Ecology, Evolution and Pathogenesis of Avian Influenza Viruses

Ecologie, Evolutie en Pathogenese van Aviaire Influenza Virussen

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam op gezag van de rector magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

vrijdag 22 december 2006 om 11.00 uur

door

Vincent Jacobus Munster

geboren te Hoorn

Promotiecommissie

Promotor:	Prof. dr. A.D.M.E. Osterhaus
Overige leden:	Prof. dr. E.H.J.H.M Claassen Prof. dr. A. van Belkum Prof. dr. R. Goldbach
Copromotor:	Dr. R.A.M. Fouchier

ISBN-10: 90-9021242-6
ISBN-13: 978-90-9021242-6

The research described in this thesis was conducted at the Department of Virology of Erasmus MC, Rotterdam, The Netherlands, with financial support from EU-grant 'Novaflu' and the Dutch Ministery of Agriculture.

Printing of this thesis was financially supported by:

Printed by PrintPartners Ipskamp B.V.

Contents

1

General introduction

Adapted from Science, 2006, 312: 384-388

Influenza A viruses have been isolated from many species including humans, pigs, horses, mink, cats, marine mammals, and a wide range of domestic birds, but wildfowl and shorebirds are thought to form the virus reservoir in nature[1]. Influenza viruses belong to the family of *Orthomyxoviridae* and are further defined based on their antigenic differences in influenza virus types A, B and C[2]. The influenza A virus genome consists of eight segments of negative-sense single-stranded RNA. The influenza A virus is a pleiomorfic particle of approximately 120 nm in diameter. The viral envelop is derived from the host cell membrane. The viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), that mediate cell entry and release of virus particles respectively, and M2, that functions as an ion channel, are transmembrane proteins that interact with the matrix protein 1 (M1) in the viral interior. The M1 protein underlies the viral lipid envelop and provides rigidity to the viral structure. The M1 protein also interacts with the ribonucleoprotein (RNP) complex and NS2 that has a role in the export of RNPs from the nucleus. Each RNP is comprised of one of the eight viral RNA (vRNA) segments associated with multiple copies of the nucleoprotein (NP) and the viral polymerase complex. The basic polymerase 2 (PB2), the basic polymerase 1 (PB1) and the acidic polymerase (PA) are the three components of the viral polymerase complex. The non structural protein 1 (NS1) is not incorporated in the viral structure but is expressed abundantly in virus infected cells. The main functions of NS1 are the regulation of the synthesis of viral proteins and interference with the host's innate immune response. The eight gene segments of influenza A virus encode 10 different proteins and are numbered 1 to 8 according to size. Gene segments 7 and 8 encode two proteins each. The recently discovered PB1-F2 protein expressed from an alternative reading frame of gene segment 2, is involved in apoptosis. However, the alternative reading-frame encoding PB1-F2 is not present in all influenza A viruses (Fig. 1)[3,4].

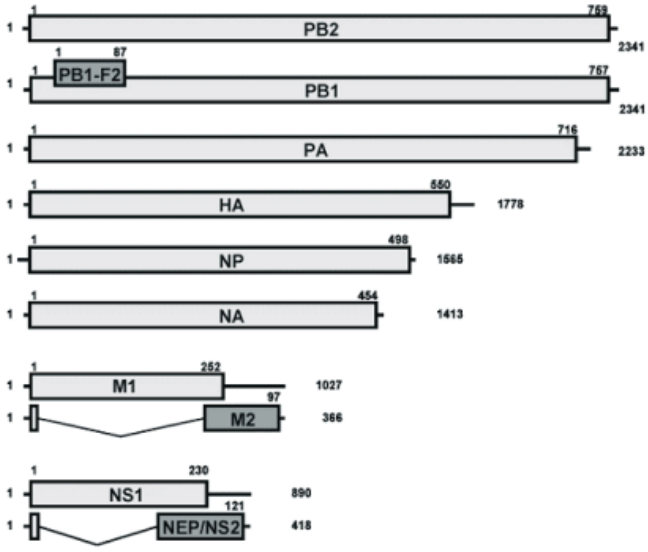


Figure 1. The genome organisation of influenza A virus. Taken from[3].

The replication cycle of influenza A viruses is initiated by binding of the influenza A virus surface glycoprotein HA to sialic acid residues on the host cell surface (Fig. 2). Upon attachment, the virus enters the cell through receptor-mediated endocytosis. Fusion of the virus membrane with the cell membrane allows the release of RNPs into the cytoplasm and subsequent transport of the RNPs into the nucleus. In the nucleus, the vRNA is transcribed into messenger RNA for the translation of viral proteins and copy RNA that serves as a template for vRNA synthesis. The newly synthesized vRNA forms RNPs with the newly translated polymerase proteins and NP and the RNPs are transported to the cell membrane. Together with the other structural proteins that have assembled there, the RNPs are packaged into new virions which bud from the cell surface. The efficient release of the viral particles is facilitated by the NA surface glycoprotein that cleaves sialic acid residues from the cell surface thereby allowing virion detachment from the cell and preventing virion aggregation (Fig. 2)[3].

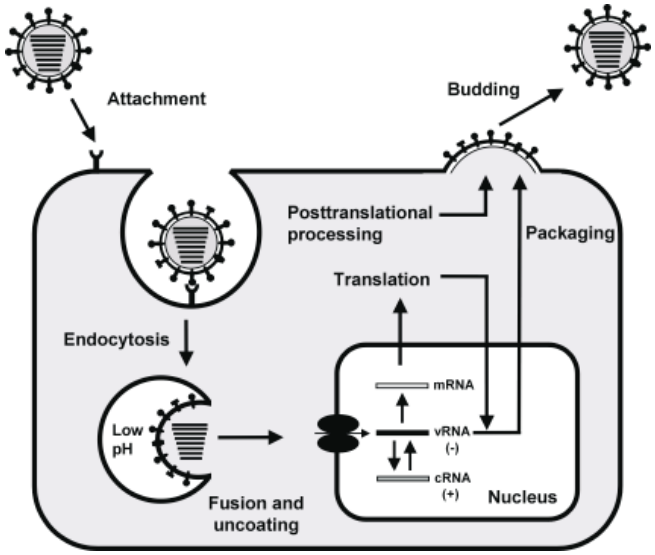


Figure 2. The replication cycle of influenza A viruses. Taken from[3].

Influenza A viruses are classified on the basis of the hemagglutinin and neuraminidase glycoproteins[1]. In wild birds and poultry throughout the world, influenza viruses representing 15 HA and 9 NA antigenic subtypes have been detected[1], which can be found in numerous combinations (also called subtypes, e.g., H1N1, H5N1)[5,6].
The HA protein is initially synthesized as a single polypeptide precursor (HA_0) which is cleaved into HA_1 and HA_2 subunits by proteases. The mature protein mediates binding of the virus to host cells, followed by fusion with endosomal membranes[1]. Influenza viruses of subtypes H5 and H7, but not other HA subtypes, may become highly pathogenic after introduction into poultry and can cause outbreaks of highly pathogenic avian influenza (HPAI, formerly termed “fowl plague”). The switch from a low pathogenic avian influenza (LPAI) virus phenotype, common in wild birds and poultry, to the HPAI virus phenotype is achieved by the introduction of basic amino

acid residues into the HA_0 cleavage site, which facilitates systemic virus replication. HPAI isolates have been obtained primarily from commercially raised poultry[7,8].
In the past decade, HPAI outbreaks have occurred frequently, caused by influenza viruses of subtype H5N1 in Asia, Russia, the Middle East, Europe, and Africa (ongoing since 1997), H5N2 in Mexico (1994), Italy (1997), and Texas (2004), H7N1 in Italy (1999), H7N3 in Australia (1994), Pakistan (1994), Chile (2002) and Canada (2003), H7N4 in Australia (1997), and H7N7 in the Netherlands (2003)[7,9].

Table 1. Prevalence of influenza A viruses in wild birds. Influenza virus prevalence in specific species is given only if tests on >500 birds have been reported. Of the 36 species of ducks, 28,955 were dabbling ducks and 1011 were diving ducks, with influenza virus prevalence of 10.1 and 1.6%, respectively. See[10] for additional comments and original data.

			Positive	
Family	**Species**	**Sampled**	**(n)**	**(%)**
Ducks	36 species	34,503	3,275	9.5
	Mallard (*Anas platyrynchos*)	1,525	1,965	12.9
	Northern Pintail (*Anas acuta*)	3,036	340	11.2
	Blue-winged Teal (*Anas discors*)	1,914	220	11.5
	Common Teal (*Anas Crecca*)	1,314	52	4.0
	Eurasian Wigeon (*Anas penelope*)	1,023	8	0.8
	Wood Duck (*Aix sponsa*)	926	20	2.2
	Common Shelduck (*Tadorna tadorna*)	881	57	6.5
	American Black Duck (*Anas rubripes*)	717	130	18.1
	Green-winged Teal (*Anas carolinensis*)	707	28	4.0
	Gadwall (*Anas strepera*)	687	10	1.5
	Spot-billed Duck (*Anas poecilorhyncha*)	574	21	3.7
Geese	8 species	4,806	47	1.0
	Canada Goose (*Branta canadensis*)	2,273	19	0.8
	Greylag Goose (*Anser anser*)	997	11	1.1
	White-fronted Goose (*Anser albifrons*)	596	13	2.2
Swans	3 species	5,009	94	1.9
	Tundra Swan (*Cygnus colombianus*)	2,137	60	2.0
	Mute Swan (*Cygnus olor*)	1,597	20	1.3
	Whooping Swan (*Cygnus cygnus*)	930	14	1.5
Gulls	9 species	14,505	199	1.4
	Ring-billed Gull (*Larus delawarensis*)	6,966	136	2.0
	Black-tailed Gull (*Larus crassirostris*)	1,726	17	1.0
	Black-headed Gull (*Larus ridibundus*)	770	17	2.2
	Herring Gull (*Larus argentatus*)	768	11	1.4
	Mew Gull (*Larus canus*)	595	0	0
Terns	9 species	2,521	24	0.9
	Common Tern (*Sterna hirundo*)	961	16	1.7
Waders	10 species	2,637	21	0.8
Rails	3 species	1,962	27	1.4
	Eurasian Coot (*Fulica atra*)	1,861	23	1.2
Petrels	5 species	1,416	4	0.3
	Wedge-tailed Shearwater (*Puffinus pacificus*)	794	4	0.5
Cormorants	1 species	4,500	18	0.4
	Great Cormorant (*Phalacrocorax carbo*)	4,500	18	0.4

Migratory birds as a natural reservoir of LPAI viruses

LPAI viruses have been isolated from at least 105 wild bird species of 26 different families (Table 1)[10]. All influenza virus subtypes and most HA/NA combinations have been detected in the bird reservoir and poultry, whereas relatively few have been detected in other species. Although many wild bird species may harbor influenza viruses, birds of wetlands and aquatic environments such as the Anseriformes (particularly ducks, geese, and swans) and Charadriiformes (particularly gulls, terns, and waders) constitute the major natural LPAI virus reservoir[1]. Anseriformes and Charadriiformes are distributed globally, except for the most arid regions of the world[11].

In birds, LPAI viruses preferentially infect cells lining the intestinal tract and are excreted in high concentrations in their feces. It has been shown that influenza viruses remain infectious in lake water up to 4 days at 22°C and more than 30 days at 0°C[12], and the relatively high virus prevalence in birds living in aquatic environments may be due in part to efficient transmission through the fecal-oral route via surface waters[1,12].

Migration is a common strategy for birds occupying seasonal habitats and may range from short local movements to intercontinental migrations. Migratory birds can carry pathogens, particularly those that do not significantly affect the birds' health status and consequently interfere with migration. Many Anseriformes and Charadriiformes are known to perform regular long-distance migrations[11], thereby potentially distributing LPAI viruses between countries or even continents. Birds breeding in one geographic region often follow similar migratory flyways, e.g., the East Asia–Australian flyway from eastern Siberia south to eastern Asia and Australia (Fig. 3A). However, the major flyways are simplifications, and there are numerous exceptions where populations behave differently from the common patterns[13,8]. Within the large continents and along the major flyways, migration connects many bird populations in time and space, either at common breeding areas, during migration, or at shared nonbreeding areas (Fig. 3). As a result, virus-infected birds can transmit their pathogens to other populations that subsequently may bring the viruses to new areas.

It is important to realize that the transmission of the viruses and their geographical spread is dependent on the ecology of the migrating hosts. For instance, migrating birds rarely fly the full distance between breeding and non-breeding areas without stopping-over and "refueling" along the way. Rather, birds make frequent stopovers during migration, and spend more time eating and preparing for migration than actively performing flights[14]. Many species aggregate at favorable stopover or wintering sites, resulting in high local densities. Such sites may be important for transmission of LPAI viruses between wild and captive birds and between different species.

Influenza viruses in ducks

Extensive surveillance studies of wild ducks in the Northern Hemisphere have revealed high LPAI virus prevalence primarily in juvenile—presumably immunologically naïve—birds with a peak in early fall before southbound migration. In North America, the prevalence falls from ~60% in ducks sampled at marshalling sites close to the Canadian breeding areas in early fall, to 0.4 to 2% at the wintering grounds in the southern U.S.A. and ~0.25% on the ducks' return to the breeding grounds in spring.

Similar patterns have been observed in Northern Europe, but influenza virus detection during spring migration can be significantly higher, up to 6.5%. Surveillance of the nesting grounds of ducks in Siberia before winter migration revealed the presence of influenza viruses in up to 8% of birds[15].

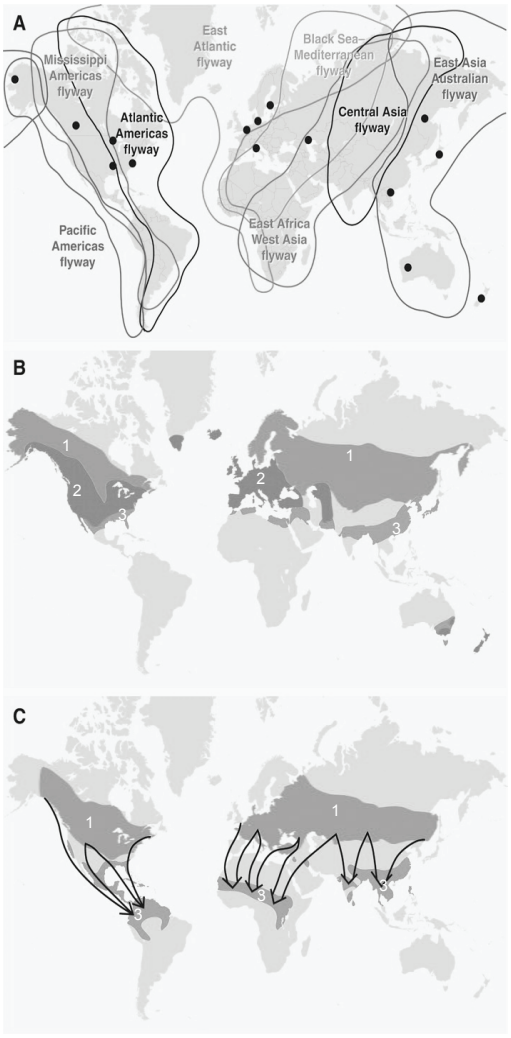


Figure 3. Migratory flyways of wild bird populations. A world map with the main general migratory flyways of wild bird populations is shown (adapted from information collected and analyzed by Wetlands International). Black dots indicate the locations of historical and current influenza virus surveillance sites from which data have been used in this manuscript (A). These global migration flyways are simplifications, and ther e are situations where populations behave differently from the common patterns. As examples, migration patterns of Mallard (*Anas platyrhynchos*) (B) and Garganey (*Anas querquedula*) in Eurasia and Africa and Blue-winged Teal (*Anas discors*) in the Americas (C) (right and left parts of the map, respectively) are provided. 1) indicates breeding areas in which species are absent during winter, 2) indicates areas in which species are present around the year, and 3) indicates areas in which species are only present in winter and do not breed. Arrows indicate the seasonal migration patterns.

Such year-round prevalence raises the possibility that LPAI virus can persist in ducks alone. This hypothesis complements earlier ones, in which additional host species or preservation of infectious influenza viruses in frozen lakes over the winter play a role in the perpetuation of avian influenza viruses[1,12].

All HA and NA subtypes, with the exception of H13 to H15, circulate in wild ducks in North America and Northern Europe. In a 26-year longitudinal study performed in Canada, influenza viruses of subtypes H3, H4, and H6 were isolated from ducks most frequently; H1, H2, H7, H10, and H11 less frequently; and H5, H8, H9, and H12 only sporadically. Although in other North American and European studies, influenza viruses of subtypes H3, H4, and H6 were also detected frequently, the detection of other virus subtypes was not significantly different[16]. Thus, the prevalence of influenza virus in general, as well as the specific distribution of subtypes, may vary between different surveillance studies depending on species, time, and place.

In the Canadian studies, cyclic patterns of influenza virus subtypes were reported: peaks in virus isolation of an HA subtype was followed 1 to 2 years later by reduced rates of isolation of this subtype. This observation awaits confirmation in other surveillance studies, but is of particular interest in relation to findings for other infectious diseases: cyclic patterns described for measles and whooping cough in humans have provided new insights in the role of spatial factors, herd immunity, and population age-structure on epidemiology[17]. Cycling of influenza virus in wild birds could provide similar new insights into the ecology of influenza viruses in their natural hosts.

Influenza virus surveillance of ducks has been performed in Japan since the late 1970s. As in other studies, influenza virus prevalence and isolated subtypes varied between years and locations[10]. The prevalence of influenza virus in wild birds elsewhere in Asia is largely unknown, but several studies have been conducted in live bird markets, where most HA and NA subtypes were found in poultry[1,18]. It is plausible that the circulation of the LPAI virus subtypes in poultry at least partially reflects that in wild birds, but no direct connection has yet been established.

Dabbling ducks of the Anas genus, with Mallards (*Anas platyrhynchos*) as the most extensively studied species, have been found to be infected with influenza viruses more frequently than other birds, including diving ducks (Table 1)[1]. Differences in virus prevalence between ecological guilds of ducks are likely in part related to behavior. Dabbling ducks feed primarily on food in surface waters; diving ducks forage at deeper depths and more often in marine habitats[11]. Dabbling ducks display a propensity for abmigration, the switching of breeding grounds between years, which is in part due to mate choice[11,14]. This behavior could provide an opportunity for influenza viruses to be transmitted between different host subpopulations. LPAI virus infection generally causes no major clinical signs in dabbling ducks, and experimental infections indicate that animals only produce a transient, low-level humoral immune response, which may be sufficient to provide partial protection against reinfection with viruses of the same subtype but is unlikely to confer protection against heterologous reinfections[19]. Different influenza virus subtypes can also infect ducks concomitantly, creating the opportunity for genetic mixing[20].

