unrelated low pathogenicity avian influenza (LPAI) viruses of the H5N1 subtype that have been detected in poultry and wild birds.

Influenza viruses have a segmented genome and the capacity to undergo gene reassortment. Theoretically, this process could occur whenever two different influenza viruses co-infect the same cell. In addition, minor changes to individual genes occur relatively frequently, leading to changes in these genes over time. Some cases of gene recombination have also occurred, most notably in outbreaks of HPAI in Chile (2002) and Canada (2004).

Avian influenza viruses are named according to their type (all avian influenza viruses are Type A), subtype (i.e. by the HA and NA glycoproteins they possess, which is written as H5N1, H7N7, etc.), by the species of animal from which they are isolated, by the geographic location from which they were isolated (often country, province or state level), and by laboratory reference number and year of isolation. (e.g. A/Goose/Guangdong/1/96 [H5N1]).

Nomenclature beyond this is not fully standardized; no internationally-agreed system has been used for naming different genetic variants within subtypes. For example, numerous new genotypes of Asian-lineage H5N1 HPAI viruses have emerged through reassortment. Although these viruses all retain a version of the "parent" H5 gene, they were initially subdivided on the basis of the makeup of other genes coding for internal proteins.

The first reported Asian-lineage H5N1 HPAI virus – A/Goose/Guangdong//1/96 – is used as a starting point for comparisons of genotypes (Chen *et al*, 2004; Guan *et al*, 2004). As these (or similar) viruses evolved, they acquired genes from other influenza viruses and the genotypes formed were named by using letters. Researchers at the University of Hong Kong were very active in this area and the letters they used (e.g. the 'Z' genotype) have been widely adopted. Unfortunately this system has not been applied consistently by all researchers.

This variation in genotype does not necessarily capture information on variations in the HA genes. This is covered by a different system of nomenclature in which multiple clades (lineages) and subclades (sublineages) of H5N1 virus haemagglutinin genes have been identified. The World Health Organization (WHO) initially suggested standardizing the nomenclature (using clades and subclades to group these viruses) in 2005 and 2006 (WHO, 2005; WHO, 2006). This has since been further revised (WHO, 2007) with at least ten clades and many subclades among the Asian-lineage H5N1 HPAI viruses currently recognized.

Because the clades and subclades are based on the genetic relationships between HA genes, individual clades may contain different genotypes (the latter is determined by the constellation of gene coding for internal proteins, not the HA gene). Therefore, use of any given system of nomenclature does not capture all of the information on the changes that are occurring. This can make comparison of molecular data difficult, especially if different labels are used by unrelated research groups to describe similar viruses or even the same strains of virus.

2.1.1 Background information on HPAI

Early cases of HPAI

HPAI is not a new disease (Alexander, 1987). Its occurrence predates the industrialization of the poultry subsector.

Although interpretation of historical data on HPAI (before the so-called 'virological era') is complicated by the potential for confusion of this disease with other diseases such as Newcastle disease, HPAI was first identified in the late 19th century and probably occurred earlier (Alexander, 1987). At least one early outbreak in 1901 spread across international borders through movement of poultry (Wilkinson and Waterson, 1975), providing forewarning of the challenges to be faced when trade in poultry and poultry products would become globalized later in the 20th century. In the United States of America in the 1920s, HPAI viruses were apparently spread via movement of poultry by rail and through live-bird markets (Alexander, 1987).

The precise origins of these early viruses and the events that facilitated the mutation of the causative viruses from low to high pathogenicity are not known (see section 2.1.2). It has been suggested that infection with HPAI was endemic in Germany, Italy and Egypt for a number of years in the late 19th and/or the first half of the 20th century (Alexander, 1987). This indicates that, in the past, not all outbreaks of the disease were rapidly contained, in contrast to those from the 1950s to the 1970s, which were generally isolated cases that were controlled rapidly. For example, the 1959 outbreak in the United Kingdom occurred on an isolated farm and resulted in the death of virtually all poultry, conditions which reduced the likelihood of spread (Alexander, 1987). Recent modelling studies support the proposition that low farm density can result in limited or even no onward transmission of infection (Truscott *et al*, 2007).

The rise in the number of cases of HPAI

There has been an increase in the number of reported outbreaks of HPAI in the past eight years (Capua and Alexander, 2004), even excluding recent outbreaks of H5N1 HPAI in Asia and elsewhere. Those associated with HPAI viruses (other than the H5N1 subtype) have occurred in the Americas (United States of America, Chile and Canada), Europe (Italy and the Netherlands) and Pakistan.

It is still not entirely clear why this increase occurred. A number of factors have been proposed, including the increase in the global poultry population, the increase in intensification of the poultry industry with a concurrent increase in free-range production, and even climate change leading to alterations in wild bird migratory paths (Capua and Alexander, 2004). None of these has been proved to be the cause of this increase, which is likely to be multifactorial.

Improved diagnostic capability and surveillance may have contributed to some of this increase. Enhanced surveillance has allowed detection of cases that might otherwise have gone undiagnosed, such as the clinically mild 'outbreak' in Texas in 2004 (Lee *et al*, 2005).