Little is known about the prevalence of influenza viruses in wild ducks in the Southern Hemisphere or potential transmission between the hemispheres. There is little connectivity between northern and southern Anatidae species, and most

species stay year round within each breeding continent. The Blue-winged Teal (*Anas discors*) is one of the few North American species that has a winter distribution that includes South America (Fig. 3C)[11]. There are several other duck species that could serve as hosts for influenza virus in South America[11], but surveillance data are not available. Similarly, only 6 of 39 Anatidae species breeding in Eurasia winter with at least part of the population south of the Sahara desert in Africa, e.g., the Garganey (*Anas querquedula*) (Fig. 3C) and the Northern Pintail (*Anas acuta*) each have African winter populations in excess of one million birds[21]. As in South America, none of the 22 Anatidae species that breed in sub-Saharan Africa spend the non-breeding season outside the continent. However, there are several species with large, widespread populations in Africa[17], and some migrate within Africa[22]. Potential areas for mixing of Eurasian and African ducks are in West Africa, near the Senegal and Niger Rivers, the floodplains of the Niger River in Nigeria and Mali, and Lake Chad[21], and influenza viruses in African Anatidae populations may thus be linked to Eurasia through migrating species. Anatidae of Oceania are mainly resident and do not perform regular seasonal migrations[11].

Influenza viruses in gulls and terns

The first recorded isolation of influenza virus from wild birds was from a Common Tern (*Sterna hirundo*) in 1961. This HPAI H5N3 virus was responsible for an outbreak in South Africa where at least 1300 of these birds died[7,23]. The most frequently detected LPAI virus subtype in gulls is H13, a subtype rarely found in other birds. The genes of gull viruses are genetically distinct from those of influenza viruses from other hosts, which suggests they have been genetically isolated for sufficient time to allow genetic differentiation (Fig. 4)[6]. This concurs with the observation that gull influenza viruses do not readily infect ducks when they are inoculated experimentally[1,24]. Although other influenza virus subtypes are also occasionally detected in terns and gulls (Table 1)[10], it is plausible that the viruses that are genetically indistinguishable from viruses of other avian hosts are most likely not endemic in gulls and terns.

Influenza viruses can be detected in a small proportion of gulls, with the highest virus prevalence reported in late summer and early fall. Most gull species breed in colonies[11], with adults and juveniles crowded in a small space, creating good opportunities for virus spread. This situation contrasts with that in dabbling ducks that do not breed in dense colonies[11], and epizootics could be more easily initiated when birds congregate in large numbers during molt, migration, or wintering.

Influenza viruses in waders

Waders in the Charadriidae and Scolopacidae families are adapted to either marine or freshwater wetland areas and often live side-by-side with ducks[25]. Long-term influenza virus surveillance studies are still sparse, but data from North America suggest a distinct role of these birds in the perpetuation of certain virus subtypes. Influenza viruses of subtypes H1 to H12 have been isolated in birds migrating through the eastern U.S.A., with a high prevalence of certain HA subtypes (H1, H2, H5, H7, H9 to H12) and a larger variety of HA/NA combinations as compared with ducks in Canada, suggesting that waders maintain a wider spectrum of viruses. Moreover, the seasonal prevalence of influenza viruses in waders seems to be reversed as compared with ducks, with higher virus prevalence (~14%) during spring migration[26].

This has led to the hypothesis that different families of wetland birds are involved in perpetuation of LPAI virus and suggests a role for waders, which may carry the virus north to the duck breeding grounds in spring. Recent genetic analyses have not revealed striking differences between influenza viruses from ducks and waders in the Americas, suggesting that these viral gene pools are not separated[27,28]. Although the wader-duck link may be a plausible scenario based on the North American data, studies in waders in Northern Europe have failed to produce similar results. Nevertheless, many wader species of the Northern hemisphere are long-distance intercontinental migrants[13] and may, therefore, have the potential to distribute influenza viruses around the globe.

Influenza viruses in other wild birds

LPAI viruses can be found in numerous other bird species (Table 1)[10], but it is unclear in which of these species influenza viruses are endemic and in which the virus is a temporary pathogen. Species in which influenza viruses are endemic share the same habitat at least part of the year with other species in which influenza viruses are frequently detected including geese, swans, rails, petrels, and cormorants. In these birds and others[10], influenza virus prevalence seems to be lower than in dabbling ducks (Table 1), but it should be noted that studies on these species are limited, and it is possible that peak prevalence has been missed because of its seasonal nature or location.

As for ducks, gulls, and waders, their behavior and ecology may be an important determinant of their role as host species. For instance, geese are mainly herbivorous and often congregate in large flocks for grazing in pastures and agricultural fields, especially during the non-breeding season. Such flocks may reach tens of thousands of birds in optimal areas and often contain several different species. Colonial breeding occurs in some goose species, but most are solitary nesters or nest in loose groups with little interaction between pairs. Given that wild geese and ducks are the ancestors of today's domestic goose and duck species and that these domestic animals in parts of the world are frequently kept alongside chickens, wild geese and ducks may form the bridge for influenza viruses between wild and domestic birds.

Genetic variation of influenza viruses in wild birds

Evolution of avian influenza viruses in their natural hosts is slow, but not negligible. Avian influenza viruses can be divided into two lineages, Eurasian and American (Fig. 4), probably as a result of long-term ecological and geographical separation of hosts. However, the avifauna of North America and Eurasia are not completely separated; some ducks and shorebirds cross the Bering Strait during migration or have breeding ranges that include both the Russian Far East and northwestern North America[11]. The majority of tundra shorebirds from the Russian Far East winter in Southeast Asia and Australia, but some species winter along the west coast of the Americas[29]. The overlap in distribution of ducks is not as profound as that of shorebirds, but a few species (e.g., Northern Pintail, *Anas acuta*), are common in both North America and Eurasia[11] and could also provide an intercontinental bridge for influenza virus. Indeed, influenza viruses carrying a mix of genes from the American and Eurasian lineages have been isolated, indicating that allopatric speciation is only partial[30-32]. The partial ecological isolation of influenza virus hosts seems sufficient to facilitate

divergent evolution of separate gene pools, but allows occasional spillover of gene segments from one gene pool to the other.
Within each genetic lineage, multiple sublineages of viral genes cocirculate, but there appear to be no consistent temporal or spatial correlations. Moreover, genetic data from duck and shorebird influenza virus isolates from the Americas suggests an active interplay between these host species[27,28]. Although certain HA subtypes are reported to be more prevalent in either shorebirds or ducks in North America, this also does not seem to have resulted in differences in the genetic composition of influenza viruses obtained from these two reservoirs[26,33].

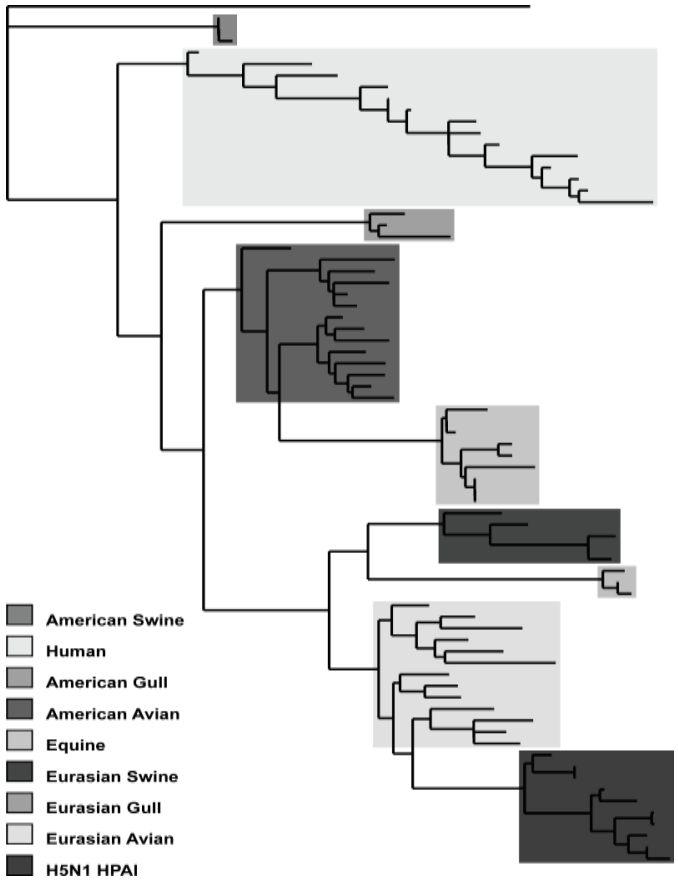


Figure 4. Phylogenetic tree for the matrix gene of influenza A viruses from a variety of hosts. Nucleotide sequences were selected from public databases and aligned, after which a maximum likelihood tree was generated using influenza virus A/Equine/Prague/57 (H7N7) as outgroup. Sequences were selected from each host to reflect the longest possible time frame and variation in locations of virus isolation. The avian influenza viruses are divided in an American lineage and a Eurasian lineage, and there are no clear patterns of host, temporal, or spatial correlation within these lineages. In contrast, the human influenza A virus lineage, the Eurasian swine lineage, and the HPAI H5N1 lineage display clear temporal patterns of virus evolution

The segmented nature of the influenza virus genome enables evolution by a process known as genetic reassortment, i.e., the mixing of genes from two or more influenza viruses. A recent study of 35 influenza virus isolates obtained from ducks in Canada indicates that genetic "sublineages" do not persist, but frequently reassort with other viruses[34]. Influenza viruses of a particular subtype do not necessarily have the same genetic make-up, even within a single year or a single host species. The high prevalence of influenza virus in some wild bird species, and the sporadic detection of concomitant infections in single birds[20] support the notion that reassortment may occur in nature. Gaining information on the actual frequency of reassortment in the wild bird reservoir and the impact of these events on LPAI virus evolution will be of considerable interest.

HPAI H5N1 viruses in wild birds

In 1997, an HPAI outbreak caused by H5N1 influenza virus occurred in chicken farms and the live bird markets of Hong Kong, which also resulted in the first reported case of human influenza and fatality attributable directly to avian influenza virus[35]. The H5N1 HPAI virus reappeared in 2002 in waterfowl at two parks in Hong Kong and was also detected in other captive and wild birds[36]. It resurfaced again in 2003 and has devastated the poultry industry in large parts of Southeast Asia since 2004. In 2005, the virus was isolated during an outbreak among migratory birds in Qinghai Lake, China, affecting large numbers of wild birds[37-39]. This single epizootic caused an estimated 10% decrease of the global population of Bar-headed Geese (Anser indicus), highlighting the potential devastating effects on vulnerable wildlife. Subsequently, the virus has appeared across Asia, Europe, and the Middle East, and in several African countries. Wild bird deaths have been reported in several of these countries, in Europe, particularly affecting Mute Swans (*Cygnus olor*) and Whooper Swans (*Cygnus cygnus*), but mortality has also been recorded in other waterfowl species, and occasionally in raptors, gulls, and herons. So far, the HPAI H5N1 strain that originated in poultry in Southeast Asia has caused mortality in >60 wild bird species[36-38,40,41]. In addition, during the devastating outbreaks in poultry, the H5N1 virus was transmitted to 241 humans, leading to 141 deaths (as of 23 August 2006)[42], and has also been isolated from pigs, cats, tigers, and leopards[41].

It is most likely that the H5N1 virus has circulated continuously in domestic birds in Southeast Asia since 1997 and, as a consequence, has evolved substantially (Fig. 2). Surveillance studies in Mainland China from 1999 onward indicated that H5N1 viruses have become endemic in domestic birds in the region and that multiple genetic lineages of the virus are cocirculating[39,43]. Poultry trade and mechanical movement of infected materials are likely modes for spreading HPAI in general[7]. For the H5N1 virus, it is without doubt that domestic waterfowl, specific farming practices, and agro-ecological environments played a key role in the occurrence, maintenance, and spread of HPAI for many affected countries[44,45].

Avian influenza in humans

It has been clear for quite some time that the pandemic outbreaks of 1957 (H2N2) and 1968 (H3N2) were caused by influenza A viruses which arose by reassortment of human and avian influenza A viruses[46,47]. Because of the host species barrier for avian influenza A viruses to infect humans (and vice versa), it was suggested that

pigs, with known susceptibility for avian and human influenza A viruses, acted as mixing vessels for the genesis of pandemic influenza A viruses. Reassortment of human and avian influenza A viruses could therefore take place in pigs, with the emergence of influenza A viruses with internal genes that allow efficient replication and transmission in humans, but with novel HA and NA subtypes against which no prior immunity exists in humans. However, in the last decade, there has been a significant change in our understanding of infections of humans with avian influenza A viruses.

Table 2. Reported cases of avian influenza infection in humans 1959-present (August 2006).

Year	Country	Subtype	Number infected	Number of Deaths	Symptoms*
1959	USA	H7N7	1	0	Unknown
1977	Australia	H7N7	1	0	Conjunctivitis
1981	USA	H7N7	1	0	Conjunctivitis
1996	England	H7N7	1	0	Conjunctivitis
1997	Hong Kong	H5N1	18	6	ILI
1999	Hong Kong	H9N2	2	0	ILI
2003	The Netherlands	H7N7	89	1	Conjunctivitis,ILI
	USA	H7N2	1	0	ILI
	Hong Kong	H5N1	2	1	ILI
	Vietnam	H5N1	3	3	ILI
2004	Canada	H7N3	2	0	ILI
	Thailand	H5N1	17	12	ILI
	Vietnam	H5N1	29	20	ILI
2005	Cambodia	H5N1	4	4	ILI
	China	H5N1	8	5	ILI
	Indonesia	H5N1	17	11	ILI
	Thailand	H5N1	5	2	ILI
	Vietnam	H5N1	61	19	ILI
2006-present	Azarbaijan	H5N1	8	5	ILI
	Cambodia	H5N1	2	2	ILI
	China	H5N1	12	8	ILI
	Djibouti	H5N1	1	0	ILI
	Egypt	H5N1	14	6	ILI
	Indonesia	H5N1	43	35	ILI
	Iraq	H5N1	2	2	ILI
	Thailand	H5N1	2	2	ILI
	Turkey	H5N1	12	4	ILI

* ILI = influenza-like illness with lower respiratory tract symptoms.

Until 1997, there were only four recorded human infections with avian influenza A viruses. This was in line with experimental infection with LPAI viruses in human volunteers which at best produced only transient infections[48]. However, in 1997, an HPAI H5N1 outbreak in poultry in Hong Kong occurred and the virus was subsequently transmitted to 18 humans, including 6 deaths[35]. This was the first indication that a purely avian influenza virus could cause respiratory disease and death in humans. This was the start of a continuing series of human infections of HPAI viruses from poultry (Table 2). It is likely that many more people have been infected with these

viruses than presented in table 2. Serological surveys after HPAI outbreaks indicate extensive seroprevalence against avian influenza A viruses, without any known occurrence of clinical disease[49-51].

Seasonal human influenza is predominantly transmitted by inhalation of infectious aerosols. For HPAI viruses infections in humans, bird-to-human, environment-to-human, and to a limited extend human-to-human transmission as likely modes of transmission[52]. The reported symptoms of avian influenza in humans range from typical influenza-like illness (ILI), to conjunctivitis, pneumonia, acute respiratory distress and other severe and life-threatening complications.

The series of human infections with avian influenza A viruses indicate that pandemic viruses could emerge without intermediate host. Avian influenza viruses could evolve into pandemic viruses either through reassortment or by progressive adaptation to efficient replication and transmission in humans, without the necessity of prior reassortment. Sequence and phylogenetic analyses suggested that the 1918 H1N1 "Spanish" influenza, which killed an estimated 20 – 50 million people worldwide, was not a reassortant virus, indicating that the H1N1 virus containing a completely avian genome, was able to establish itself in the human population. Moreover, sequencing analysis of the gene segments of the H1N1 "Spanish" influenza and other avian viruses which directly infected humans, has identified mutations that are a result of progressive adaptation to the human (mammalian) host[53-55].

Influenza vaccines

Vaccination is the most effective way of seasonal influenza control. Influenza A virus vaccines are used to induce virus neutralizing antibodies. These antibodies are primarily directed against the HA and NA surface glycoproteins, providing protective immunity against infection. Whole inactivated vaccines, split vaccines, subunit vaccines and live-attenuated influenza vaccines are available for vaccination of humans against the yearly influenza A virus epidemic. The rapid antigenic evolution of the HA of human influenza A viruses, the so-called antigenic drift, necessitates an intensive global surveillance to monitor the antigenic properties of the circulating virus strains in order to maintain antigenic similarity between the vaccine strains and the circulating wild-type virus[56]. Whereas human influenza A vaccines have to be closely matched to the circulating field strain in order to elicit an effective response, the overall breadth of response generated by vaccines used in poultry is wider and can be generated with relatively little homology to the field strain. Inactivated whole-virus water-in-oil emulsion vaccines are the conventionally used vaccine preparation. Alternatives for the traditional vaccine preparation have been developed recently. Using molecular biology, recombinant fowlpox vaccines, recombinant infectious laryngotracheitis virus and recombinant Newcastle disease virus containing an H5 insert have been developed[57-59]. Vaccination with inactivated whole-virus vaccines or recombinant vaccines prevents clinical disease and mortality, decrease susceptibility of poultry to infection with avian influenza viruses and decrease shedding from the respiratory and intestinal tract. Data on the quantification of vaccination in poultry indicate that vaccination not only protects poultry from disease signs but is also effective in reducing the transmission level of the virus, and could therefore prevent major outbreaks[60]. However, these vaccines do not completely prevent infection, and appropriate biosecurity measures should be implemented to prevent spread

between vaccinated flocks.

Outline of this thesis

Wild bird populations play an essential role in the maintenance and perpetuation of avian influenza A viruses. Detailed information about the epidemiology, ecology and evolution of avian influenza A viruses in their natural hosts in Northern Europe was limited. Several studies were designed to fill this gap in our knowledge by screening tens of thousands of wild bird cloacal or fecal specimens (chapter 2 – 7). The majority of specimens were collected by ornithologists during the fall migration and wintering of different Anseriformes (duck, geese and swans) species in The Netherlands and Sweden (chapter 2, 6, 7). However, emphasis was also placed on birds within other bird orders, such as the Charadriiformes (gulls, terns and waders) leading to the discovery of a novel HA subtype, H16 (chapter 4) and a new avian influenza host (chapter 5). The second part of this thesis focuses on H5 and H7 avian influenza viruses, and the problems that arise once avian influenza A viruses of the H5 and H7 subtypes are introduced into poultry. The relation between LPAI and HPAI H5 and H7viruses is described by comparing genetic and antigenic variation of strains currently circulating in wild birds and the strains causing the HPAI outbreaks of the last decade in Europe (chapter 7). The close antigenic relation between H7 LPAI strains and the HPAI strain causing the 2003 H7N7 HPAI outbreak in The Netherlands led to development of a prototype vaccine based on the HA of one of the H7 LPAI strains isolated during our wild bird surveillance (chapter 8, 9). The genetic and phenotypic differences between human H7N7 strains from either a conjunctivitis case or the fatal case isolated during the H7N7 HPAI outbreak in The Netherlands allowed detailed comparison of the molecular basis of the pathogenicity of the human H7N7 HPAI isolates *in vivo* and *in vitro* (chapter 10, 11). To determine the relation between attachment of HPAI H5N1 virus and the pathogenesis of HPAI H5N1 in humans, the distribution of attachment of HPAI H5N1 to the cells of the human lower respiratory tract was studied (chapter 12). The findings presented in chapter 2 – 12 are evaluated in a summarizing discussion (chapter 13).

2

Spatial, temporal and species variation in prevalence of influenza A viruses in wild migratory birds

Submitted

Vincent Munster
Chantal Baas
Pascal Lexmond
Jonas Waldenström
Anders Wallensten
Thord Fransson
Guus Rimmelzwaan
Walter Beyer
Martin Schutten
Björn Olsen
Albert Osterhaus
Ron Fouchier

Influenza A surveillance in wild birds is essential to improve our understanding of the complex ecology, epidemiology, genetics and evolution of avian influenza A viruses. Here we present data on the prevalence of influenza A viruses in the wild bird population from our ongoing influenza surveillance studies. From 1998 until 2006 more than 36,000 samples collected from 321 different bird species of 18 orders were screened for the presence of influenza A virus using molecular diagnostic tools. 332 avian influenza A viruses were isolated from 992 samples that were positive by RT-PCR. In total 55 different viral subtypes were detected, including all neuraminidase subtypes identified to date and all hemagglutinin subtypes with the exception of H14 and H15. Temporal and spatial variation in influenza A virus prevalence was observed within and between bird species. The ecology and epidemiology of avian influenza A virus in wild birds in Northern Europe is discussed in relation to host ecology, and contrasted with results from previously published studies.

Introduction

Birds of wetlands and aquatic environments such as the Anseriformes (particularly ducks, geese, and swans) and Charadriiformes (particularly gulls, terns, and shorebirds) are thought to constitute the major natural reservoir for avian influenza A virus[1,61]. Influenza A viruses of all hemagglutinin (HA) and neuraminidase (NA) subtypes (H1 to H16 and N1 to N9) and most HA/NA combinations have been identified in the wild bird reservoir. Anseriformes and Charadriiformes are distributed globally, except for the most arid regions of the world, and represent an almost global coverage of influenza A virus host species[6,26]. In birds, influenza viruses preferentially infect cells lining the intestinal tract and are excreted in high concentrations in their feces, and transmission is thought to be achieved primarily via the fecal-oral route[1]. This route likely represents an efficient way to transmit viruses between waterfowl, by shedding the virus via feces into the surface water[1].

The prevalence of avian influenza A viruses in their natural hosts depends on geographical location, seasonality, and species. For instance, the prevalence of avian influenza A viruses in ducks in North America varies from less than 1 % during spring migration up to 30 % prior to and during fall migration, but large variations in virus prevalence have been observed in different surveillance studies[1]. The peak in prevalence during fall migration is believed to be related to the large numbers of young immunologically naïve birds of that breeding season[1,61]. Although there is extensive data on surveillance studies of influenza A viruses in ducks and shorebirds in North America[16,26], limited up-to-date information is available for Eurasia, Africa, South America, and Oceania and only for limited numbers of species[15,62,63,64]. Because of the apparent species-specific niches of certain HA subtypes such as H13 and the recently discovered H16[6,24,65,66], yet unidentified influenza A viruses may exist in nature. Information about influenza A viruses in Eurasia in addition to North America is of particular interest because the influenza A viruses found in Eurasian wild birds are genetically distinct from those of wild birds in the Americas[1,6]. The direct zoonotic potential of several Eurasian lineage avian influenza A viruses are currently the cause of serious concern[52,55,67,68]. The increasing problems with outbreaks of highly

pathogenic avian influenza (HPAI), the potential spread of HPAI H5N1 by wild birds over large geographic areas, and the threat that certain avian influenza A viruses pose to public and animal health emphasize the need for more information on the ecology of avian influenza A viruses circulating in the wild bird reservoir. Our current knowledge of the epidemiology of avian influenza A viruses, virus ecology in relation to host ecology, the temporal and spatial patterns of avian influenza A viruses in their natural hosts, the role of potential new hosts in the influenza A virus ecology and the interaction between wild birds and poultry, is still very limited.

Traditionally, influenza A virus surveillance studies in wild birds have been performed by direct virus isolation from fecal samples or cloacal swabs in embryonated hen's eggs[69]. This method is labor intensive due to handling time of each of the individual samples, and is quite sensitive to laboratory contaminations, in particular if blind-passage is used routinely during virus isolation attempts. Currently this diagnostic method is being replaced in many laboratories by molecular diagnostic tests, such as conventional RT-PCR or real-time RT-PCR methods targeting highly conserved gene segments of influenza A virus. Such molecular methods allow the rapid identification of influenza A virus positive specimens from large collections of samples, which can then be used for targeted virus isolation attempts[70,71]. In this study we present data on the prevalence of influenza A viruses from our ongoing wild bird surveillance studies. From 1998 until 2006, we screened more than 36,000 samples collected from 321 different bird species using molecular diagnostics. Most samples were collected from birds in Northern Europe, but samples from North and South America, Asia, Africa, Antarctica and the Arctic were also included. We used this data to describe temporal and spatial patterns in influenza A virus prevalence in different wild migratory bird species.

Materials and methods

Specimens

Birds were trapped by expert ornithologists using duck decoy, duck traps, wader funnel traps, mist nets, clap nets, cannon nets or Helgoland traps. Cloacal swabs were collected using sterile cotton swabs (Greiner, The Netherlands) of two different sizes depending on the size of the birds. The cloacal swabs were stored in transport media (Hanks balanced salt solution containing 0.5 % Lactalbumin, 10 % glycerol, 200 U/ml penicillin, 200 µg/ml strepromycin, 100 U/ml polymyxin B sulfate, 250 µg/ml gentamycin, 50 U/ml nystatin (ICN, The Netherlands) and shipped to the laboratory where they were stored at –80 ºC upon analyses. Before shipment, the samples were stored at 4 ºC for less than a week, at –80 ºC if such freezers were available nearby the sampling site and at –20 ºC only if rapid transport or storage at –80 ºC was practically impossible.

Sample location

Samples were obtained from 321 different bird species belonging to 18 different orders and a wide variety of sampling locations. The majority of samples were obtained consistently over the years from the same sites in The Netherlands (Krimpen a/d Lek, 51º54'N 4º41'E and Lekkerkerk, 51º54'N 4º38'E) and Sweden (Ottenby Bird

Observatory, Öland, 56º12'N 16º24'E). Besides the consistent sampling at these sites, samples were also collected during short-term sampling expeditions at different sites in Europe, Asia, Africa, North America, South America, Antarctic, and the Arctic. In 2005 numerous sampling sites were added in response to potential HPAI H5N1 threats.

RNA isolation and virus detection

RNA isolation and RT-PCR was performed as described previously for samples obtained until 2002[80]. From 2003 onwards, RNA was isolated using a MagnaPure LC system with the MagnaPure LC Total nucleic acid isolation kit (Roche Diagnostics, Almere, The Netherlands) and influenza A virus was detected using a real-time RT-PCR assay targeting the matrix gene[71]. To ensure efficient influenza A virus detection, the published probe sequence was changed to 6-FAM-TTT-GTG-TTC-ACG-CTC-ACC-GTG-CC-TAMRA-3', based on the avian influenza A virus sequences available from public databases. Amplification and detection was performed on an ABI7700 with the TaqMan EZ RT-PCR Core Reagents kit (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) using 20 μl of eluate in an end volume of 50 μl. Pools of five individual samples were prepared and processed in parallel with several negative and positive control samples in each run. Upon identification of influenza A virus positive pools, the RNA isolation and RT-PCR procedures were repeated for the individual samples within each positive pool and individual RT-PCR positive samples were subsequently used for virus isolation.

Virus isolation and characterization

For influenza A virus RT-PCR positive samples, 200 μl of the original material was inoculated into the allantoic cavity of 11-day-old embryonated hens' eggs. The allantoic fluid was harvested two days after inoculation and influenza A virus was detected using hemagglutination assays with turkey erythrocytes. When no influenza A virus was detected upon the initial virus isolation attempt, the allantoic fluid was passaged once more in embryonated hens' eggs. The HA subtype of virus isolates were characterized using a hemagglutination inhibition (HI) assay with turkey erythrocytes and subtype-specific hyperimmune rabbit antisera raised against all HA subtypes[6].

Sequence analysis

The NA subtype of virus isolates was characterized by RT-PCR and sequencing. RT-PCR was performed using primers specific for the conserved non-coding regions of NA, essentially as described by others[81]. PCR products were purified from agarose gels using the Qiaquick Gel Extraction kit (Qiagen, Leusden, The Netherlands) and sequenced. Sequencing was performed using the Big Dye terminator sequencing kit version 3.0 (Amersham Pharmacia Biotech, Roosendaal, the Netherlands) and a 3100 genetic analyzer (applied BioSystems, Nieuwerkerk aan den IJssel, the Netherlands), according to the instructions of the manufacturer. Nucleotide sequences were aligned using the Clustal W program running within the Bioedit software package, version 5.0.9. NA nucleotide sequences were analyzed with the basic local alignment search tool (BLAST) available from Genbank[82] to identify the NA subtype.

Statistics

The 95% confidence interval analysis and the Pearson X2-test were used for the analyses of the dataset used in this study.

Results

Avian influenza A virus surveillance in wild birds

The influenza A virus surveillance study described here was initiated in 1998 in collaboration with a large network of ornithologists. The sample collection includes many bird species that have been reported to be permissive to avian influenza A virus (in particular Anseriformes and Charadriiformes) in earlier influenza A virus surveillance studies[1,16,26,64]. To obtain more detailed information on the prevalence of avian influenza viruses in birds belonging to other orders, relatively large numbers of samples of birds from different bird families and various geographical locations were also included. This was done in part because most of the earlier studies relied solely on virus isolation in embryonated hens' eggs as screening method, to see if molecular detection methods would yield different results. Birds were caught with a wide variety of different methods, depending on species and location; duck decoy and duck traps for duck species, funnel traps and mist nets for shorebird species, mist nets and Helgoland traps for passerine species, and cannon nets and clap nets for goose and swan species. Whereas sampling efforts predominantly were concentrated to the Netherlands and Sweden, relatively large numbers of samples were obtained from other locations in Northern Europe (7 countries, multiple sites). Limited numbers of samples were obtained from Africa (Nigeria, Ghana), North America (USA, Canada), South America (Argentina), Asia (Kazakhstan, South Korea), the Arctic (Norway, Iceland) and the Antarctic Peninsula. All samples were taken from healthy birds, as part of the ornithologists' own research projects. From 1998 until 2006 we have sampled 36796 birds belonging to 18 orders and 321 species (Table 1). Virus isolation was performed on all samples that were positive by RT-PCR. Of the 992 RT-PCR positive samples, 332 virus isolates were recovered, yielding an overall recovery rate of 33.5%. For all virus isolates the HA and NA subtypes were characterized. All influenza A virus isolates were obtained from birds belonging to the orders of Anseriformes and Charadriiformes.

Prevalence of influenza A viruses in Anseriformes

Within the different duck, goose and swan species the overall influenza A virus prevalence for each species is presented in Table 2. Influenza A viruses were detected in 649 of 8938 (7.3 %) Mallards (*Anas platyrhynchos*), 76 of 2538 (3.0 %) Eurasian Wigeons (*Anas penelope*), 60 of 940 (6.4 %) Common Teals (*Anas crecca*), 13 of 448 (2.9 %) Northern Pintails (*Anas acuta*), 8 of 298 (2.7 %) Gadwalls (*Anas strepera*), and 5 of 135 (3.7 %) Northern Shovelers (*Anas clypeata*) which belong to the dabbling ducks. The overall prevalence in dabbling ducks was 6.1 %. In Mallards and Teals, the influenza A virus prevalence was higher than in Wigeons, Pintails, Gadwalls and Shovelers combined (7.2% vs. 3.0%, Pearson X2-test , $P<0.001$). The sampled dabbling ducks all migrate along the East-Atlantic flyway. The ring recovery for the most extensively studied duck species, Mallards ringed at Ottenby Bird

Observatory (Öland, Sweden) in 2002 and 2003, and Mallards, Eurasian Wigeons and Common Teals from 1998 - 2005 ringed in The Netherlands is shown in Figure 1[72].

Table 1. Overview of the bird samples analyzed in this study. The table includes data for specific orders and families only if ≥ 50 samples of that order or family were tested.

	Order	Family	Species	N Samples
	Sphenisciformes	Spheniscidae	3	190
	Procellariiformes	Procellariidae	1	107
	Pelicaniformes	Pelecanidae	1	64
		Phalacrocoracidae	2	1202
	Anseriformes	Anatidae	28	20,901
	Falconiformes	Accipitridae	5	70
	Galliformes	Phasianidae	10	50
	Gruiformes	Rallidae	4	1,029
	Charadriiformes	Alcidae	4	907
		Laridae	11	4,099
		Scolopacidae	36	2,754
		Haematopodiae	1	88
		Charadriidae	8	296
	Columbiformes	Columbidae	7	109
	Piciformes	Picidae	5	25
		Ramphastidae	5	43
	Passeriformes	Sylviidae	25	1,138
		Alaudidae	3	177
		Turdidae	10	939
		Estrildidae	13	211
		Emberizidae	11	121
		Paridae	9	400
		Corvidae	7	57
		Motacillidae	8	204
		Prunellidae	1	123
		Sturnidae	4	220
		Muscicapidae	17	204
		Timaliidae	2	188
		Ploceidae	9	178
		Pycnonotidae	5	97
		Regulidae	2	195
		Troglodytidae	1	88
Others	7	33	63	322
Total	18	65	321	36,796

Influenza A viruses were also detected in ducks belonging to other guilds: 2 of 37 (5.4 %) Common Eiders (*Somateria mollissima*), 2 of 335 (0.6 %) Common Shelducks (*Tadorna tadorna*) and 2 of 62 (3.2 %) Tufted Ducks (*Aythya fuligula*), which belong to the stifftails, shelducks and pochards respectively. Influenza A viruses were not detected in 20 other ducks, belonging to 8 additional species. Nearly all of the duck

samples were obtained during fall migration in Sweden and The Netherlands and wintering sites in The Netherlands.

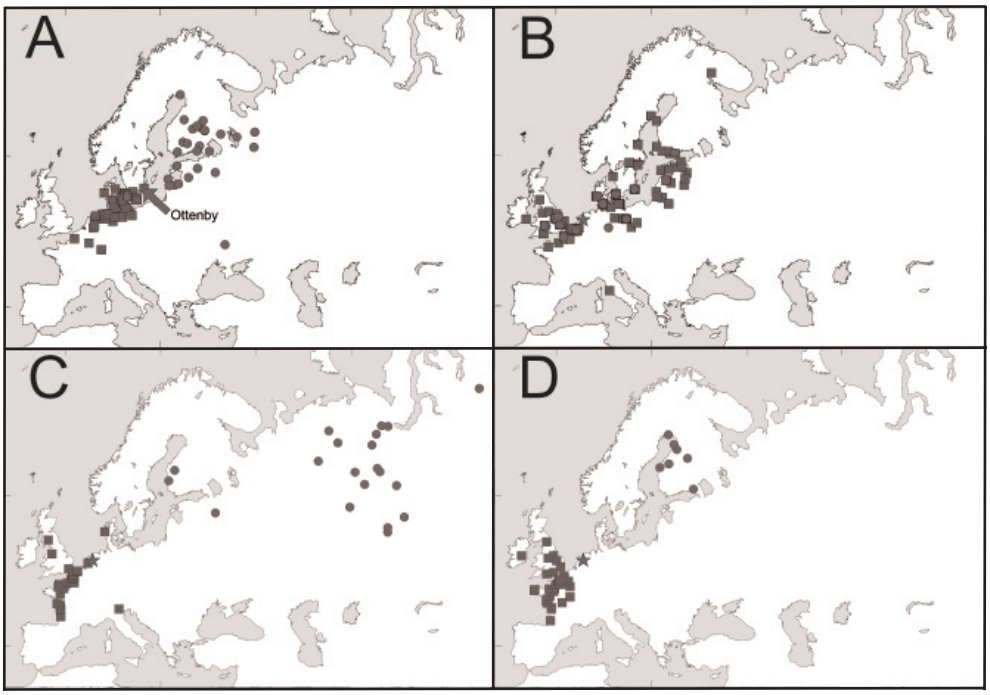


Figure 1. Summer and winter distribution of different duck species ringed in The Netherlands and Sweden. A: Recoveries of Mallards (*Anas platyrhynchos*) ringed at Ottenby Bird Observatory, Sweden (arrow), and found in 2002 and 2003 in the period May-August (n=29, circles) and November-February (n=54, squares). B: Ringing sites of Mallards (*Anas platyrhynchos*) found in The Netherlands (asterisks) from 1976-2005 in the period May-August (n=61, circles) and November-February (n=311, squares). C: Recoveries of Wigeons (*Anas Penelope*) ringed in The Netherlands (asterisks) and found from 1998-2005 in the period May-August (n=20, circles) and November-February (n=38, squares). D: Recoveries of Teals (*Anas crecca*) ringed in The Netherlands (asterisks) and found from 1998-2005 in the period May-August (n=7, circles) and November-February (n=36, squares)[72]. The squares represent winter recoveries (November – February) and circles represent summer recoveries (May – August).

Influenza A viruses belonging to subtypes H1-H13 were obtained from Mallards, H1, H4, H6 and H9 were obtained from Eurasian Wigeons, H1, H3, H6 and H8 were obtained from Common Teals, H9 from Gadwalls, H2 from Northern Pintails and H11 from Northern Shovelers. The overall HA subtype distribution in mallards was different from that in all other ducks (Pearson X2-test, $P<0.001$). Note, however, that there were only relatively few virus isolates obtained from other duck species (N=26). The differences in subtype distribution of influenza A viruses obtained from Mallards compared with influenza A viruses obtained from other ducks and other species is shown in Figure 2. Whether the subtype distribution differs between the different duck species remains an important question to be addressed in these and other ongoing surveillance studies.