Much of the increase in importance of HPAI stems from the severe effects of the cases that did occur. Three large outbreaks in Italy (1999), the Netherlands (2003) and Canada (2004) involving areas of high poultry density led to the destruction of tens of millions of poultry, including many healthy birds (Capua *et al*, 2003; Power, 2005; Stegemen *et al*, 2004).

Source of viruses for outbreaks of HPAI

Given the level of attention paid to HPAI recently, it is sobering to reflect on how little has been demonstrated about the mode of introduction and spread of the disease. Wild aquatic birds have been proposed as the most likely source of LPAI viruses that converted to HPAI viruses in many HPAI outbreaks (excluding, for the moment, those caused by Asian-lineage H5N1 HPAI viruses) (see for example Alexander, 2007) but the evidence, in most cases, is circumstantial. Table 1 provides a summary of some of these outbreaks of HPAI, and includes information on both the possible source of the virus and factors contributing to the emergence of the highly pathogenic strain. These cases demonstrate that the precise source of infection in most outbreaks was not identified.

The lack of information on source(s) also applies to outbreaks involving Asian-lineage H5N1 HPAI viruses. Few in-depth investigations have been conducted on disease outbreaks caused by these viruses, especially in developing countries, and even when these have been performed, the route of entry or source of virus has not been proved. For example, tracing of contacts and movements was performed in Japan (Nishiguchi *et al*, 2005) and the Republic of Korea (Wee *et al*, 2006) following outbreaks in 2003-04, but even after these detailed studies, the authors could only speculate on how the virus entered these countries. Similarly, recent epidemiological studies on outbreaks in Israel (2006) and the United Kingdom (2007) were not able to prove how the virus was introduced (Balicer *et al*, 2007; Defra, 2007).

Table 1   Selected HPAI outbreaks – possible sources and environmental factors

Outbreak	Virus subtype	Source	Possible contributing factors
Australia 1975-76	H7N7	Unknown	Dam on farm attracted wild birds Untreated drinking water used for poultry Substandard biosecurity (Turner, 1981)
Australia 1985	H7N7	Unknown	Dam on farm attracted wild birds Untreated drinking water used for poultry Substandard biosecurity (Westbury, 1997)
Australia 1992	H7N3	Unknown	Adjacent to free-range duck farm Substandard biosecurity (Westbury, 1997)
Australia 1994	H7N3	Unknown	Many wild birds on watercourse due to drought Untreated drinking water used for poultry (Westbury, 1997)
Australia 1997	H7N4	Unknown	Wild birds in vicinity Free-range farmed emu chicks infected subclinically on one of three infected farms Dead bird pick-up vehicle travelled to multiple farms including the infected farms (Selleck *et al*, 2003)
Canada 2004	H7N3	Unknown	Migratory birds in area prior to the outbreak (Power, 2005)
Italy 1997-98	H5N2	Unknown	Marketing of infected birds Rearing of birds in the open Presence of mixed species (Capua *et al*, 2003)
Italy 1999-2000	H7N1	Original source unknown – presumably wild bird	Widespread infection with low pathogenicity virus prior to emergence of highly pathogenic strain (Capua *et al*, 2003)
Mexico 1994	H5N2	Unknown	Widespread circulation of low pathogenicity viruses prior to emergence of highly pathogenic strain (Villarreal, 2006)
Netherlands 2003	H7N7	Unknown but likely to be wild bird introduction	LPAI virus similar to the HPAI virus detected in wild mallards (Munster *et al*, 2005)
USA 1983-84	H5N2	Unknown but likely to be wild bird introduction	Infection of poultry with low pathogenicity viruses Live–bird markets (Suarez and Senne, 2000)
USA 2004	H5N2	Link to live-bird markets	Similar LPAI viruses circulating for some time prior to emergence of HPAI strain (Lee *et al*, 2005)

An association between wild birds and infection with LPAI viruses in farmed turkeys in Minnesota, United States, has long been recognized (Halvorson *et al*, 1985). It is also considered a probable risk factor for repeated outbreaks of both LPAI and HPAI in northern Italy (Capua *et al*, 2003), along with the high density of poultry and the large number of live birds imported to the area.

In Italy, H7N3 LPAI viruses virtually identical to those found in wild birds in 2001 were subsequently detected in farmed turkeys in 2002-03 (Campitelli *et al*, 2004). A close relationship has been demonstrated between H5 and H7 LPAI viruses isolated from wild mallards and those from outbreaks of HPAI in Europe, including the 2003 H7N7 outbreak in the Netherlands (Munster *et al*, 2005). The origin of the 2004 outbreak of HPAI in Canada is not known, but this and other cases developed in poultry farms located in areas with seasonally high populations of migratory wild birds.

Outbreaks of HPAI in Australia from the 1970s to the 1990s were on farms implementing inadequate biosecurity measures. This included several cases in which untreated drinking water from ponds or rivers frequented by wild waterbirds was supplied to poultry (Westbury, 1997). It is pertinent to note that no new cases of HPAI have been diagnosed in Australia in the past ten years, which is probably attributable (at least in part) to implementation of enhanced biosecurity measures on commercial farms. These were introduced in response to earlier cases of HPAI, and to a major outbreak of Newcastle disease on large intensive farms. It may also reflect a reduction in the number of wild birds due to prolonged drought (Turner, 2004).