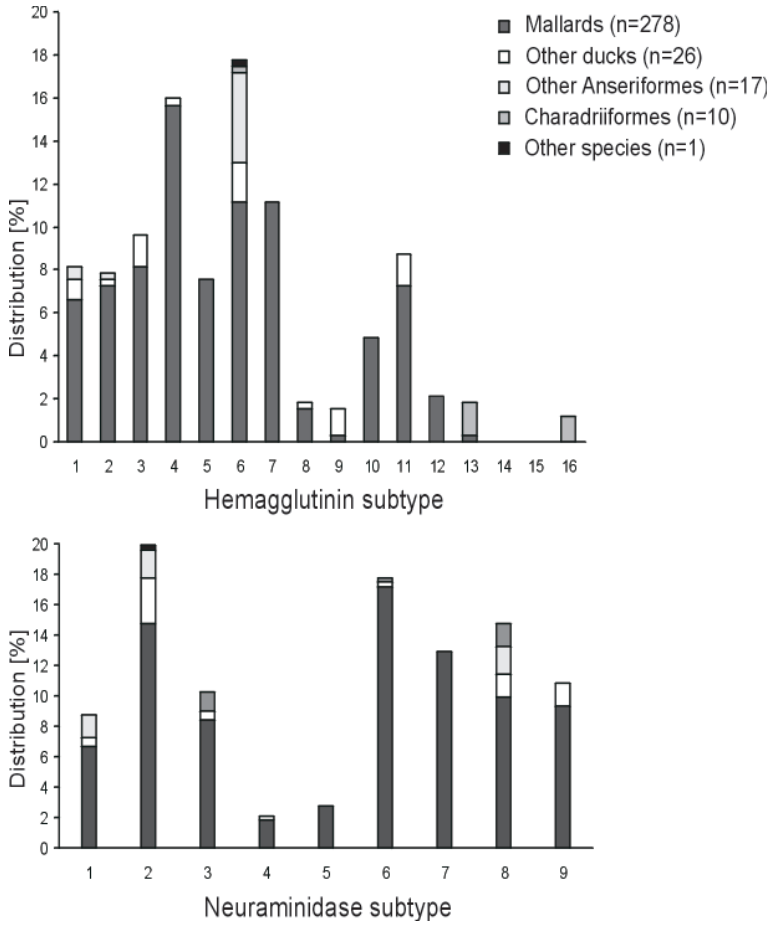


Figure 2. Distribution of HA and NA subtypes in influenza A virus isolates obtained from wild birds. Data from all 332 virus isolates were included with the distribution of HA subtypes shown in the top panel and the NA subtypes in the bottom panel.

We have made serious efforts to collect samples within the order of *Anseriformes* other than duck species. Samples were obtained from 8 goose species and 3 different swan species (Table 2). Influenza A viruses were detected in samples obtained from 82 of 3821 (2.1 %) White-fronted Geese (*Anser albifrons*), 8 of 1139 (0.7 %) Barnacle Geese (*Branta leucopsis*), 11 of 455 (2.4 %) Greylag Geese (*Anser anser*), 4 of 413 (1.0 % *)* Brent Geese (*Branta bernicla*), 2 of 315 (0.6 %) Bean Geese (*Anser fabalis*), 6 of 285 (2.1 %) Pink-footed Geese (*Anser brachyrhynchus*), 3 of 153 (2.0 %) Bewick's Swans (*Cygnus colombianus*) and 1 of 47 (2.1 %) Mute Swans (*Cygnus olor*)(Table 2). We did not detect influenza A virus in 376 Canada geese (*Branta canadensis*), 100 Egyptian geese (*Alopochen aegyptiacus*) and 26 Whooper swans (*Cygnus cygnus*). HA subtypes detected in the virus isolates from the goose and swan species were H1 (9.5 %), H2 (4.8 %), H6 (81 %) and H9 (4.8 %). Thus, the vast majority of influenza A virus isolates obtained from goose and swan species were of the H6 subtype; H6N1 (18 %), H6N2 (35 %) and H6N8 (47 %).

Table 2. Prevalence of influenza A virus of wild birds sampled in this study. This table includes all species in which we ever detected influenza A virus by RT-PCR.

Order	Family	Species	Sampled	Positive prevalence (n)	n (%)
Anseriformes	Ducks	9 species	13,731	817	6.9
		Mallard (*Anas platyrhynchos*)	8,938	649	7.3
		Eurasian Wigeon (*Anas penelope*)	2,538	76	3
		Common Teal (*Anas crecca*)	940	60	6.4
		Northern Pintail (*Anas acuta*)	448	13	2.9
		Gadwall (*Anas strepera*)	298	8	2.7
		Northern Shoveler (*Anas clypeata*)	135	5	3.7
		Common Shelduck (*Tadorna tadorna*)	335	2	0.6
		Tufted Duck (*Aythya fuligula*)	62	2	3.2
		Common Eider (*Somateria mollissima*)	37	2	5.4
	Geese	6 species	6,428	113	1.8
		White-fronted Goose (*Anser albifrons*)	3,821	82	2.1
		Barnacle Goose (*Branta leucopsis*)	1,139	8	0.7
		Greylag Goose (*Anser anser*)	455	11	2.4
		Brent Goose (*Branta bernicla*)	413	4	1
		Bean Goose (*Anser fabalis*)	315	2	0.6
		Pink-footed Goose (*Anser brachyrhynchus*)	285	6	2.1
	Swans	2 species	200	4	2
		Bewick's Swan (*Cygnus colombianus bewickii*)	153	3	2
		Mute Swan (*Cygnus olor*)	47	1	2.1
Charadriiformes	Gulls	3 species	2,602	22	0.8
		Black-headed Gull (*Larus ridibundus*)	1,583	14	0.9
		Common Gull (*Larus canus*)	226	2	0.9
		Herring Gull (*Larus argentatus*)	753	5	0.7
		Greater Black-backed Gull (*Larus marinus*)	40	2	5
	Waders	2 species	234	2	0.9
		Red Knot (*Calidris canutus*)	229	1	0.4
		Red-necked Stint (*Calidris ruficollis*)	5	1	20
	Auks	1 species	817	3	0.4
		Guillemot (*Uria aalge*)	817	3	0.4
Gruiformes	Rails	1 species	237	1	0.4

Prevalence of influenza A viruses in Charadriiformes

The Charadriiformes are represented in our surveillance studies by the Laridae (gull and tern species), the Haemanoptopodiae (Oystercatchers), the Charadriidae, and Scolopacidae (wader species) and the Alcidae (auks). Within the Laridae family a total of 4099 samples were obtained from 9 gull species and 2 tern species. These samples originated from many locations, predominantly in Northern Europe (Table 1). Sampling sites were concentrated in 5 countries (The Netherlands, Sweden, Finland, Latvia and Estonia). Influenza A viruses were detected in 14 of 1583 (0.9 %) Black-headed Gulls (*Larus ridibundus*), 2 of 226 (0.9 %) Common Gulls (*Larus canus*), 5 of 753 (0.7 %) Herring Gulls (*Larus argentatus*) and 2 of 40 (5 %) Greater Black-backed Gulls (*Larus marinus*)(Table 2). Influenza A virus was not detected in 1402 Lesser Black-backed Gulls (*Larus fuscus*), 58 Kittiwakes (*Rissa tridactyla*), 14 other birds of 3 *Larus* species, and 22 Terns (*Sterna paradisaea* and *Sterna hirundo*). The influenza A virus prevalence within the gull species varied greatly with respect to colonies sampled, sample timing and geography. Virus prevalence of 60

% was detected in small flocks of juvenile Black-headed Gulls less than 1 year old during fall migration caught in Sweden, Ottenby[6], while the overall prevalence in gulls is only 0.8% (Table 2). Moreover, influenza A virus was undetectable in many different colonies during breeding season over multiple years in the Netherlands and Sweden. Positive samples from gulls were predominantly obtained in June, July, and August. Influenza A virus subtypes isolated from the different gull species were H6N8 (10 %), H13N6 (10 %), H13N8 (40 %) and the recently characterized novel subtype H16N3 (40 %).

Within our surveillance studies we have sampled 47 wader species and obtained 3159 samples. The majority of the samples (60 %) were taken during fall migration on a variety of different sampling sites in Europe, the rest of the samples were taken either during spring migration (35 %) or at the breeding grounds (5%). We obtained one positive sample from a Red Knot (*Calidris canutus*) out of 229 sampled birds caught at Delaware Bay, USA in early May 2005. Moreover we obtained one positive sample from a Red-necked Stint (*Calidris ruficollis*) out of 5 red-necked stints, sampled in South Korea (Table 2). All other waders were negative for influenza A virus.

Within the Alcidae family we obtained 907 samples from 4 bird species. Three influenza A virus positive samples were obtained from 817 sampled Guillemots (*Uria aalge*) which were all H6N2 viruses[30]. Influenza A virus was not detected in samples of 90 other birds belonging to three Alcidae species.

Prevalence of influenza A viruses in other bird species

Influenza A viruses were rarely detected in other species. An influenza A virus was detected in 1 out of 237 Common Coots (*Fulica atra*) sampled. More than 10,000 samples were collected from wild birds in 14 orders other than the previously described Anseriformes, Charadriiformes, and Gruiformes. No influenza A viruses were detected in any of these samples. The samples were obtained from a large variety of sampling sites (Europe, Africa, Antarctica and the Arctic) and throughout the year.

Temporal and longitudinal variation in prevalence of influenza A viruses in Mallards

For the most abundantly studied species in our studies, Mallards, we compared the prevalence of influenza A virus from 1999–2005 in The Netherlands. The sample size was approximately evenly distributed from 1999 until 2004 (484, 588, 459, 512, 450 and 384 samples respectively), but in 2005 the sample size increased to 1520 samples as the result of increased surveillance efforts in response to the emerging HPAI H5N1 threat (total number of samples 1999 – 2005 was 4397). In 2005 the number of sampling sites for mallards in The Netherlands increased from 2 to 8. Throughout the years the peak prevalence varied from 0.93 % in September 2002 to 20.76 % in October 2001. The peak prevalence for most years was in the months September and October, with the exception of 2000 (January) (Fig. 3). Similar fluctuations in peak prevalence between years were observed in Eurasian Wigeons (0.83 % in December 2002 and 20 % in September 2005) and Common Teals (4 % in November 2000 and 30 % in November 2005) (data not shown).

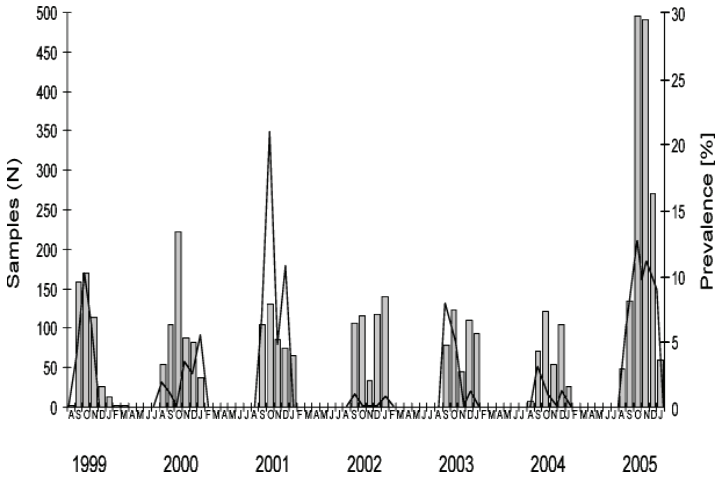


Figure 3. Annual influenza A virus prevalence in mallards during fall migration in The Netherlands from 1999 to 2005. Bars indicate the number of samples collected per month (left y-axis), and the line indicates the percentage of samples positive for influenza A virus by RT-PCR (right y-axis).

Despite the variation in sample size and peak prevalence between years, we calculated a trend line for influenza A virus in Mallards in The Netherlands and compared this with a similar trend line for influenza A virus in Mallards in Sweden 2002–2005 (Wallensten *et al*, manuscript in preparation). The winter and summer distribution of these mallard populations is shown in Figure 1. The overall influenza A virus prevalence in Mallards in Sweden was approximately 3 fold higher as compared to the prevalence in The Netherlands (Figure 4). For both countries, influenza A virus prevalence is already relatively high upon arrival of the birds on the sampling sites in August, only to drop to lower prevalence after November.

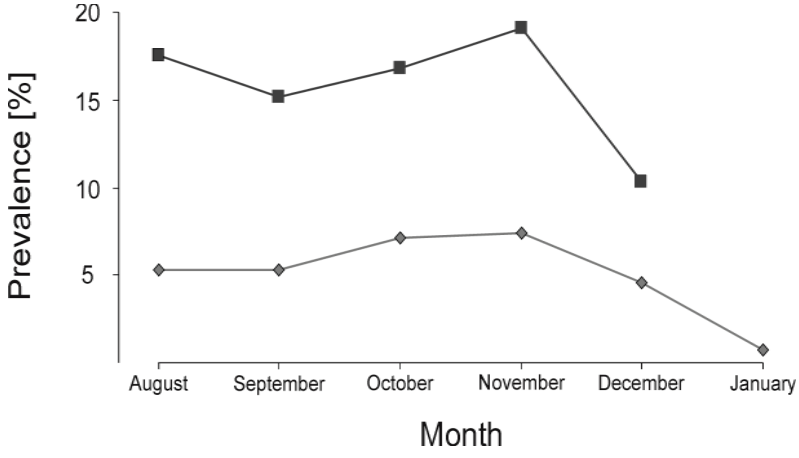


Figure 4. Influenza A virus prevalence in Mallards caught in Sweden and The Netherlands during fall migration. The filled squares (■) represent the proportion (%) of influenza A virus positive mallards caught and sampled in Sweden between 2002 and 2005 at Ottenby Bird Observatory and the filled diamonds (♦) represent mallards caught at various locations in The Netherlands from 1998 until 2005.

Population demography and prevalence of influenza A viruses in dabbling ducks

Information about the age structure and gender of the sampled ducks was obtained via the involved ornithologists. The prevalence of influenza A viruses with respect to age and sex was determined for a subset of the duck samples (Mallards and Eurasian Wigeons). The prevalence was different between juvenile (1st year), and adults (consecutive years). The influenza A virus prevalence was 6.8 % for juvenile ducks (n=2038) and 2.8% for adult ducks (n=895). Juvenile birds thus had a generally larger chance to be influenza A virus positive than adult ducks (RR: 2.24, 95% CI: 1.61 to 3.71). No apparent differences in prevalence were observed between male (n = 4737) and female (n = 3114) ducks. (RR: 1.13, 95% CI: 0.944 to 1.35). For other bird species, either the sample size or information on age and gender was too limited to perform similar analyses.

Table 3. Proportion of influenza A virus hemagglutinin and neuraminidase subtypes among 332 virus isolates. Totals are shown in boldface, the virus subtypes with high prevalence (> 9% of isolates) are shown in boldface and underlined.

	N1	N2	N3	N4	N5	N6	N7	N8	N9	**Total**
H1	6.0	1.2				0.3	0.3	0.3		**8.1**
H2	0.6	0.3	4.2		0.6		0.6		1.5	**7.8**
H3		0.6	0.3		0.3	2.1		6.3		**9.6**
H4		1.5	0.6			**13.6**			0.3	**16.0**
H5		3.0	1.8			0.3			2.4	**7.5**
H6	1.8	**9.9**	0.3		0.3	0.3		5.1		**17.8**
H7			0.3				**10.5**		0.3	**11.1**
H8				1.8						**1.8**
H9		1.5								**1.5**
H10		0.9		0.3	0.3	0.6	1.2	0.6	0.9	**4.8**
H11	0.3	0.9	1.5			0.3	0.3	0.6	4.8	**8.7**
H12					1.2			0.3	0.6	**2.1**
H13						0.3		1.5		**1.8**
H16			1.2							**1.2**
Total	**8.7**	**19.9**	**10.2**	**2.1**	**2.7**	**17.8**	**13.0**	**14.8**	**10.8**	

Prevalence of HA subtypes and HA/NA subtype combinations in wild birds

We determined the distribution of the HA and NA subtypes for the 332 obtained influenza A virus isolates (Table 3). The H4 (16 %) and H6 (17,8 %) were the most abundantly detected HA subtypes. H1 (8,1 %), H2 (7,8 %), H3 (9,6 %), H5 (7,5 %), H7 (11,1 %), H10 (4,8 %) and H11 (8,7 %) were intermediate detected subtypes. The H8 (1,8 %), H9 (1,5 %), H12 (2,1 %), H13 (1,8 %) and H16 (1,2 %) were the least frequently detected HA subtypes of influenza A viruses. H14 and H15 were never detected. Viruses of the H13 and H16 subtypes were primarily obtained from Charadriiformes (Figure 2). Viruses of subtype H6 were obtained relatively frequently from wild birds other than Mallards. It was difficult to compare the HA subtype distribution between different avian hosts, because the number of virus isolates obtained from wild birds other than Mallards was limited. However, the overall subtype distribution in Mallards was found to be different from that in all other ducks ($P<0.001$). Note, that there are only few positive other duck species

(N=26). All H5 and H7 isolates were low pathogenic avian influenza (LPAI) viruses as determined by sequencing of the HA gene[73].
The most frequently detected NA subtype was N2 (19.9 %) followed by N6 (17.8 %), N8 (14.8 %), N7 (13 %), N9 (10.8 %), N3 (10.2 %), N1 (8.7 %), N5 (2.7 %) and N4 (2.1 %)(Table 3). As for HA, there were no clear differences in distribution of NA subtypes between different hosts. Subtypes N5 and N7 were only found in viruses isolated from Mallards. From Charadriiformes we only obtained viruses of the N3, N6 and N8 subtypes and from geese and swans we only obtained viruses of the N1, N2 and N8 subtypes (Fig. 2).
In total 55 different HA/NA subtype combinations were detected within our wild bird surveillance (Table 3). The most frequently detected subtype combination was H4N6, which comprised 14 % of all isolated influenza A viruses, closely followed by H7N7 (11 %) and H6N2 (10.2 %). Viruses containing H8 matched only with N4 and viruses containing H16 only with N3 (Table 3).