2.1.2   Mutation from LPAI virus to HPAI virus

A key event in the genesis of all HPAI viruses is conversion (mutation) of an H5 or H7 LPAI virus to an HPAI virus. This has occurred in the past following multiplication of LPAI viruses of these subtypes in chickens but it is not known whether this is an essential prerequisite.

Virulence of avian influenza viruses is a polygenic trait (Suarez *et al*, 2004) that usually results from insertion or substitution of multiple basic amino acids at the cleavage site of the HA protein. These are not normally present in LPAI viruses. This mutation allows the HA protein to be cleaved by a broad range of proteases, allowing the viruses to multiply systemically (Alexander, 2000). Other novel mechanisms for conversion of LPAI viruses to HPAI viruses have been described in outbreaks of HPAI in Chile (2002) and Canada (2004). These arose through recombination between the HA gene and that of another gene coding for an internal protein, leading to insertion of additional amino acids at the HA cleavage site (Suarez *et al*, 2004; Pasick *et al*, 2005). Modification of the cleavage site appears to be an essential condition, but is not the only factor that determines virulence (Londt *et al*, 2007).

Even though the molecular events surrounding mutation from an LPAI virus to an HPAI virus are known, the factors that lead to this mutation are not clear for many outbreaks of H5 and H7 avian influenza viruses, including the first of the Asian-lineage H5N1 HPAI viruses.

An HPAI virus has been generated experimentally by repeat passage of a LPAI virus through chickens by air sac and intracerebral inoculation (Ito *et al*, 2001) but the exact triggers for this change under natural conditions are not known. In some earlier outbreaks of HPAI, it was evident that the change from a LPAI virus to an HPAI virus followed introduction of LPAI virus to large flocks of commercial poultry. This change apparently occurred within a matter of days in some outbreaks (as was the case of the 2004 Canadian outbreak [Bowes *et al*, 2004]). On the other hand, in some Central American countries, low pathogenicity H5N2 strains have circulated in poultry for a number of years without developing into highly pathogenic strains. Even in Mexico, where mutation of a LPAI H5N2 virus to an HPAI virus occurred in 1994 and this HPAI virus strain was subsequently eliminated, H5N2 LPAI viruses continue to circulate (Villarreal, 2006) but have not reverted to high pathogenicity.

The Asian-lineage H5N1 HPAI viruses differ somewhat from those in earlier HPAI outbreaks in that the HPAI viruses first detected in 1996 were not eliminated, and have circulated in highly pathogenic form for over 11 years (Sims *et al*, 2005). These viruses have evolved considerably over this time, but no closely related low pathogenic precursor strains of the H5 subtype have been isolated, (Duan *et al*, 2007; Mukhtar *et al*, 2007). Presumably, such a virus existed prior to 1996 but has never been detected or reported (Sims *et al*, 2005). The HA and NA genes in LPAI viruses most closely related to those in Goose/Guangdong/1/96 were isolated experimentally from aquatic birds in Japan (Mukhtar *et al*, 2007).

All subsequent Asian-lineage H5N1 avian influenza viruses have remained highly pathogenic and their origins can be traced back to viruses similar to those found in geese in Guangdong in 1996 (Sims *et al*, 2005). Even those viruses that have infected domestic ducks subclinically in many parts of Asia retain the multiple basic amino acids at the cleavage site and are highly pathogenic for terrestrial poultry (Chen *et al*, 2004).

No evidence has been provided to indicate that the first Asian-lineage H5N1 HPAI virus emerged in an intensive poultry farm. If this conversion from LPAI to HPAI occurred in geese (the type of poultry from which it was first isolated), this would not have involved industrialized production facilities because geese in southern China were then reared mainly in small flocks with only a few large semi-intensive production units.

The lack of markers of adaptation to chickens (e.g. a deletion in the stalk of the NA glycoprotein (Matrosovich *et al*, 1999) in the original 1996 strains of H5N1 HPAI virus also suggests, but does not prove, that limited circulation of this virus occurred in chickens before 1997. A stalk deletion in the NA was not detected in viruses of this lineage until March 1997, when it was detected in a virus isolated from a dead chicken on a farm in the Hong Kong Special Administration Region (SAR) (Bender *et al*, 1999).

These observations, coupled with evidence that other HPAI viruses emerged in the early 20th century prior to intensification of the poultry industry, indicate that the circulation of a LPAI virus in industrialized poultry rearing systems, although considered an important factor in the emergence of some HPAI strains, is not an essential prerequisite for the genesis of an HPAI virus.

2.2   SPREAD OF H5N1 HPAI IN ASIA AND BEYOND

Transborder spread of H5N1 HPAI was first reported in late 2003 and early 2004, yet H5N1 HPAI viruses were already present in Viet Nam (where the first H5N1 HPAI isolates were detected in 2001) and China (1996). Even by 2002, numerous Asian-lineage H5N1 HPAI virus genotypes had already emerged (Sims *et al*, 2005).