Discussion

The recent improvements on rapid and sensitive molecular diagnostic tests have facilitated the high-throughput screening of wild birds for the presence of influenza A virus. Over an 8-year period (1998–2005) we sampled more than 36,000 wild birds belonging to 18 different orders and 321 different species. Despite the difference in screening methodology used in this study as compared to most previous studies, and the wide range of wild bird species tested here, only few “new” avian influenza A virus host species were identified. These were: Barnacle Goose, Bean Goose, Brent Goose, Pink-footed Goose, Bewick’s Swan, Common Gull, Guillemot and Red-necked Stint. It is reassuring that the use of classical methods for virus detection in numerous surveillance studies has not resulted in an apparent biased detection towards viruses that can be isolated easily in embryonated hens’ eggs. However, in our hands virus detection by RT-PCR was more sensitive than virus detection by isolation in embryonated hens’ eggs, since we could isolate viruses from only one third of the RT-PCR positive samples. Even when transport and storage conditions and temperature were optimal and time between sample collection and processing was minimal, not all RT-PCR positive samples yielded virus isolates.
Our large-scale surveillance of wild birds for influenza A virus confirmed the high prevalence in fall in various duck species belonging to the guild of dabbling ducks (*Anas* species) as observed in previous studies conducted in the Northern Hemisphere[1]. As in most surveillance studies, Mallards were the most extensively studied species within our study. The surveillance of Mallards at two different geographical locations (The Netherlands and Sweden) allowed the temporal and geographical comparison of virus prevalence data. Influenza A virus prevalence varied between the two locations at the same time point, where the prevalence in Mallards during fall migration in Sweden was ~3-fold higher as compared to the prevalence in Mallards in The Netherlands, indicating that not the absolute time point but timing relative to migration was a determinant of influenza A virus prevalence. High virus prevalence early in fall migration likely gradually declines as the migration proceeds, thus forming a North-South gradient of influenza A virus prevalence even within a single species.

This would explain geographical differences in influenza A virus prevalence between Northern and more Southern latitudes observed in different surveillance studies[16,22]. Influenza A viruses were already present in migratory Mallards in the Netherlands in August and prevalence peaked in September and October, with fluctuations between different years. Influenza A virus prevalence was generally higher in juvenile ducks as compared to adults. The estimated yearly turnover rate of Mallards in Northern Europe is roughly 1/3; 56% of the juvenile Mallards die during their first year and the mortality in adult birds is ~40 %[266]. Thus, one third of the mallard population consists of juvenile birds, which are immunologically naïve and thus probably more susceptible to influenza A virus.

The influenza A virus prevalence in Mallards was comparable to the prevalence in other dabbling ducks such as Eurasian Wigeons, Common Tealls, Gadwalls and Northern Shovelers, but smaller numbers of samples were obtained from the latter species. Influenza A viruses were also detected in ducks belonging to other guilds than the dabbling ducks (Common Shelduck, Tufted Duck and Common Eider) but the prevalence was lower as compared to the dabbling ducks. In total, we analyzed 13771 samples from 17 duck species, and detected influenza A virus in 811 of 13297 dabbling ducks and 6 of 440 other ducks (Pearson X2, p= <0.001). This study also included other species in the order of the Anseriformes, such as a variety of goose and swan species (7130 samples from 11 species). The overall influenza A virus prevalence within goose and swan species was also lower as compared to the dabbling ducks, ranging from 0.7 % to 2.4 %, depending on species. Several factors could contribute to the high virus prevalence in dabbling ducks as compared to other species. The dabbling behavior itself is likely to be an important factor; when ducks are shedding virus in surface waters via feces they may efficiently transmit virus to other ducks that feed on the same waters. Influenza A virus can stay infectious for prolonged periods in surface water dependent on temperature, salinity and pH[12]. The prolonged presence of influenza A viruses in surface water may enable the spread of viruses in different host sub-populations that otherwise would be separated in time and space. Whereas dabbling ducks feed on fresh surface water, diving ducks forage deeper under the surface and more often in marine habitats, and most goose and swan species graze in pastures and agricultural fields. Such differences in feeding behavior could potentially lead to less efficient transmission of influenza A viruses and thus account for the differences in influenza A virus prevalence in these species. Population size could be a second important factor enabling the annual co-circulation of multiple virus subtypes within the same (meta-) population of birds. The importance of population size, age structure and herd-immunity for the epidemiology of infectious diseases has been described for numerous pathogens, for which measles in humans is exemplary[17]. The dabbling duck populations are estimated at between 5,000,000 and 10,000,000 in Northern Europe alone[21]. Mallards are the most abundant species (5,000,000) followed by Eurasian Wigeon (1,250,000) and Common Teal (400,000)[21]. Interestingly, we observed the highest influenza A virus prevalence in two of these species; Mallards and Common Tealls. The population estimates for the different goose species in Northern Europe are significantly lower as compared to the dabbling ducks; White-fronted Goose (600,000), Brent Goose (300,000), Greylag Goose (200,000), Barnacle Goose (176,000), Bean Goose (80,000) and Pink-footed Goose (34,000)[21]. The smaller population sizes could limit

the potential of perpetuation of influenza A virus in these species in general, and in particular the continuous co-circulation of multiple influenza A virus subtypes. When we plotted the influenza A virus prevalence in duck, goose and gull species (species with >200 samples tested) as a function of the population size of these species (Figure 5), the population size did not appear to be the main correlate of virus prevalence (R^2 = 0.0001). The relative clustering of the data points from the duck, goose and gull species (Figure 5), suggest that other factors (taxonomy, behavior, etc.) could determine influenza A virus prevalence.

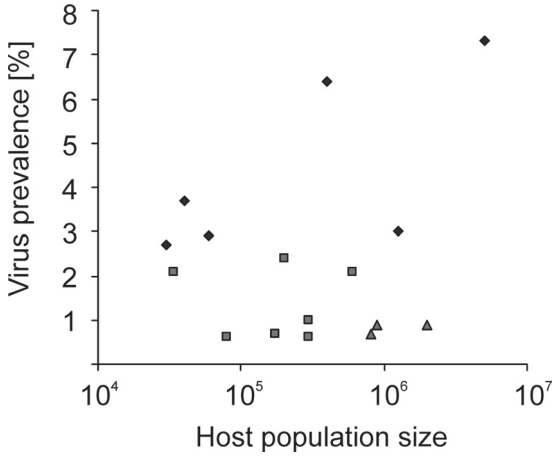


Figure 5. Relation between influenza A virus prevalence in avian hosts and their population sizes. Species were categorized in dabbling ducks (diamonds; Mallard, Eurasian Wigeon, Common Teal, Northern Pintail, Gadwall and Northern Shoveler), geese (squares; White-fronted Goose, Barnacle Goose, Greylag Goose, Brent Goose, Bean Goose, and Pink-footed Goose) and gulls (triangles; Black-headed Gull, Common Gull, and Herring Gull) and virus prevalence in these species was plotted against their respective population sizes[21]. There was no correlation between influenza A virus prevalence and population size, but a clustering of data points according to the species categories.

Influenza A viruses were detected in a variety of gull species; Black-headed Gulls, Common Gulls, Greater Black-backed Gulls and Herring Gulls. In total, 4099 samples collected from 11 different gull species were analyzed. The peak influenza A virus prevalence in gulls was in June, July, and August, somewhat earlier as compared to the peak in ducks, geese and swans. However, the prevalence within the gulls varied greatly with respect to the populations sampled, timing and geography. Virus prevalence of 60 % was detected in small flocks of juvenile Black-headed Gulls less than 1 year old during fall migration caught in Sweden, Ottenby[6], while the overall prevalence in Black-headed Gulls was only 0.9%. It is unclear which factors contribute to the influenza A virus prevalence patterns. The population size of 2,000,000 Black-headed gulls in Northern Europe seems to be sufficiently large for the continuous circulation of influenza A viruses. Behavioral factors influencing influenza A virus ecology in gulls could include colony breeding, gregariousness during migration and wintering, feeding patterns, and the mixing of different populations of birds. Because most samples collected at the breeding grounds of Black-headed Gulls were negative for influenza A virus, and most positive samples were obtained in the late summer

and early fall from this species, it appears that influenza A viruses primarily circulate in gulls shortly after they have left their breeding grounds. More data is needed for a better understanding of the epidemiology of influenza A virus in gulls.

Surveillance studies performed along the East Coast of North America suggested a distinct role for wader species in the perpetuation and maintenance of certain influenza A virus subtypes[26]. In these studies, the seasonal prevalence of influenza viruses in waders was reversed as compared to ducks, with highest virus prevalence (~14%) during spring migration. In addition, certain HA subtypes (H1, H2, H5, H7, H9 - H12) were most frequently obtained from waders, and a larger variety of HA/NA combinations were obtained from waders as compared to ducks. This has led to the hypothesis that different families of wetland birds are involved in perpetuation of influenza viruses and that waders may carry the virus north to the duck breeding grounds in spring. Within our surveillance study, no such pattern was observed in Northern Europe. We sampled 2996 birds belonging to 39 wader species, but not a single influenza A virus was detected in European waders. In contrast, influenza A virus was detected in 1 shorebird sample obtained from Delaware bay (USA) and 1 from South Korea. Although the majority of our wader samples were collected during fall migration, a reasonable sample size was collected during spring migration. Thus, we have not obtained any evidence that waders play a role in the perpetuation of influenza A virus in Europe. The recently intensified year-round surveillance in a wide variety of wader species, which also includes serological data collection, may allow a definitive conclusion on the role of waders in the ecology of influenza A virus in Northern Europe.

Although historically influenza A viruses have been obtained from more than 105 species of 26 different families[61], we did not detect significant influenza virus prevalence in species other than those belonging to the orders Anseriformes and Charadriiformes. We therefore suggest that although multiple bird species can be infected with avian influenza virus, they do not contribute significantly to the overall virus ecology; infections of these hosts, although potentially with high peak prevalence, may be only transient.

Influenza A virus subtypes H1 through H12 were isolated frequently from Mallards, and several of these virus subtypes were also detected in Eurasian Wigeons, Common Teals, Gadwalls and Northern Shovelers. The absence of subtypes H14 and H15 in our collection was probably due to the geographical separation of virus hosts (H14 in Southern Russia and H15 in Australia[74,75]). We detected influenza A virus of the H13 subtype in one duck, which was probably a spillover from the gull reservoir (see below). The HA subtype distribution was found to be different between Mallards and other duck species, although the comparison was made with relatively few virus isolates from ducks other than Mallards. Because all HA subtypes isolated from Anseriformes were also isolated from Mallards, it is likely that Mallards play a pivotal role in the perpetuation of influenza A virus subtypes H1 through H12 in Europe. Subtypes H5 and H7 were rarely detected in longitudinal studies in ducks in Canada whereas in this European study they represent 8,2 % and 12,1 % of the influenza A viruses obtained from ducks. The most common virus subtypes in Europe, H3, H4, H6, were also common subtypes in the Canadian studies.

All influenza A viruses isolated from gulls belonged to the H6, H13 and H16 subtypes. The predominant isolation of H13 and H16 influenza A viruses from different gull

species confirms the common notion that these viruses belong to the influenza A virus "gull lineage"; H13 and H16 viruses are genetically distinct from those of influenza viruses from other hosts, and seem to have adapted to replication in particular in gull hosts[2,24].

Interestingly, influenza A viruses of the H6 subtype seem to have a broader host range as compared to other influenza A virus subtypes in our study. Of the influenza viruses obtained from birds other than dabbling ducks and gulls, 79 % were H6 influenza viruses. H6 viruses were isolated from gulls and represented the predominant virus subtype in auks, swans and geese. Although viruses of the H6 subtype were also abundant in ducks, the relative abundance of this subtype in ducks does not explain the large variety of species from which these viruses were isolated; H4 and H7 viruses were also detected frequently in ducks but rarely in other birds. H6 influenza A viruses have been transmitted from wild birds to poultry on several occasions[76,77], providing further evidence for the ability of these influenza A viruses to be transmitted between different bird species.

With the existence of 16 HA subtypes and 9 NA subtypes, there are 144 potential HA/NA subtypes of influenza A virus. Although certain HA/NA combinations have not yet been detected in wild birds, with the addition of the current study most combinations have now been found. With the recently increased surveillance efforts, the detection probability of rare subtypes and subtype combinations, such as H8, H14 and H15 has increased. However, functional constraints that limit the number of subtype combinations could exist; to date, influenza A viruses of the H8 subtype have only been found in combination with N4, potentially indicating an incompatibility of H8 to pair with other NA subtypes.

HPAI H5N1 viruses have caused large-scale outbreaks in poultry in SE Asia since 1997, and have also been transmitted to a variety of mammalian species, including humans[35,52,68]. Until 2005, wild migratory birds probably did not play any role of significance in the epidemiology and spread of HPAI H5N1, although the virus was detected sporadically in wild birds. A large-scale outbreak in wild migratory birds occurred in April - June 2005 at Lake Qinghai in China[37,38,78], after which the HPAI H5N1 virus rapidly spread westwards across Asia, Europe, the Middle East and Africa. Since then, affected wild birds have been reported in several countries[41], but even in areas with significant outbreaks in poultry the virus prevalence in wild birds is low and their role in spreading the disease is unclear. It is likely that the influenza A surveillance studies in wild birds like those presented here could provide "early warning" signals for the introduction of HPAI H5N1 into new regions[79]. The current increased interest in influenza virus surveillance in wild and domestic birds further provides a unique opportunity to increase our understanding not only of HPAI epidemiology but also of the ecology of LPAI viruses in their natural hosts.

Acknowledgments
We thank the following ornithologists J. Berkhouwer, J. de Boer, T. Boudewijn, L. Bruinzeel, D. Buehler, F. Cottaar, C. Dove, B. Ebbinge, M. van Eerden, C. Eising, G. Escudero, T. Groothuis, B. Herrman, J. Hooijmeijer, H. Horn, M. Inia, D. Jonkers, J. Jukema, N. Kajiwara, G. Keijl, M. Klaassen, D. Kuiken, F. Leighton, M. Loonen, E. Maassen, F. Majoor, L. Mendes, G. Muskens, U. Ottosson, B. Pellegrom, T. Piersma, K. Polderdijk, J. Smit, M. Stervander, P. Tavernier, H. Vader, M. van Eerden, A. van Loon, T. de Vaal, C. Verbeek, S. Verhulst, B. Voslamber, and H. Zantinge and their respective organisations; Alterra Wageningen, Eendenkooi Stichting, NIOZ, Ottenby Bird Observatory, Sovon, University of Groningen, Wetlands International, vogelringstation Amsterdamse Waterleiding duinen, ringstation Castricum, ringstation Schiermonnikoog and Zuid-Hollands Landschap for their continuous support of the wild bird influenza A surveillance program. We thank T. Bestebroer, J. Guldemeester, S. Herfst and Ger van der Water for excellent technical assistance.

3

Mounting evidence for the presence of influenza A virus in the avifauna of the Antarctic region

Antarctic Science, 2006, 18: 353-356

Anders Wallensten*
Vincent Munster*
Albert Osterhaus
Jonas Waldenström
Jonas Bonnedahl
Tina Broman
Ron Fouchier
Björn Olsen

* These authors contributed equally to the results of this study

Penguin blood samples collected at Bird Island, sub-Antarctic South Georgia, and faecal samples taken from penguins at several localities along the Antarctic Peninsula were analyzed in order to investigate if influenza A virus is present in penguin populations in the South Antarctic region. Serology was performed on the blood samples while the faecal samples were screened by a RT-PCR method directed at the matrix protein gene for determining the presence of influenza A virus. All faecal samples were negative by PCR, but the blood samples gave serologic indications that influenza A virus is present among these penguins species, confirming previous studies, although the virus has still not been isolated from any bird in the Antarctic region.

Introduction

Influenza A virus has been isolated from at least 105 species of birds representing a span of 26 bird families[61]. The virus is most frequently found in bird species associated with aquatic environments, and is especially prevalent in dabbling ducks of the Anatidae family. However, the virus has also been detected or isolated in geese (Anseridae), shorebirds (Scolopacidae and Charadriidae) and gulls (Laridae)[61]. All the known hemagglutinin and neuraminidase subtypes of influenza A strains, including the recently characterised H16[6], have been isolated from wild birds, and nearly all of them from dabbling ducks. Generally, ducks, geese, shorebirds and gulls are infected with low pathogenic strains of influenza A virus that do not cause disease symptoms, and hence these hosts are considered as the main natural reservoir of influenza A viruses[83], while infections in other families of birds appear sporadic.
Influenza A virus has so far been isolated in birds on all continents with the exception of Antarctica. To date there is only serological data suggesting presence of influenza A viruses in the Antarctic avifauna. Three different studies have found elevated antibody titers against influenza A viruses in either penguins or skuas in Antarctica[84,85,86]. Here we present data from two expeditions to the Antarctic and sub-Antarctic region, that adds to the knowledge on the presence of the virus in the most remote of all continents.

Material and Methods

Serological analysis of penguin sera

Sera was collected from 76 blood samples obtained from Gentoo Penguins (*Pygoscelis papua*) that were sampled at the Bird Island in the sub-Antarctic South Georgia (54˚,00´S, 38˚,03´W) in 1996. Penguin blood samples were collected as whole blood, centrifuged for 10 minutes at 14 000 rpm and serum was separated. The sera were analyzed using the commercially available Influenza type A virus antibody ELISA kit (European Veterinary Laboratory, Woerden, The Netherlands) according to the manufacturers' instructions. For the detection of specific anti-NP antibodies, we used horseradish peroxidase-conjugated goat anti-wild bird immunoglobulin (Ig) G (Bethyl Laboratories, Inc., TX, USA) instead of the supplied anti-bird conjugate[87]. Prior to running the ELISA, the recognition of penguin sera by the anti-wild bird

conjugate was confirmed by using 96 well high binding EIA plates (Corning, NY, USA) coated with penguin sera. Subsequently, penguin sera samples were diluted 1:100 in PBS buffer with pH 7.4 (Gibco, Breda, The Netherlands) and were applied to the wells of a NP-coated ELISA plate and were let to incubate at 37ºC for 60 minutes. The plate was washed four times with PBS. Thereafter, 100 µl of anti-bird conjugate diluted 1:100 in conjugate buffer was applied to each well, followed by incubation at 37ºC for 60 minutes. After incubation and washing as above, plates were developed with 100 µl TMB substrate for 15 minutes. The reaction was stopped by applying 50 µl of stop solution (0.5 M sulfuric acid) and the optical density (OD) was read at 450 nm and 620 nm. Blank, positive and negative controls were included on each plate. To compute the positive/negative (P/N) value of each sample, we divided the mean OD of positive antigen-containing wells by the OD of negative antigen-containing wells. Samples with a P/N value ≥ 2 were considered positive.

Analysis of penguin faecal samples

Five penguin colonies along the Antarctic Peninsula (Table 1) were visited during a week in February 2002. Penguins of three different species, Gentoo Penguin (*Pygoscelis papua*), Adélie Penguin (*Pygoscelis adeliae* Hombron & Jacquinot), and Chinstrap Penguin (*Pygoscelis antartica* Forster), were caught in their breeding colonies using hand nets. In total 190 penguins were sampled (Table 1). Each trapped individual was sampled through the insertion of a sterile cotton wool swab into the bird's cloacae. The swabs were then placed in transport media (Hanks balanced salt solution containing 10% glycerol, 200 U/ml penicillin, 200 mg/ml streptomycin, 100 U/ml polymyxin B sulfate, and 250 mg/ml gentamycin, (MP biomedicals, Zoetermeer, The Netherlands) and stored in refrigerator temperatures during the remainder of the expedition (four days of travel after completion of fieldwork), and subsequently stored at–70ºC until analyses.

Virus detection was performed by RNA isolation, RT-PCR and dotblot detection as previously described[80]. In brief, RNA was isolated using a high pure RNA isolation kit (Roche Diagnostics, Almere, The Netherlands) and viral sequences were amplified in a one-step RT-PCR using primers M52C (5'- CTT CTA ACC GAG GTC GAA ACG –3') and M253R (5'- AGG GCA TTT TGG ACA AAG/T CGT CTA -3') targeting the matrix gene segment. RT-PCR products were transferred to dotblots and visualized using ECL detection reagents and exposure to hyperfilm (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) upon hybridization with the biotinylated oligonucleotide Bio-M93C (5'- CCG TCA GGC CCC CTC AAAGCC GA –3').

Results and discussion

Apart from being geographically separated, sample material also differed between studies, and consequentially two analysis methods were used. Of the 76 blood samples from Bird Island, 9 (11.8%) gave positive serology results for influenza A virus. Eight of these birds were adult individuals (out of 33 tested adults) and one was juvenile (out of 40 tested juveniles), while the remaining three penguins were not aged. These results contrast to the 190 faecal samples from the Antarctic Peninsula, that all were negative when analyzed by RT-PCR. The PCR methodology used is

routinely used in other studies, with a generally high detection rate[63,88],. Therefore, we see the lack of positive amplicons as evidence that none of the sampled penguins were infected and excreting influenza A virus at the time of sampling.
This pattern with serologic findings, but without isolation of virus is similar to the results from the few previous studies from the region. One of the earlier studies failed to isolate virus from tracheal and cloacal swabs obtained from 550 Adélie penguins in the Eastern Antarctic (66º17´S, 110º32´E), but found serological evidence of influenza A virus subtype H7 in 6 out of 285 blood samples[85]. Similarly, Austin and Webster (1993) did not isolate virus from Adélie Penguins or Skuas (*Catharacta spp.*) on Ross island (77º51´S, 166º´E) neither in 1978 nor 1986, but found evidence of subtype H10 in 26% (24 out of 91 birds) of serological samples from Adélie Penguins as well as elevated titers of serum antibodies in Antarctic Skuas (*Stercorarius skua maccormicki*)[84]. Finally, Baumeister *et al.* (2004) reported serological evidence of influenza A virus in Gentoo Penguins, Chinstrap Penguins, Skuas and Giant Petrels (*Macronectes giganteus*) at Potter Peninsula (62º15´S, 58º39´E) and in samples from Adélie Penguins and Skuas at Hope Bay (63º24´S, 56º59´E) on the Antarctic Peninsula[86]. They also found serological evidence in samples from Giant Petrels at Harmony Point (63º24´S, 56º59´E) on Nelson Island, South Shetland Islands. Attempts have also been made to detect influenza A virus in penguin guano, using sensitive RT-PCR, but without success[89]. Hence, our study like the few earlier studies finds serologic indications of influenza A virus in Antarctic birds, but yet there is no successful isolation of the virus.

Table 1. Number of faecal sampled penguins at the different localities.

	Robert Island	Paulet Island	Brown Bluff	Hannah Point	Necco Harbour	No. sampled birds
Longitude	59.270	55.767	56.917	60.600	62.533	
Latitude	62.280	63.583	63.533	62.250	64.850	
Breeding pairs in colony	no data	60,000	250	1000	250	
Gentoo Penguin (*Pygoscelis papua*)			41	41	49	131
Adélie Penguin (*Pygoscelis adeliae*)		30				30
Chinstrap Penguin (*Pygoscelis antarctica*)	29					29
Total	29	30	41	41	49	190

Negative virus isolation results could be interpreted in different ways. First of all, virus prevalence could peak at other time periods of the year than that of the sampling. Similarly, if the virus is confined to certain penguin colonies, or, if it only infects a low proportion of individuals in each colony, failure to detect virus could be due to chance events when choosing colonies or individuals to sample. Our study is fairly large, 190 samples from five localities and for instance, at Poulet Island we would have expected to find positive samples if the prevalence had been > 5-10%, given our sample size of 30 birds and a population larger than 3000 individuals[90]. The moderate prevalence of seroconverted penguins (2-26%) in earlier serological studies and in our study (11.8%), may together with the negative PCR results indicate that penguins are not

a primary host for influenza A virus but rather a species that is occasionally infected by means of spill over from other species. Given the crowded nature of a penguin colony, one would expect most individuals within a colony to contract the disease if a contagious virus was introduced into a large susceptible population, which would result in high seroprevalence. On the other hand, observed low seroprevalences may reflect a lack of yearly epizootics, where low seroprevalence will be found in in-between years when the cohort of juvenile birds has not been exposed.

There is no obvious reason why influenza A viruses should be absent from the Antarctic avifauna. Antarctica is not as remote as it may look at first glance; a large proportion of a variety of bird species migrate from northerly latitudes to breed there, including procellarids, cormorants and terns. The most extreme long-distant migrant, the Arctic Tern (*Sterna paradisea* Pontoppidan), migrates from its Arctic breeding grounds to spend part of its non-breeding season in the Antarctic waters during the summer. Interestingly, influenza A virus has been isolated from this species[91,92]. These and other birds could potentially introduce the virus into penguin populations, where transmission would be rapid in the dense colonies. There are also indications that the virus can persist for several months in cold water[93,94], and would therefore be well adapted for the temperatures prevailing during the summer. On the other hand climatic factors such as high UV-radiation and the lack of fresh-water[95] may have a negative impact on the existence of influenza A viruses in Antarctica as well as the absence of dabbling ducks as a primary host species for the virus.

To conclude, no isolations of influenza A virus from Antarctic birds have been made so far, even though more than 1000 birds have been tested at different locations, at different times and by different methods. However, four independent studies report serologic evidence of past infections, indicating that the virus either is endemic to the region in penguins or in an as far unknown host, or that the virus has been repeatedly introduced from other continents. There is clearly a need for additional sampling and screening of wildlife in the region, especially now when a highly pathogenic influenza A virus of the subtype H5N1 is spreading rapidly in wild bird populations in Eurasia and Africa causing morbidity and mortality among birds[61] and we therefore believe that publishing data, both positive and negative, is of utmost importance enabling future expeditions to make better judgements upon choice of timing, species to sample and methods to be used.

Acknowledgements

Counts of the number of breeding penguins and the coordinates for sample localities were kindly provided by the SCAR group of experts on birds.

4

Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from Black-headed Gulls

Journal of Virology, 2005, 79: 2814-2822

Ron Fouchier
Vincent Munster
Anders Wallensten
Theo Bestebroer
Sander Herfst
Derek Smith
Guus Rimmelzwaan
Björn Olsen
Albert Osterhaus

In wild aquatic birds and poultry around the world, influenza A viruses carrying 15 antigenic subtypes of hemagglutinin (HA) and 9 antigenic subtypes of neuraminidase (NA) have been described. Here we describe a previously unidentified antigenic subtype of HA (H16), detected in viruses circulating in Black-headed Gulls in Sweden. In agreement with established criteria for the definition of antigenic subtypes, hemagglutination inhibition assays and immunodiffusion assays failed to detect specific reactivity between H16 and the previously described subtypes H1 to H15. Genetically, H16 HA was found to be distantly related to H13 HA, a subtype also detected exclusively in shorebirds, and the amino acid composition of the putative receptor-binding site of H13 and H16 HAs was found to be distinct from that in HA subtypes circulating in ducks and geese. The H16 viruses contained NA genes that were similar to those of other Eurasian shorebirds, but genetically distinct from N3 genes detected in other birds and geographical locations. The European gull viruses were further distinguishable from other influenza A viruses based on their PB2, NP and NS genes. Gaining information on the full spectrum of avian influenza A viruses and creating reagents for their detection and identification will remain an important task for influenza surveillance, outbreak control, and animal and public health. We propose that sequence analyses of HA and NA genes of influenza A viruses be used for the rapid identification of existing and novel HA and NA subtypes.

Introduction

Influenza virus types A, B and C belong to the family of *Orthomyxoviridae* and have many biological properties in common[96]. A key difference between them is their host-range; whereas influenza viruses of types B and C are predominantly human pathogens that have sporadically been isolated from seals and pigs respectively[97,98], influenza A viruses have been isolated from many animal species including humans, pigs, horses, mink, marine mammals and a wide range of domestic and wild birds[1,99]. Wild birds, predominantly ducks, geese and shorebirds form the reservoir of influenza A viruses in nature. Avian influenza viruses preferentially infect cells lining the intestinal tract of birds and are excreted in high concentrations in their feces. While avian influenza viruses are generally nonpathogenic in wild birds, they sometimes cause significant morbidity and mortality upon transmission to other species, including domestic birds and mammals[1,99].

Influenza A viruses are classified on the basis of the antigenic properties of the hemagglutinin (HA) and neuraminidase (NA) glycoproteins expressed on the surface of virus particles. To date, influenza A viruses representing 15 HA and 9 NA subtypes have been detected in wild birds and poultry throughout the world[65,74,75,100]. These antigenic subtypes are distinguished by double immunodiffusion assays with hyperimmune animal sera because such tests revealed antigenic relationships amongst influenza A virus isolates which were not apparent with other methods[100,101]. Disadvantages of immunodiffusion assays for this purpose are that the development of monospecific reagents (antisera and antigens) can be very time-consuming, that the outcome is heavily dependent on the quality of the reagents used, and that the

assay provides qualitative rather than quantitative information.
The influenza A virus HA protein is encoded by viral gene segment 4 and is initially synthesized as a single polypeptide precursor (HA_0). The mature HA forms homotrimers and each monomer is generated upon cleavage of HA_0 into HA_1 and HA_2 subunits by trypsin-like or furin-like proteases. The HA of influenza A virus mediates early steps of the viral replication cycle, receptor binding and membrane fusion. The cellular receptors are sialic acids of cell surface glycoproteins and glycolipids (reviewed in reference[102]).
During surveillance studies of influenza A virus in wild birds in Northern Europe[63,103], we have isolated influenza A viruses from Black-headed Gulls (*Larus ridibundus*) in Sweden which could not be classified by using antisera raised against the 15 known HA subtypes. Genetic analyses of the HA genes and antigenic analyses with hemagglutinin inhibition (HI) assays provided strong support for the classification of a novel HA subtype. This was subsequently confirmed by double immunodiffusion assays with hyperimmune rabbit antisera. We thus propose that the HA of these Black-headed Gull viruses represent a new HA subtype, H16, which is genetically distantly related to HA of subtype H13. Moreover, we suggest that, with the rapidly increasing number of HA nucleotide sequences available from public databases, novel HA subtypes can be defined on the basis of their amino acid sequences.

Materials and methods

Virus detection

Black-headed Gulls (*Larus ridibundus*) were caught by using funnel traps at bird observatory Ottenby in Öland, Sweden, in August 1999. Additional Black-headed Gulls were caught by hand on their breeding grounds at various locations in Sweden and The Netherlands. Cloacal swabs were collected with cotton swabs and stored in transport media at -70ºC. Transport media consisted of Hanks balanced salt solution supplemented with 10 % glycerol, 0.5 % lactalbumin, 200 U of penicillin/ml, 200 μg streptomycin/ml, 100 U polymyxin B sulfate/ml, 250 μg gentamicin/ml and 50 U nystatin/ml (all from ICN, Zoetermeer, The Netherlands). RNA isolation, reverse transcription (RT)-PCR, and dotblot detection were performed as described elsewhere[80]. In brief, RNA was isolated by using a high pure RNA isolation kit (Roche Molecular Biochemicals) and viral sequences were amplified in a one-step RT-PCR using primers M52C (5'- CTT CTA ACC GAG GTC GAA ACG –3') and M253R (5'- AGG GCA TTT TGG ACA AAG/T CGT CTA -3') targeting the matrix gene. RT-PCR products were transferred to dot blots and visualized with enhanced chemiluminescence detection reagents and exposure to hyperfilm (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) upon hybridization with the biotinylated oligonucleotide Bio-M93C (5'- CCG TCA GGC CCC CTC AAA GCC GA –3').

Virus isolation and characterization

For RT-PCR-positive samples, 200 μl of the original specimens was inoculated into the allantoic cavities of 11-day-old embryonated chicken eggs and hemagglutination titers in allantoic fluids were determined with turkey erythrocytes by standard

procedures[104]. Virus isolates were characterized by hemagglutination[105] and neuraminidase[106] inhibition assays with subtype-specific hyperimmune rabbit antisera raised against HA and NA preparations of virus isolates representing all influenza A virus subtypes and by nucleotide sequence analysis (see below).

Nucleotide sequence analyses and phylogenetic trees

RT-PCR specific for the conserved noncoding regions of avian influenza viruses was described by others[81]. PCR products were purified using the Qiaquick gel extraction kit (Qiagen, Leusden, The Netherlands) and sequenced directly or upon cloning in pCR2.1 (Invitrogen, Groningen, The Netherlands). Sequencing was performed with the Dyenamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech, Roosendaal, the Netherlands) and a 373 genetic analyzer (Applied BioSystems, Nieuwerkerk aan den IJssel, the Netherlands). Primer sequences are available upon request. Nucleotide sequences are available from GenBank (accession no. AY684874 to AY684913). Nucleotide and amino acid sequences were aligned by using Clustal W running under Bioedit 5.0.9[107]. DNA maximum-likelihood trees were generated with Phylip 3.6[108] with 100 bootstraps and 3 jumbles. The consensus tree was used as the user tree in Dnaml to recalculate branch-lengths and trees were rerooted at the midpoint. To circumvent computational limitations, we generated a tree representing all NA sequences available from public databases by using the unweighted pair group method with arithmetic mean (UPGMA) clustering method of the Neighbour program of Phylip. Amino acid sequence alignments were bootstrapped 100 times and distance matrices were generated using Kimura's distance. The consensus of 100 UPGMA trees was calculated, and this consensus tree was used for recalculation of branch lengths with the Fitch program of Phylip[108]. For Dnaml trees, we used sequences representing Eurasian avian influenza A viruses when available. The accession numbers that were used are as follows: for NA, AF523393, AJ416628, AY180837, AY207530, AY207552, AY340077, AY684892-AY684895, L06574, M11445, M24740), HA (AF091313, AJ427297, AY083840, AY338460, D90304, D90307, L43916, M21647, M25283, M26089-M26091, and M35997; for internal genes, see reference[55]. The full list of accession numbers for all full-length NA (N = 649), HA_0 (N = 541), HA_1 (N = 1959) and HA_2 (N = 608) sequences available from public databases in June 2004, used for Fig. 2 and Fig. 5A, is available on request.

DNA immunization of rabbits

The HA gene segments of A/Black-headed Gull/Sweden/5/99 and A/Gull/Maryland/704/77 were cloned in expression plasmid pcDNA3. Both constructs were verified by sequencing. Rabbits were immunized with 100 μg plasmid DNA mixed with 100 μl Lipofectin as previously described for chickens[109]. After 4 immunizations at weeks 0, 3, 6 and 9, the HI antibody titers were detectable but low (~12). After two more immunizations at weeks 12 and 19 with 500 μg of plasmid DNA, the HI antibody titers were ~96, and the animals were euthanized at week 20 for serum collection.

Reference sera

Hyperimmunized rabbit antisera were generated against the following influenza

reference strains: A/Puerto Rico/8/34 (H1N1), A/Fort Monmouth/1/47 (H1N1), A/Swine/Shope/1/56 (H1N1), A/Duck/Alberta/35/76 (H1N1), A/Singapore/1/57 (H2N2), A/Hong Kong/1/68 (H3N2), A/Equine/Miami/1/63 (H3N8), A/Duck/Ukraine/1/63 (H3N8), A/Duck/Czechoslovakia/1/56 (H4N6), A/Tern/South Africa/61 (H5N3), A/Duck/Hong Kong/205/77 (H5N3), A/Turkey/Massachusetts/65 (H6N2), A/Shearwater/Australia/1/72 (H6N5), A/Equine/Prague/1/54 (H7N7), A/Seal/Massachusetts/1/80 (H7N7), A/Turkey/Ontario/6118/68 (H8N4), A/Turkey/Wisconsin/1/66 (H9N2), A/Chicken/Germany/N/49 (H10N7), A/Duck/England/1/56 (H11N6), A/Duck/Memphis/546/74 (H11N9), A/Duck/Alberta/60/76 (H12N5), A/Gull/Maryland/704/77 (H13N6), A/Mallard/Gurjev/263/82 (H14N5), A/Duck/Australia/341/83 (H15N8), A/shearwater/West Australia/2576/79 (H15N9). Reference strains were propagated in embryonated chicken eggs, and afterwards, the allantoic fluid was cleared by centrifugation for 10 min at 1000 x g and filtration through a 0.45-μm-pore-size filter. The virus was pelleted by centrifugation for 1.5 h at 150.000 x g at 4ºC and resuspended in phosphate-buffered saline (PBS). Virus was treated overnight at 4ºC with 1% Triton-X-100, loaded on a layer of 25% sucrose in PBS and centrifuged for 1.5 hours at 250.000 x g at 4ºC. The top layer containing HA and NA proteins was inspected for purity and quantity on 12.5 % Sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie brilliant blue. Animals were immunized with approximately 500 μg HA/NA proteins in 2.4 ml of a Specol water-in-oil emulsion[110] at 1-month intervals using 0.4 ml antigen intramuscularly and 2 ml antigen subcutaneously. After 3 immunizations, HI antibody titers against the homologous viruses were determined, and if necessary the rabbits were immunized once more.