As described previously, the first of these H5N1 HPAI viruses in Asia was identified in 1996 in sick geese from Guangdong Province in southern China (Xu *et al*, 1999). Related H5N1 HPAI viruses then caused the outbreak in HKSAR in 1997, in which both poultry and humans were affected. This was followed by further outbreaks in HKSAR (in poultry only) from 2001 to early 2002 (Sims *et al*, 2003a) and poultry and captive wild birds in 2002-03 (Ellis *et al*, 2004). By this time, infection with a range of Asian-lineage H5N1 HPAI viruses had been detected in an arc extending from Hanoi to Shanghai (Sims *et al*, 2005) and even to provinces further north (see for example A/chicken/Hebei/718/01[H5N1], Genbank *Taxonomy ID:* 367715). Remarkably, apart from HKSAR, no disease was officially reported in poultry in any of these places (Sims *et al*, 2005).

From 2003 onwards, infection and disease spread widely to three continents, initially through East and Southeast Asia in 2003-04, and then into southern Russia, the Middle East, Europe, Africa and south Asia in 2005-06.

The precise events that led to broad transborder spread of these viruses are not known, but among the likely contributory factors are the changes that allowed the H5N1 HPAI viruses to multiply subclinically in ducks (see section 3.1), presumably leading to a high baseline level of infection in the region from which the viruses could spread. The presence of high quantities of virus in domestic ducks in a system where live poultry are moved long distances and sold in live-bird markets (Li *et al*, 2003) without being tested for infection would facilitate spread of infection within, and perhaps in some cases, between countries.

The adaptation to ducks may also have favoured subsequent spread of H5N1 by wild *Anatidae* (Gilbert *et al*, 2006a; Sturm-Ramirez *et al*, 2005). Limited laboratory studies on wild ducks indicate that they can be infected subclinically with Asian-lineage H5N1 HPAI viruses, even after inoculation with very low doses of virus. The clinical outcome depends on a number of factors, including the age of the ducks at the time of inoculation, the species of duck and the strain of virus (Brown *et al*, 2006). The duration of excretion in most subclinically infected wild ducks was short, but still long enough to allow for transfer from one site to another. There is also considerable circumstantial evidence from Europe, Russia and Mongolia to indicate that wild birds played a significant role in the spread of these viruses (ProMED-mail, 2005; Gilbert *et al*, 2006b; Irza, 2006) (see section 2.3).

Historically, once established in an area, spread of HPAI was associated with movement of contaminated objects or infected poultry (Alexander, 2000; Wilkinson and Waterson, 1975). This is still considered to be the case today, but the market chains and extent of movement of poultry and products are often more complex than those seen early last century, complicating investigations and analyses. In most cases, epidemiological studies are conducted some time after the disease occurs, leaving investigators the task of determining and ranking probabilities on the basis of imperfect and sometimes complex historical information.

Epidemiological investigations should be conducted on all new outbreaks through tracing studies in an attempt to determine the most likely source of infection. A large number of outbreaks occurring simultaneously over a wide area suggests undetected cases of infection. This occurred in early 2004, when many seemingly disconnected cases of H5N1 HPAI were reported in a number of Asian countries. When such situations arise, further investigations are needed to establish the pathways of infection and the connections between the cases. This type of analysis has not been done for many of the outbreaks of HPAI in Asia and, until such information is available, suggestions on the mode of spread for many of these outbreaks remain speculative.

The recent outbreak of equine influenza in Australia provides some insights into the different routes of transmission of influenza viruses in animal populations. Equine influenza virus had never previously been reported in Australia and therefore horses in Australia (other than a very small percentage of horses vaccinated for international travel) were highly susceptible to infection. The virus was almost certainly introduced to the country via an imported infected stallion, with initial spread probably occurring via fomite transfer. This was exacerbated by subsequent movement of infected horses and by additional fomite transfer. Local short distance spread, possibly aerogenous, also occurred. This epidemic demonstrated that different mechanisms may be involved in the first introduction of virus to those associated with subsequent spread. This has also been proposed in recent investigations of outbreaks of H5N1 HPAI in poultry in eastern Turkey (Bayraktar, 2007), where it is suspected that wild bird introduction was followed by spread through local poultry trade.

2.3   MODE OF ENTRY OF H5N1 HPAI TO COUNTRIES AND PLACES

When infections occur in new locations, the sources to consider include live poultry, poultry products, vehicles, objects and materials (including feed and water) used on farms or in markets contaminated with virus, people (e.g. failure to wash hands after handling infected poultry, contaminated clothing or footwear), wild birds and trade in other types of birds. These various routes are examined in detail in Chapters 3 and 4.

There has been considerable speculation about the mode of entry of H5N1 HPAI virus into unaffected countries, especially concerning the relative role of trade in poultry and movement of free-flying wild birds. It is highly likely that both have played a role, although the role of wild

birds has been questioned because surveillance studies on clinically normal wild birds have resulted in limited detection of H5N1 HPAI viruses.

Few detailed studies on the mode of introduction of H5N1 viruses have been conducted. Evidence from HKSAR demonstrated how trade in live poultry could be a potential source of infection. Repeatedly, geese and ducks sent to a central slaughtering facility from mainland China to HKSAR between 1999 and 2001 were found to be infected on arrival (Agriculture, Fisheries and Conservation Department, 2001; Cauthen *et al*, 2000; Guan *et al*, 2002a). However, H5N1 viruses have also been found in free-flying non-domesticated birds in HKSAR, and these birds could potentially act as a means of entry to non-biosecure farms or zoological collections.

The large volume of trade in live chickens and other terrestrial poultry between Guangdong province in China and HKSAR was also implicated (but never proved) as a source of infection from 2001 to 2003. During this period, the H5N1 virus genotypes found on farms in HKSAR were less varied than those detected in HKSAR's live-poultry markets (Guan *et al*, 2002b; Li *et al*, 2004a). These markets were supplied with poultry from farms in HKSAR and from Guangdong province, suggesting that local Hong Kong farms were not the only source of infection for these poultry markets.

The outbreak in the United Kingdom in February 2007 implicated trade in poultry meat as the most likely source of infection.

One study (Kilpatrick *et al*, 2006) assigned relative probabilities to trade in poultry[1] and in wild birds and also to natural movements of wild birds as the source(s) of infection in various countries. The study suggested that most of the introduction of H5N1 HPAI virus to Europe was probably via natural movements of wild birds. However, not all of the conclusions in this paper concur with those from field investigations – for example, the study predicted trade as the most likely source for Kazakhstan, but this was not fully supported by field observations (Gilbert *et al*, 2006b, citing a World Organisation for Animal Health [OIE] investigation). The Kilpatrick paper also suggested spread of virus from Thailand to Indonesia but, genetically, these viruses belonged to different clades. Furthermore, from the known or suspected time of introductions, infection probably occurred in Indonesia before it occurred in Thailand.

Regarding the relative contributions of anthropogenic versus wild bird factors in transborder spread of these viruses, much is still unknown. Debate on the role of wild birds intensified when viruses closely related to those found in wild birds at Lake Qinghai, China, in 2005 (belonging to subclade 2.2), spread across Russia to Europe and into Africa (see for example Feare, 2007).

Available information on wild birds and their potential to spread H5N1 viruses was reviewed in an European Food Safety Agency risk assessment, and this demonstrated the many gaps in the available data – both virological and ornithological (European Food Safety Agency, 2006). This risk assessment concluded that migratory birds pose a potential threat for the introduction of virus to Europe, but considerable uncertainty was associated with the conclusions.

Many of the original Russian outbreaks in domestic poultry in 2005 occurred in remote communities adjacent to wetlands having limited contact with the outside world, and where poultry and wild waterbirds mixed. Russian veterinary authorities have since concluded that wild birds were responsible for much of the spread within their country and have based their preventive programmes on this, providing vaccination coverage for non-biosecure flocks in the

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[1] The main indicator used for risk from trade in poultry was legal trade in live poultry from infected countries, much of which involves day-old chicks.

vicinity of sites where wild aquatic birds congregate (Irza, 2006). This does not mean that spread through trade did not occur once virus was established in a particular area.

Spread from Russia to other countries bordering the Black Sea is also considered to be due to wild bird movements. This followed extremely harsh winter conditions that led to dispersal of wild birds into western Europe in 2006 (Gilbert *et al*, 2006b).

Other cases in wild birds occurred in places with very few poultry or no evidence of infection in poultry. Wild bird cases in 2005 in Mongolia provided the best circumstantial evidence for spread of H5N1 HPAI by wild birds over long distances, given the very low domestic poultry population in that country. However, it is not possible to rule out other extremely remote possibilities such as use of live decoy ducks for hunting, which has been reported but is unlikely to have occurred (Williams, 2005; Feare, 2006). Infection was detected in Mongolia again in 2006, coinciding with the return of migratory birds in the northern spring (OIE, 2006).

In a number of countries, including China, cases of infection in poultry have occurred on relatively isolated farms. Subsequent investigations have revealed no apparent contacts between these farms and other potential sources of infection, apart from wild birds (F. Guo, personal communication with Les Sims). Wild birds have also been proposed as a possible source of infection for three out of four outbreaks in Japan in 2003-04 (Nishiguchi *et al*, 2005). This conclusion was based on the distance between outbreaks and lack of connection between the first three cases, the unprotected water supplies to affected farms, and the location of the farms in mountainous areas attractive to wild birds. Similar conclusions were made for infections in the Republic of Korea in 2003 based on circumstantial evidence (Wee *et al*, 2006). The return of H5N1 viruses to the country in 2006 coincided with movements of migratory birds and, although wild birds were not proved to be the source, they were considered to be the likely route of introduction (Lee *et al,* 2008).

The evidence for wild bird involvement in all the above cases, while compelling in some instances, remains circumstantial. It is also evident that wild birds have sometimes provided a convenient scapegoat for those wanting to deflect attention away from inherent problems in poultry production and marketing systems and their contribution to the spread of H5N1 HPAI.

The situation in Africa is less clear. Viruses isolated in Nigeria also belong to subclade 2.2. This particular subclade does not appear to be widely established in poultry or in places with high poultry density (see for example Smith *et al*, 2006a). If these introductions were due to illegal trade in poultry products, it is difficult to understand why such outbreaks did not occur in earlier years and involve genotypes more commonly found in poultry such as clade 1 or subclade 2.3, given that these strains of H5N1 HPAI viruses have been circulating widely in Asia for some time.