Double immunodiffusion assays

Double immunodiffusion assays were performed essentially as described previously[111]. Approximately 5 mm thick 1 % agarose-gels, containing 2 % polyethylene glycol in phosphate-buffered saline, were used in wells which were punched with a Pasteur pipet approximately 1 cm apart. Antisera and antigens were loaded in these wells, and afterwards, the gels were placed in a humidified chamber for 48 hours at room temperature. The gel was washed twice with PBS for 24 h and once with water for 4 h. Gels were covered with filter paper and dried overnight. Dried gels were stained for 10 min in 0.5 % (wt/vol) Coomassie brilliant blue, 40 % (vol/vol) ethanol, 10 % (vol/vol) glacial acetic acid and destained in 15 % (vol/vol) ethanol, 5 % (vol/vol) glacial acetic acid. Sera were concentrated 8 times by precipitation with ammonium sulfate and 10 times by filtration through Mircocon centrifugal filter devices with a 100.000-Da molecular mass cutoff (Millipore Corporation, Bedford, MA, USA). We used 20 μl concentrated polyclonal antiserum obtained from rabbits hyperimmunized by DNA vaccination (see above). As antigens, we used 20 μl of whole-virus lysates, prepared by pelleting virus from the allantoic fluid of infected chicken eggs and resuspension it in 2.5 % of the original volume of PBS with 1 % Triton-X100.

Results

Influenza A virus in Black-headed Gulls in Europe

Within the framework of our ongoing influenza A virus surveillance studies in wild

birds[63,103], we obtained 10 samples from Black-headed Gulls in 1999 that were screened for influenza A virus by RT-PCR specific for the matrix gene. These samples were obtained from juvenile birds that were less than one year old and were sampled between August 10 and 14 at the Ottenby Bird Observatory in Öland, Sweden. Cloacal swabs obtained from six of these birds, 8-224, 8-251, 8-291, 8-292, 8-298 and 8-510 were positive for influenza A virus by RT-PCR and agarose gel electrophoresis or dot blot hybridization (data not shown). We next attempted to isolate influenza A virus from the RT-PCR-positive samples in 11-day-old embryonated chicken eggs. From samples 8-224, 8-291, 8-292 and 8-298, influenza A virus isolates were obtained upon the first inoculation of eggs as determined by the presence of hemagglutinating activity in the allantoic fluid. We were unable to detect hemagglutinating activity in the allantoic fluid of eggs inoculated with samples 8-251 and 8-510, but after blind passage of the allantoic fluid in embryonated eggs, an isolate was obtained from sample 8-251. Further attempts to isolate virus from sample 8-510 were not successful. The virus isolates were named A/Black-headed Gull/Sweden/1/99 (8-251), A/Black-headed Gull/Sweden/2/99 (8-298), A/Black-headed Gull/Sweden/3/99 (8-224), A/Black-headed Gull/Sweden/4/99 (8-291) and A/Black-headed Gull/Sweden/5/99 (8-292).

Between June 17 and July 6 2000, we performed the same testing of 90 cloacal swab samples obtained from juvenile Black-headed Gulls in Pieterburen, The Netherlands. Of these, three were positive by RT-PCR but only one was positive upon repeated attempts to isolate a virus (sample 17-73, A/Black-headed Gull/Netherlands/1/00). Additional Black-headed Gull samples (5 from The Netherlands, December 1998 and 351 from Venan, Hoenborop, Kirokskar, Malmö, Kalmar, Umeå and Lund in Sweden in June 2000) were all negative by RT-PCR (data not shown).

Table 1. Antigenic characterization of Northern-European gull influenza A viruses by HI assays with antisera raised in rabbits and ferrets[a].

Virus	Subtype	Titer for virus (subtype) with sera from:				
		Hyperimmunized rabbits			Postinfection ferrets	
		A/Duck/Memphis/546/76	A/Gull/Maryland/704/77	A/BHG/Sweden/2/99	A/BHG/Sweden/2/99	A/BHG/Sweden/5/99
A/Duck/Memphis/546/76	H11	**3,480**	<10	<10	<10	<10
A/Gull/Maryland/704/77	H13	<10	**2,560**	<10	<10	<10
A/BHG/NL/1/00	H13	<10	640	NT	NT	NT
A/BHG/Sweden/1/99	H13	<10	2,560	<10	<10	<10
A/BHG/Sweden/2/99	H16	<10	<10	**2,560**	**320**	20
A/BHG/Sweden/3/99	H16	<10	<10	7,680	320	20
A/BHG/Sweden/4/99	H16	<10	<10	2,560	640	80
A/BHG/Sweden/5/99	H16	<10	<10	3,840	80	**480**

[a] Homologous HI titers are in boldface type. NT, not tested; BHG, Black-headed Gull.

HI assays with influenza A viruses isolated from Black-headed Gulls

The six influenza A virus isolates obtained from Black-headed Gulls in Sweden and The Netherlands were used in HI assays with hyperimmunized rabbit antisera representing all HA subtypes. Virus isolates A/Black-headed Gull/Netherlands/1/00 and A/Black-headed Gull/Sweden/1/99 reacted specifically with the rabbit antiserum

raised against A/Gull/Maryland/704/77 (H13N6, homologous HI antibody titer of 2,560), giving rise to HI antibody titers of 640 and 2,560 (Table 1), indicating that these viruses contained a HA of subtype H13. The other four virus isolates did not react specifically with the H13 antiserum (Table 1) or any of the antisera in the H1-H15 reference panel (data not shown).
We generated a new hyperimmunized rabbit antiserum raised against one of the unidentifiable virus isolates, A/Black-headed Gull/Sweden/2/99. To this end, virus was propagated in embryonated eggs and purified from the allantoic fluid. The virus was treated with Triton-X100 and the HA and NA proteins were separated from viral ribonucleoprotein complexes by centrifugation through 25 % sucrose. Rabbits were immunized three times with these partially purified HA and NA protein preparations. Using the hyperimmune rabbit antisera raised against A/Black-headed Gull/Sweden/2/99, we obtained a homologous HI titer of 2,560 and titers of 2,560 to 7,680 against A/Black-headed Gull/Sweden/3/99, A/Black-headed Gull/Sweden/4/99 and A/Black-headed Gull/Sweden/5/99. This antiserum did not react with the H13 viruses A/Black-headed Gull/Sweden/1/99 and A/Black-headed Gull/Netherlands/1/00, and only marginally with A/Gull/Maryland/704/77 (Table 1). Low, non-specific, HI antibody titers of 20-80 are occasionally observed between antisera and antigens representing distinct HA subtypes (data not shown).
We also raised postinfection antisera in ferrets against A/Black-headed Gull/Sweden/2/99 and A/Black-headed Gull/Sweden/5/99. Such antisera, collected two weeks after infection, are generally more specific than hyperimmune rabbit antisera. Neither of these ferret antisera reacted with the H13 isolates tested. When tested against the four unidentifiable Gull viruses, these antisera displayed some antigenic heterogeneity; the antiserum raised against A/Black-headed Gull/Sweden/2/99 gave rise to higher HI antibody titers against A/Black-headed Gull/Sweden/2/99 (titer of 320), A/Black-headed Gull/Sweden/3/99 (titer of 320) and A/Black-headed Gull/Sweden/4/99 (titer of 640) as compared to A/Black-headed Gull/Sweden/5/99 (titer of 80), whereas the ferret antiserum raised against A/Black-headed Gull/Sweden/5/99 gave the opposite results (homologous titer of 480 and heterologous titers of 20, 20 and 80, respectively).
Thus, whereas influenza viruses A/Black-headed Gull/Sweden/1/99 and A/Black-headed Gull/Netherlands/1/00 were identifiable as H13 viruses using HI assays, the other four viruses isolated from Black-headed Gulls were not. The latter four viruses were antigenically related, but displayed some antigenic heterogeneity with ferret antisera.

Sequence analyses and phylogeny
We next sequenced the HA open reading frame (ORF) for all six European Black-headed Gull viruses and compared them to sequences available from public databases. Upon analysis with the basic local alignment search tool (BLAST) available from GenBank, the HA ORF of A/Black-headed Gull/Sweden/1/99 and A/Black-headed Gull/Netherlands/1/00 revealed high percentages of nucleotide and amino acid sequence identity to the HA of H13 influenza A viruses, whereas the four unidentifiable influenza A viruses revealed low homology at the nucleotide and amino acid level, in agreement with the HI assay results (data not shown). We generated DNA maximum-likelihood trees for the HA ORFs of the six European Black-headed Gull

viruses and those of all complete H13 HA ORFs available from public databases. This phylogenetic tree revealed that the HA ORFs of A/Black-headed Gull/Sweden/1/99 and A/Black-headed Gull/Netherlands/1/00 were genetically most closely related to the HA ORF of another Eurasian Gull virus, A/Gull/Astrakhan/227/84, and less related to the American H13 viruses A/Gull/Maryland/704/77 and A/Pilot Whale/Maine/328/84[66]. The four unidentifiable Black-headed Gull viruses from Sweden were found on a separate branch in this phylogenetic tree, distinct from the H13 viruses (Fig. 1A). The HA gene of A/Black-headed Gull/Sweden/5/99 was genetically distinct from the HA genes of the other 3 unidentifiable Black-headed Gull viruses, in agreement with the antigenic heterogeneity of these virus isolates in HI assays with postinfection ferret antisera. The HA genes of A/Black-headed Gull/Sweden/3/99 and A/Black-headed Gull/Sweden/4/99 were identical at the nucleotide level and differed by only one silent nucleotide substitution from the HA gene of A/Black-headed Gull/Sweden/2/99.

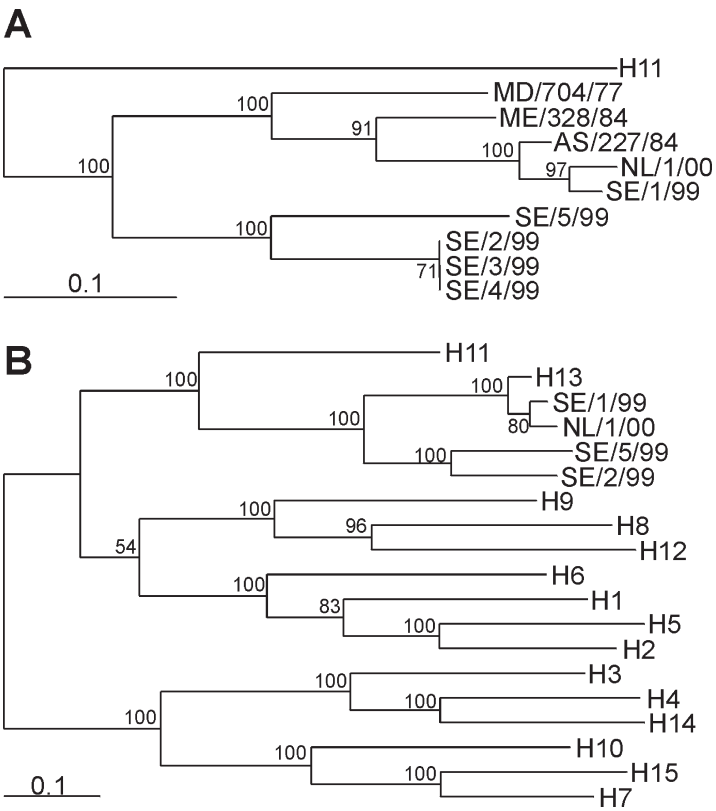


Figure 1. Phylogeny of HA genes from Swedish and Dutch Black-headed Gull influenza A viruses. DNA maximum likelihood trees were generated using the HA_0 ORFs of prototype H13 influenza A viruses and all isolates described in this study (A), or representative sequences for all HA subtypes, when available from Eurasian avian origin (B). DNA maximum likelihood trees were built using 100 bootstraps and 3 jumbles, and branchlengths were recalculated for the resulting consensus tree. The scale bars roughly represent 10 % of nucleotide changes between close relatives. MD; A/Gull/Maryland, ME; A/Pilot whale/Maine, AS; A/Gull/Astrakhan, NL; A/Black-headed Gull/Netherlands, SE; A/Black-headed Gull/Sweden. Small numbers in trees represent bootstrap values.

To determine the significance of the branching of the Black-headed Gull HAs in the phylogenetic tree, we next constructed a new maximum-likelihood phylogenetic tree in which all HA subtypes were included by using HAs from Eurasian avian origin when available (Fig. 1B). This tree showed that the length of the branches between the HA genes of viruses of subtype H13 and those of the unidentifiable viruses (maximum-likelihood tree distances ranging from 0.39 - 0.42) were similar to the length of the branches between the subtypes H2 and H5 (maximum-likelihood tree distance 0.40), H7 and H15 (maximum-likelihood tree distance 0.35) and H4 and H14 (maximum-likelihood tree distance 0.42), suggesting that the unidentified viruses represent a previously undescribed HA subtype, H16.

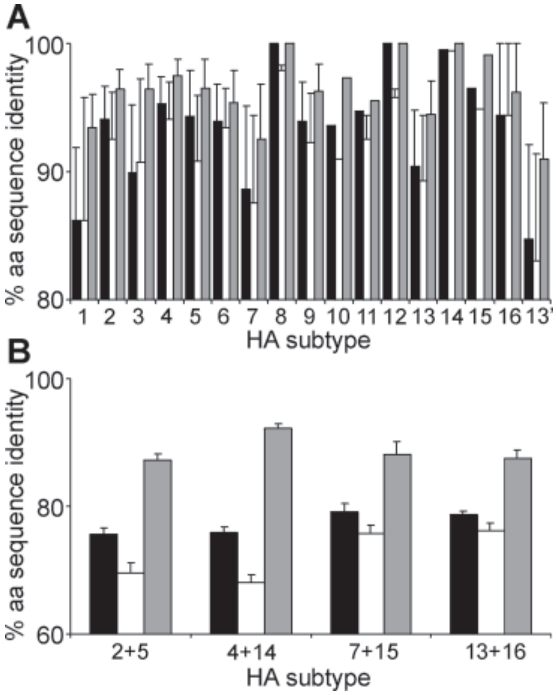


Figure 2. Amino acid sequence identity between pairs of HA sequences. The average percentages amino acid sequence identity and standard deviations are shown for all pairs of HA sequences within a subtype (A) or for all pairs of HA sequences from two closely related subtypes (B). Black, white and gray bars represent HA_0, HA_1, and HA_2 sequences respectively. HA subtype 13' represents the combined group of H13 and H16 HA sequences. The percentages and standard deviations were calculated using all HA sequences available from public databases upon sequence alignment per subtype (A) or per two subtypes (B) and subsequent calculation of pairwise hamming distances[112].

The identification of a novel HA subtype, H16, as evidenced by HA amino acid sequence comparisons. We obtained all HA sequences available from public databases, irrespective of host species or geographical or temporal information, and aligned these sequences per HA subtype. Alignments were made individually for HA_0, HA_1 and HA_2, thereby including as many sequences representing an entire HA_0, HA_1 or HA_2 domain as possible for analysis. Hamming amino acid distance

matrices were generated for each alignment of sequences[112], and the average percent amino acid sequence identity and the standard deviation were calculated for each HA subtype (Fig. 2A). The average percentage amino acid sequence identity for viruses characterized as HA subtypes H1-H15 ranged from 86-100 % for HA_0 and HA_1 and from 92-100 % for HA_2. The lowest average percentage amino acid sequence identity was found for viruses within subtypes H1, H3 and H7. The relatively high diversification of these HA ORFs is likely due to the fact that many of these viruses are isolated frequently not only from avian hosts, but also from nonavian hosts: H1 from humans and pigs, H3 from humans, pigs and horses and H7 from horses. When the H13 HA sequences were grouped separately from the putative H16 HA sequences, the average percent amino acid sequence identity between viruses within both subtypes was in the same range as for other avian HA subtypes (H13, 90, 89, and 95% for HA_0, HA_1, and HA_2, respectively; putative H16: 94, 94 and 96% for HA_0, HA_1,and HA_2, respectively). In contrast, when the H13 HA sequences were grouped together with the putative H16 sequences, the average percentage amino acid sequence identity between the HA sequences were 85, 83, and 91% for HA_0, HA_1 and HA_2, respectively. Thus, the combined group of H13 and H16 HA sequences would be more diverse than any known HA subtype identified to date, including those that diversified in non-avian hosts.

This same conclusion was reached when minimum rather than average percentage amino acid sequence identity was calculated (data not shown). The minimum percentage amino acid sequence identity between any pair of sequences in the H13-H16 combined subtype were 78, 74, and 86%, respectively, for HA_0, HA_1 and HA_2. The minimum percentage amino acid sequence identity was lower only for some pairs of HA_0 and HA_1 sequences of isolates of subtype H1 that have diversified in multiple hosts (75%).

We next compared the average percentage amino acid sequence identity and standard deviation between strains of the most closely related HA subtypes; H2 versus H5, H4 versus H14, H7 versus H15 and H13 versus putative H16 (Fig. 2B). The average percentage amino acid sequence identity between HA ORFs of isolates belonging to the H13 and putative H16 subtypes were 79, 76, and 88% for HA_0, HA_1 and HA_2 respectively. These percentages were in the same range as those observed for other related HA subtypes (HA_0; 76 to 79%; HA_1; 68 to 76%; HA_2; 87 to 92%).

Thus, the phylogenetic trees and the analyses of HA amino acid sequences representing all previously described HA subtypes provide strong evidence for the classification of the HAs of A/Black-headed Gull/Sweden/2/99, A/Black-headed Gull/Sweden/3/99, A/Black-headed Gull/Sweden/4/99 and A/Black-headed Gull/Sweden/5/99 as a novel subtype, H16.

The identification of a novel HA subtype, H16, by classical criteria

The classical way to identify a novel HA subtype is by using a double immunodiffusion assay[75,100,111]. In such assay, antibodies and antigens are loaded in separate wells in a gel-based matrix, after which diffusion gradients will form. When the diffusion gradients of antigens and antibodies cross, a line of immunoprecipitation may form to indicate the specific reaction between the antibodies and antigens. Because precipitations due to influenza virus antigens other than HA are undesired for HA typing, monospecific antisera or highly purified HA protein preparations (or both)

are required. We generated hyperimmune rabbit antisera by DNA vaccination with cloned versions of the HA genes of influenza viruses A/Black-headed Gull/Sweden/5/99 and A/Gull/Maryland/704/77. The antisera were concentrated 80-fold by a combination of precipitation with ammonium sulfate and microfiltration because of the low titers of specific antibodies in the original antisera as determined in HI assays (HI antibody titers of 96). As antigens, we used whole-virus lysates prepared after 40-fold concentration of virus from the allantoic fluid of infected embryonated chicken eggs.