Recently it has been suggested that there were multiple incursions of H5N1 virus into Nigeria (although all strains in Africa belonged to subclade 2.2, there are minor differences between these viruses). This means it is not possible to rule out wild birds as a source of infection (Ducatez *et al*, 2006).

The spread of virus to other countries in West Africa may well have occurred through the movement of poultry and poultry products or fomite spread, but this, too, remains speculative. The sources of virus for Egypt, the Sudan and Djibouti remain unknown, but genetic studies have revealed that those in the Sudan and Egypt are not identical (Ducatez *et al*, 2006), possibly suggesting different origins. One case of infection in a wild duck was detected in Egypt before outbreaks were reported in poultry (Earhart *et al*, 2007).

2.3.1　Factors complicating analysis of modes of introduction

Analysis of routes of entry to uninfected places has been complicated by the limited capacity in some countries to investigate diseases, imprecise information on illegal movements of poultry or poultry products and delays in reporting of outbreaks when they first occurred. Even when the disease has been recognized early and full investigations undertaken, it has not always been possible to determine how the virus gained entry to the country and then to flocks of poultry.

Analysis has also been hampered because the first case of disease or infection recognized (the index case) in many countries was unlikely to be the first case of infection. HPAI can occur in smallholder or scavenging poultry without being diagnosed because mortality in village flocks from other causes occurs regularly (see for example FAO, 1998; Johnston, 1990). These deaths are not always reported and even if local authorities are advised, there is no guarantee that all cases will be investigated or reported further up the chain to central or provincial veterinary authorities. This under-reporting is most clearly evident in places where human cases have occurred in the absence of reported avian infections ('human sentinels') (Sims, 2007). In addition, targeted village surveillance in Indonesia and studies in Cambodia have also demonstrated significant under-reporting of mortalities in poultry (Elly, 2007; Desvaux *et al*, 2006).

Outbreaks have also gone unreported in large commercial chicken farms (Nishiguchi *et al*, 2005) although it is usually harder to hide such cases for extended periods because the number of infected birds increases rapidly. This leads to high mortalities, a large virus load, and a high probability of spread once infection is established in a large flock in an area with high concentrations of interlinked poultry farms. The possibility of failure to report disease needs to be taken into account when analysing outbreak data. Indeed, the sale of sick poultry is recognized as one of the so-called 'coping strategies' for farmers and villagers when their poultry or those of their neighbours are showing signs of disease.

Conclusions on the source of infection are often based on a process of elimination or on associations and probabilities rather than definitive evidence. Disease investigations are time consuming and require appropriately resourced and trained investigators, preferably focussing on investigation rather than control when the disease outbreak occurs. Unfortunately, investigations in countries with limited veterinary resources have often been relegated to a secondary role, or even neglected entirely, due to the competing demands of control programmes. This situation is improving in many countries as a result of increased funding of veterinary services, and it is expected that the quality of data on disease outbreaks and prevalence of infection will improve in the future (Sims, 2007). Even in developed countries, control and eradication of the disease have taken priority over investigations when outbreaks first occur (Balicer *et al*, 2007).

2.3.2   Molecular epidemiology – establishing linkages between viruses

Sequencing of the genes of H5N1 HPAI and other avian influenza viruses has assisted greatly in establishing links (and differences) between H5N1 virus isolates from different countries. In some cases, these data demonstrate independent introductions of virus to different countries. For example, molecular investigations suggest initial introductions of separate clades of H5N1 HPAI virus to Thailand (clade 1) and Indonesia (subclade 2.1) (i.e. viruses of different origin), followed by spread within each country (Chen *et al*, 2006c; Smith *et al*, 2006b). Subsequently, additional incursions have been detected in Thailand (Smith *et al*, 2006b) but not in Indonesia, where only the initial strains have persisted and evolved. The precise origin or mode of entry of these viruses is not known. The closest known related viruses are those isolated in China in 2003 (WHO, 2007).

Strains of H5N1 HPAI virus isolated in Japan and the Republic of Korea in 2003-04 are very closely related, suggesting a common (unknown) origin (Mase *et al*, 2005). The closest related virus was detected in poultry in southern China. Preliminary data also suggest that viruses introduced to these countries in 2006-07 were closely related to each other, but not to the 2003-04 viruses (i.e. the 2007 viruses apparently belong to subclade 2.2 whereas those in 2003-04 belong to subclade 2.5 [WHO, 2007]). This indicates new incursions of virus rather than persistence of the earlier strain.

Genetic data also suggests that multiple incursions of H5N1 HPAI occurred in Viet Nam (Smith *et al*, 2006a) and HKSAR, with the latter exposed to many different genotypes since 1997. This reflects the broad genetic diversity of H5N1 viruses in the region over this period, starting with the original goose viruses (Li *et al*, 2004a). Based on sequence data, it has been suggested that some strains of H5N1 HPAI virus in Viet Nam may have originated in China (Chen *et al*, 2006c). Movement in the opposite direction cannot be excluded, but does not match the predominant trade pattern for poultry, much of which is illegal and driven by price differences for poultry between China and Viet Nam (Sipress, 2006).

Molecular studies have also demonstrated that evolution of H5N1 viruses is continuing, as shown by the emergence of new sublineages of virus. For example, viruses belonging to a new subclade (subclade 2.2) of H5N1 viruses were detected in wild birds in Qinghai province in China in 2005 (Chen *et al*, 2005; Chen *et al*, 2006a; Zhou *et al*, 2006) and these have now been found in Europe and Africa. Other new lineages that have been detected in the last few years include clade 7 viruses in Shanxi province in China in 2006, clade 4 viruses in Guiyang province and subclade 2.3 viruses that have been detected in southeast China and Southeast Asia since 2005 (WHO, 2007).

The subclade 2.2 viruses from Qinghai in 2005 appeared to be reassortants, with four different variants detected during the outbreak (Chen *et al*, 2006a). Many of these viruses have lysine (K) instead of glutamate (E) at position 627 in the PB2 protein (the E627K mutation), generally regarded as a marker of passage through a mammalian host and, until 2005, rarely seen in avian isolates (Taubenberger *et al*, 2005). The presence of this mammalian signature in viruses in subclade 2.2 – first found in wild birds – is yet another demonstration of our limited knowledge and understanding of how these viruses evolve. The closest related viruses were detected in wild ducks at Poyang Lake in Jiangsu province in China in March 2005 (Chen *et al*, 2006c).

For infection and disease in wild birds with subclade 2.2 H5N1 HPAI viruses, it is not clear whether the outbreak in wild birds in May 2005 in Qinghai was the first such outbreak associated with this subclade, or merely the first one identified (due to its size). There are thousands of lakes in remote parts of the Qinghai plateau and across northeastern Asia where mortalities in small numbers of wild birds could have occurred unnoticed. Unfortunately, when reviewing the genesis of this H5N1 HPAI epizootic, absence of data has repeatedly been mistaken for absence of infection by the popular media, and even by some scientists. Unless the quality and range of surveillance programmes in apparently uninfected locations are considered, incorrect conclusions can be drawn about the infection status of countries or parts of countries (Sims, 2007).

It has been suggested that the failure to detect infected live wild birds in West Africa indicates that they probably played no role in the introduction of virus to Nigeria (Brown, 2006). Instead, the considerable movement of poultry and poultry products into Nigeria has been proposed as a reason to support the role of trade as the more likely source of introduction. As with many countries, it is still not known whether the first cases of infection detected in Nigeria were the first cases to occur and molecular data also suggests that wild birds cannot be ruled out.

2.4   TRANSMISSION OF VIRUS TO FARMS AND THE RISKS ASSOCIATED WITH DENSELY-POPULATED POULTRY AREAS

Once an avian influenza virus is introduced to a new location, infection may be extinguished unless it gains entry to a farm and is able to spread to other farms and/or markets.

HPAI epidemics in Europe, Canada and Southeast Asia have demonstrated the potential risks and major detrimental effects of HPAI in areas with a high density of poultry (referred to as 'densely populated poultry areas' or DPPA [Marangon *et al*, 2004]). These areas are highly susceptible to HPAI due to the fact that a breakdown in a single, large farm can directly and indirectly infect or affect neighbouring farms. In many of these areas, there is considerable movement of vehicles and people from farm to farm (Capua and Alexander, 2004) leading to conditions that facilitate spread of a virus once established. Control measures implemented by veterinary authorities in areas of high poultry density can also contribute to the spread of virus to neighbouring farms, presumably through contaminated dust particles disturbed during the culling process or possibly through inadvertent carriage of virus by investigators checking farms for excess mortality or other evidence of infection (Power, 2005). Airborne spread of virus over short distances has probably occurred, especially from heavily infected farms (Brugh and Johnson, 1986; Power 2005). Transmission by flies and vermin is also possible, given the fact that virus has been isolated from blow flies in Japan (Sawabe *et al*, 2006) and that these viruses can multiply in a range of mammalian species, including mice, without prior adaptation.

In areas with high poultry density, a 'stamping out' policy involving the culling of poultry on infected farms, neighbouring farms, contact premises, or in a zone of a certain diameter around an infected farm, can lead to the destruction of millions of poultry, as was seen in the Netherlands (some 30 million head of poultry culled or died) (Stegeman *et al*, 2004) and Canada (17 million head) (Bowes *et al*, 2004) following outbreaks of HPAI in 2003 and 2004.

Many of the areas in Asia affected by HPAI also have high poultry densities, but these are often scattered across many households rather than being concentrated in large flocks.

These risks can be reduced through implementation of strict on-farm biosecurity procedures (see Chapter 5) and use of other preventive measures such as prophylactic vaccination (see Chapter 6). Reliance of on-farm biosecurity measures does not prevent all virus incursions, especially if not all appropriate measures are taken, as has been seen in outbreaks in the Republic of Korea in late 2006 and in the United Kingdom in early 2007. The biosecurity status of the worst farms in a region can influence the fate of other well-run farms. These risks are exacerbated if farmers do not report disease in a timely manner, because of greater viral loads and opportunities for transmission.

Using well designed and properly implemented preventive measures, poultry reared under intensive conditions can remain free from infection even when farms are concentrated into relatively small areas. This has been demonstrated in HKSAR using a combination of enhanced biosecurity and vaccination since 2003.

However, in some production systems, notably those that allow poultry to forage freely, biosecurity cannot be enhanced significantly without changing the nature of the farming system. These farms remain at risk in areas where the virus is active. This is exacerbated if there is uncontrolled movement of traders and middlemen between farms and markets, leading to potential fomite transfer of viruses. In these systems, there is also considerable potential for wild birds to make contact with scavenging poultry such as grazing ducks, especially in wetlands that attract aquatic birds. Developing appropriate preventive measures for this type of farming

represents a major challenge for animal health authorities, but is essential if these flocks are to remain free from infection.

2.5   SPEED OF DETECTION AND RESPONSE TO VIRUS INCURSIONS

As with all emergency diseases, the speed of detection of HPAI is considered to be a major determinant of the extent of subsequent spread. In many countries in Asia, it appears that late recognition or late acknowledgement of the presence of H5N1 HPAI led to widespread dispersal of the viruses before effective, coordinated action was taken to control the disease. In places with various combinations of moderate to high poultry (and human) densities, poorly regulated large live-bird markets and/or high populations of domestic ducks, infection became 'endemic' (Chen *et al*, 2006c). Such countries included Thailand, Viet Nam, China and Indonesia, all of which have since implemented expensive control strategies to reduce the impact of the virus. Most have apparently been successful in reducing the level of infection, while some are in the process of developing infection-free compartments and/or zones.

There are a number of factors that influence the timeliness of reporting of – and thus the response to – H5N1 HPAI. Industrialized farms often have the technical support and infrastructure to detect and respond to outbreaks of the disease far more rapidly than small commercial farms and villages. This is due in part to the lack of access to veterinary services for these smaller holdings. Veterinary paraprofessionals are available in some countries for village level poultry, but their level of expertise in poultry disease diagnosis and control is often limited and their numbers small. Furthermore, in some countries, villagers do not necessarily seek assistance from veterinary paraprofessionals in the event of disease outbreaks in their scavenging poultry (Sims, 2007). A number of donor-funded projects are now in place aimed at increasing the level of support from veterinary paraprofessionals to smallholders and village level flocks.

Villagers do not always report disease when it occurs given the regular die-offs of poultry that occur from diseases such as Newcastle disease, infectious bursal disease, duck virus enteritis and, now, HPAI. Such die-offs are considered part of the normal production process in a low-input production system.

While the control of H5N1 HPAI relies on early reporting of disease, farmers across all production sectors have been guilty of failing to report suspicious cases. In some places, this has been due to genuine mistaken identity or failure to recognize that infection was present (especially in subclinically infected ducks), but in other cases concerns about the consequences of reporting have led to hiding of disease (see for example Nishiguchi *et al*, 2005).

Although it has been suggested that compensation is an incentive for farmers to report disease (World Bank, 2006), field observations suggest this relationship is weak when livelihoods are at stake or when high value birds such as fighting cocks are involved. Compensation usually only provides part payment for the loss of culled poultry and in most countries does not extend to reimbursement for consequential losses, which can far exceed the value of the destroyed poultry. Therefore, although absence of a compensation system means there is no incentive to report, the payment of compensation does not guarantee that reports will be forthcoming. For example, HKSAR provided generous compensation to farmers, but this still did not prevent one farmer from selling infected poultry to market in 2002 (Sims *et al*, 2003b). Some studies are currently in progress to examine the factors that motivate farmers to report disease and these are expected to assist in determining strategies for enhancing cooperation and early reporting.

2.6   CONCLUSIONS

HPAI is not a new disease and occurred well before the industrialization of poultry production. The number of outbreaks of HPAI has increased dramatically in the last eight years, in part due to spread of Asian-lineage H5N1 HPAI, but also as a result of outbreaks of diseases due to other HPAI viruses. Although a number of possible causes for this increase have been proposed, the reasons are not known and it is probably multifactorial.

Aquatic birds are the natural hosts of avian influenza viruses but are not normally infected with HPAI viruses. H5N1 HPAI has proved the exception to this rule by causing both clinical and sub-clinical infection in domestic ducks.

HPAI viruses are derived from LPAI viruses, often after multiplication in commercial poultry. Asian-lineage H5N1 HPAI viruses were first detected in 1996 and all viruses in this lineage isolated since then have been highly pathogenic. The circumstances that led to the emergence of this lineage of HPAI viruses are not known.

Asian-lineage H5N1 HPAI viruses have now spread across three continents. The mode of spread of these viruses is still not entirely clear. Trade in poultry and poultry products and movement of wild birds have played a role, although the relative contributions of these in the initial introduction into countries previously free from infection has not always been determined. Much of the evidence supporting these various modes of introduction is circumstantial and complicated in many cases by failure to detect the initial incursion of virus. Molecular epidemiology has assisted greatly in assessing links between various isolates

Speed of detection has also played a role in determining the effectiveness of control and eradication programmes. Many factors influence the timeliness of reporting, which is key to determining the speed of responses. Producers in all production sectors have not reported disease, sometimes due to failure to recognize the disease but also because of fears regarding the consequences of reporting. Compensation has been proposed as an incentive to report, and the absence of a compensation system provides little incentive to report. However, field experiences suggest that availability of compensation (which does not cover consequential losses or even the full value of poultry) does not necessarily lead to reporting of disease.