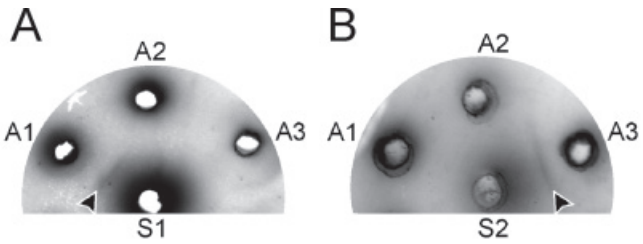


Figure 3. Double radial immunodiffusion assay with hyperimmune rabbit antisera raised against HA of Gull influenza A viruses. Concentrated hyperimmune antisera raised against H13 HA of A/Gull/Maryland/704/77 (panel A) or HA of A/Black-headed Gull/Sweden/5/99 (panel B) were loaded in wells S1 and S2 respectively. Lysates prepared from concentrated virus stocks of A/Gull/Maryland/704/77 (H13), A/Shoveler/Netherlands/18/99 (H11), and A/Black-headed Gull/Sweden/5/99 were loaded in antigen wells A1, A2 and A3 respectively. Precipitates were allowed to form for 48 hours, after which the gels were dried and stained with coomassie brilliant blue. Specific precipitates were observed only between wells A1 (H13, A/Gull/Maryland/704/77) and S1 (anti-H13, A/Gull/Maryland/704/77) and wells A3 (H16, A/Black-headed Gull/Sweden/5/99) and S2 (anti-H16, A/Black-headed Gull/Sweden/5/99), as indicated with arrowheads.

The antiserum raised against the H13 HA of A/Gull/Maryland/704/77 (Fig. 3A, serum S1) gave rise to antigen-antibody complexes when tested against virus lysate A/Gull/Maryland/704/77 (antigen A1), but not with virus lysates A/Shoveler/Netherlands/18/99 (H11, antigen A2) and A/Black-headed Gull/Sweden/5/99 (putative H16, antigen A3). Conversely, the antiserum raised against the putative H16 HA of A/Black-headed Gull/Sweden/5/99 (Fig. 3B, serum S2) gave rise to antigen-antibody complexes when tested against virus lysate A/Black-headed Gull/Sweden/5/99 (antigen A3) but not with virus lysates A/Gull/Maryland/704/77 (antigen A1) and A/Shoveler/Netherlands/18/99 (antigen A2). Thus, the HA of A/Black-headed Gull/Sweden/5/99 represents a previously unidentified HA subtype, H16, according to established criteria[100].

Predicted amino acid sequence of H16 HA

In figure 4, the predicted amino acid sequences of the HA0 ORFs of the two antigenic variants of H16, obtained from A/Black-headed Gull/Sweden/2/99 (564 aa) and A/Black-headed Gull/Sweden/5/99 (566 aa) are shown. The predicted HA0 amino acid sequences are 88.3 % identical, and those of the HA_1 and HA_2 subunits 88.6 and 92.3 % identical, respectively. The HA ORFs of A/Black-headed Gull/Sweden/2/99 and A/Black-headed Gull/Sweden/5/99 contain 9 and 8 potential N-linked glycosylation sites (N-X-T/S) respectively. With the exception of potential N-linked glycosylation site number 4 (Fig. 4), all these sites are in common with H13 HA[66,113]. The protease

cleavage site in H16 HA does not contain multiple basic amino acid residues (SIVER*GLFG, SVGER*GLFG), and is somewhat different from the cleavage site in H13 HA (AISNR*GLFG). Recently, differences in the receptor-binding site between HAs originating from Laridae (gulls) and Anatidae (ducks, geese) were described for aa positions 154, 191, 215, 222, 223, 227 to 229, and 231[114]. With the exceptions of amino acid positions 154 and 223, the following amino acid residues in the receptor-binding site of H16 HA were in agreement with these observations: 191Ala, 215Leu, 222Gly, 227Arg, 228Ser, 229Trp, and 231Lys (Fig. 4).

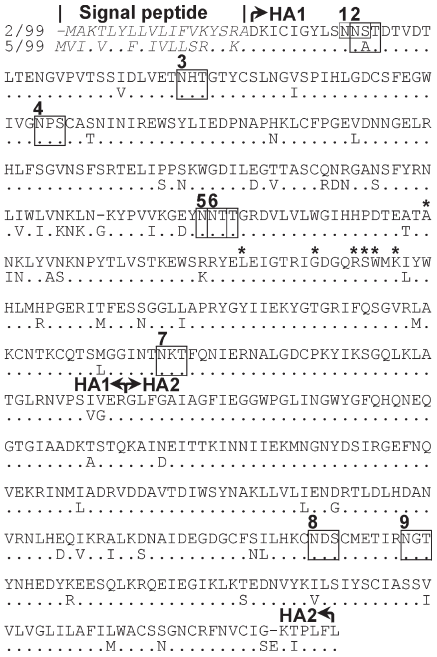


Figure 4. Alignment of predicted amino acid sequences of HA, subtype H16. An alignment of representative H16 amino acid sequences (2/99; A/Black-headed Gull/Sweden/2/99, 5/99; A/Black-headed Gull Sweden/5/99) is shown with periods representing identical amino acid residues and dashes representing gaps. Potential N-linked glycosylation sites (N-X-T/S) are boxed and numbered. Asterisks above the sequences indicate amino acid positions at which differences were observed between HA genes from Laridae and Anatidae (positions 191, 215, 222, 227, 228, 229, 231 in H3 HA)[114]. The predicted signal peptide and HA_1 and HA_2 domains are indicated.

Sequence analyses of the NA genes of influenza A viruses isolated from European Black-headed Gulls. We next amplified the NA gene segments of the Black-headed Gull influenza A viruses by RT-PCR with primers targeting the conserved noncoding regions and sequenced the obtained PCR products. Upon BLAST analysis, the NA gene of A/Black-headed Gull/Netherlands/1/00 revealed closest resemblance to NA sequences of subtype N8, the NA gene of A/Black-headed Gull/Sweden/1/99 to NA sequences of subtype N6 and the NA genes of the other 4 Black-headed Gull viruses to NA sequences of subtype N3 (data not shown). A UPGMA phylogenetic tree, based on an amino acid sequence distance matrix in which all NA sequences available from

public databases were included, was in agreement with these BLAST results (Fig. 5A). In this tree, each of the European Black-headed Gull viruses was found on a branch with previously identified Gull viruses: A/Black-headed Gull/Sweden/1/99 (H13N6) with A/Gull/Maryland/704/77 (H13N6) and A/Gull/Astrakhan/227/84 (H13N6), A/Black-headed Gull/Netherlands/1/00 (H13N8) with A/Herring Gull/Delaware/677/88 (H2N8), and the other Black-headed Gull viruses (H16N3) with A/Gull/Heuwiese/899-6/80 (H7N3). Whereas the NA genes of Black-headed Gull viruses of subtype N6 and N8 are closely related to NA genes obtained from influenza A viruses isolated from other avian hosts (ducks, poultry), the N3 NA genes obtained from the H16 Black-headed Gull viruses form a distinct genetic lineage within the N3 NA cluster, together with the N3 genes of A/Tern/Astrakhan/775/83 (H13N3) and A/Gull/Heuwiese/899-6/80 (H7N3). Both the DNA maximum-likelihood tree (Fig. 5B) and the UPGMA tree based on the amino acid sequence distance matrix (Fig. 5A) revealed that the genetic distance between the European shorebird N3 sequences and the duck/poultry N3 sequences is smaller than the genetic distance between related NA subtypes such as N1 and N4, N6 and N9 and N5 and N8. In neuraminidase inhibition assays with hyperimmune rabbit antisera[106], the NA of the H16 Black-headed Gull viruses gave a weak positive reaction with an antiserum raised against A/Duck/Hongkong/207/77 (H5N3) (data not shown), indicating that they are antigenically related.

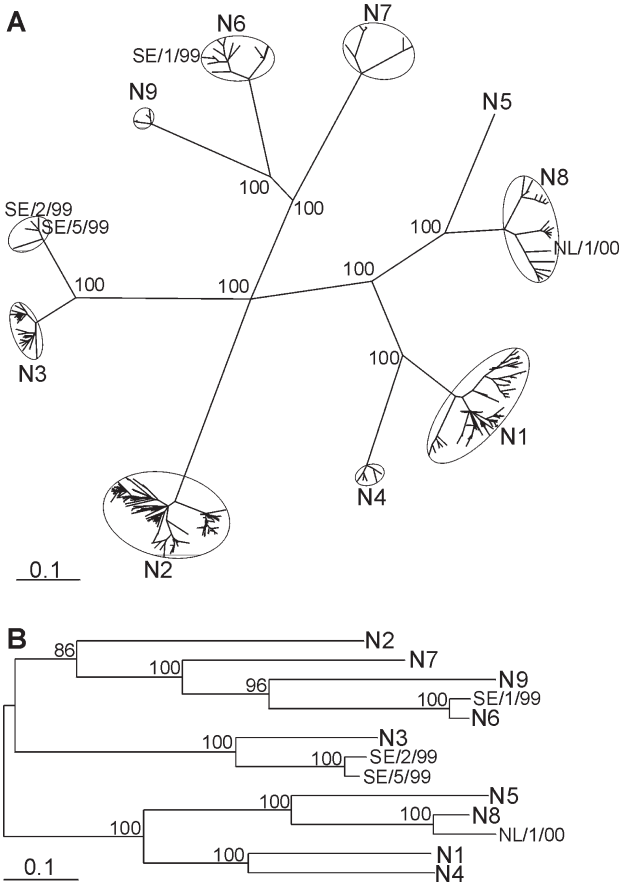


Figure 5. Phylogeny of NA genes from Swedish and Dutch Black-headed Gull influenza A viruses. A tree was generated with all 649 full length NA amino acid sequences available from public databases (A). Aligned sequences were bootstrapped 100 times, and the Kimura amino acid distance matrices were converted to trees using a UPGMA clustering algorithm. Very similar trees were obtained when distance matrices were generated using Hamming distances or the Jones-Taylor-Thornton model[108,112]. The consensus tree was used for recalculation of the branchlengths with the Fitch program of Phylip. Representative nucleotide sequences, when available from Eurasian avian origin, were used to generate a DNA maximum likelihood tree (B). This tree was built using 100 bootstraps and 3 jumbles, after which branchlengths were recalculated for the consensus tree. NL/1/00, SE/1/99, SE/2/99, SE/5/99 represent the NA sequences of Black-headed Gull viruses of subtypes H13N8, H13N6, H16N3 and H16N3 from The Netherlands and Sweden respectively. Scale bars roughly represent 10% of changes between close relatives. Small numbers in trees represent bootstrap values.

Sequence analyses of the internal genes of influenza A viruses isolated from European Black-headed Gulls

We sequenced the internal genes of the H13N6, H13N8 and representative H16N3 viruses (A/Black-headed Gull/Sweden/2/99 and A/Black-headed Gull/Sweden/5/99). These nucleotide sequences were analyzed in DNA maximum-likelihood phylogenetic trees in which sequences of prototypic influenza A virus isolates[55] from different hosts and geographical locations were included (Fig. 6). The PB2, NP and NS genes of the Black-headed Gull viruses were closely related to those of previously characterized gull viruses, whereas the PB1 and MA genes were closer to sequences of Eurasian avian origin and the PA genes closer to sequences of either American or Eurasian avian origin. GenBank BLAST analyses confirmed these results (data not shown). Thus, some of the internal genes of these European Black-headed Gull viruses are genetically distinct from those of the prototypic gull viruses, such as A/Gull/Maryland/704/77. The internal genes of the H13 and H16 European Black-headed Gull viruses are genetically closely related, although there was considerable heterogeneity between the NS genes of H13 and H16 viruses (Fig. 6F).

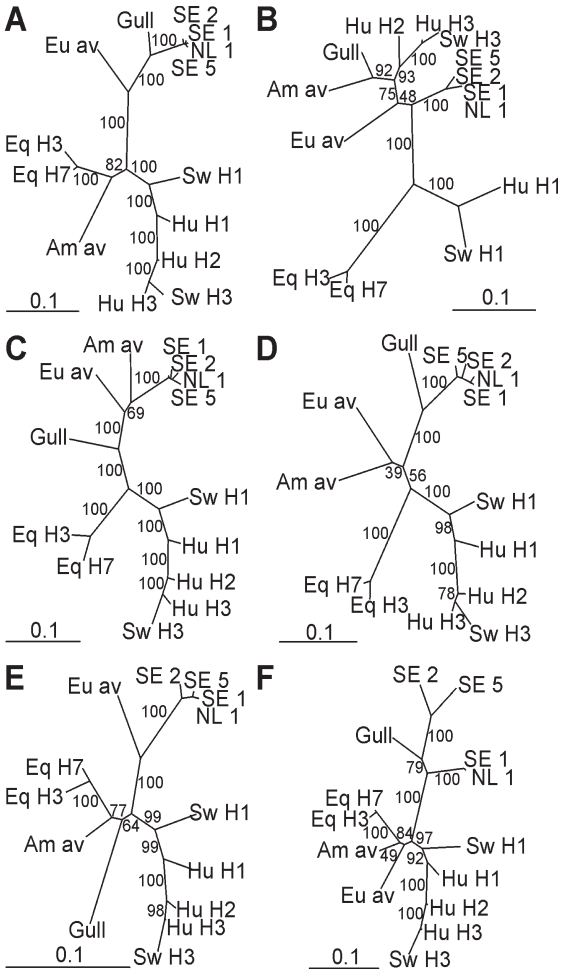


Figure 6. Phylogenetic trees representing the internal genes of influenza A viruses. Trees were constructed based on a 2262 nucleotide fragment of gene segment 1 (PB2) (A), 2267 nucleotides of gene segment 2 (PB1) (B), 2142 nucleotides of gene segment 3 (PA) (C), 1486 nucleotides of gene segment 5 (NP) (D), 947 nucleotides of gene segment 7 (MA) (E) and 794 nucleotides of gene segment 8 (NS) (F). Sequences obtained from influenza viruses A/Black-headed Gull/Netherlands/1/00 (H13N8) (NL 1), A/Black-headed Gull/Sweden/1/99 (H13N6) (SE 1), A/Black-headed Gull/Sweden/2/99 (H16N3) (SE 2) and A/Black-headed Gull/Sweden/5/99 (H16N3) (SE 5) were compared with those of reference strains available from GenBank, representing the known genetic lineages of influenza A virus[55]. The DNA maximum likelihood trees were built using 100 bootstraps and 3 jumbles, and branchlengths were recalculated for the resulting consensus tree. Scale bars roughly represent 10% of nucleotide changes between close relatives. Small numbers in trees represent bootstrap values.

Discussion

Here we identified a previously undescribed HA subtype of influenza A virus, H16, obtained from Black-headed Gulls in Sweden. Phylogenetic analyses of the HA nucleotide sequences, and comparison of the predicted amino acid sequences of all HA genes available from public databases provided strong evidence for the classification of the HA of four of the Swedish Black-headed Gull viruses as H16. This classification was subsequently confirmed in a double radial immunodiffusion assay, according to classical criteria. The closest relative of the H16 HA gene was the HA gene of subtype H13.

HA of subtype H13 so far has been found exclusively in shorebirds such as gulls and in a pilot whale (potentially a spill-over from shorebirds), but not in other avian species that are natural hosts of influenza A virus such as ducks and geese[24]. Since the HA of subtype H16 has remained undetected until 1999, it is possible that the HA of subtype H16 is present only in a limited number of avian hosts. It is of interest to note that many amino acid residues, predicted to be in the receptor-binding site of HA, are in common in H13 and H16 Gull influenza A viruses, but distinct in HA of influenza A viruses isolated from ducks and geese[114]. The receptor-binding site of HA could thus provide a molecular basis for the putative host-preference of H13 and H16 influenza A viruses, and could explain why these viruses have a specific ecological niche.

With the exception of the NS genes, the internal genes of the H13 and H16 European Black-headed Gull viruses were genetically closely related (Fig. 6). The PB2, PB1, PA, NP and MA genes were 97 to 99% identical at the nucleotide level, but for the NS gene this was as low as 86 percent. It is unclear what the origin is of these distinct NS gene segments. In BLAST searches, the NS genes of all 4 European Black-headed Gull viruses revealed more nucleotide sequence identity to those of other Gull viruses than to allele A and allele B avian NS genes. The fact that they still clustered within the gull lineage suggests that these NS genes have diversified in gulls or other shorebirds, rather than that they were recently introduced in gulls from other avian hosts, as could be speculated for the NS genes of some American Gull viruses[115,116]. The PB1, PA and MA genes of the European Black-headed Gull viruses were genetically distinct from those of American gull viruses[1,117,118]. In contrast, the PB2, NP, and NS genes of the European Black-headed Gull viruses were genetically most closely related to those of American gull viruses[1,115,116,119,120]. Thus, the influenza A viruses isolated from gulls can be distinguished genetically from other avian influenza A viruses based on the PB2, NP and NS genes, but not necessarily based on PB1, PA and MA.

The four influenza A viruses of subtype H16 contained NA genes that were closely related to NA of subtype N3. Genetically, these 4 NA genes clustered together with the NA genes from influenza A viruses isolated from Eurasian shorebirds (A/Tern/Astrakhan/775/83 (H13N3) and A/Gull/Heuwiese/899-6/80 (H7N3)). This Eurasian shorebird lineage of N3 genes is genetically distinct from the N3 genes detected in other wild birds and poultry around the world. The genetic distance between Eurasian shorebird N3 and other N3 sequences is somewhat smaller than between related NA subtypes (N1-N4, N5-N8, N6-N9), and there is some reactivity in NI assays between antigens and antisera raised against the different N3 lineages. Thus, there

is no strong argument to classify the Eurasian shorebird N3 genes as a separate NA subtype, based on existing criteria.
Although the double radial immunodiffusion assay has proven to be valuable for the classification of antigenic subtypes of HA and NA of influenza A virus, the question arises whether it still provides the best classification today. The immunodiffusion assay was recommended for classification at a time when antigenic characterization was easier than nucleotide sequence analysis. A disadvantage of the immunodiffusion assay is that high-titer monospecific antisera or highly purified antigen preparations (or both) are required to prevent undesired immunoprecipitation reactions between additional influenza virus antigens and the antisera. The second disadvantage of such serological tests, is that they provide quantitative rather than qualitative information because the serological assays (HI, neuraminidase inhibition, or immunodiffusion assays) depend on the amount of antigen and quality and quantity of antibodies used. Weak antisera may identify too many different subtypes and strong or less specific antisera too few, which may yield discrepant results in different laboratories.
Today, sequence analysis is a standard technique in most laboratories, and may be a preferred method for classification. A wide variety of algorithms for clustering or inference of genetic relationships are available for analyses of nucleotide or amino acid sequences[121]. Sequences can be grouped in existing or novel clusters based on preset criteria that could include the maximum distance between sequences within a cluster (subtype) and the minimum distance between sequences of different clusters (subtypes). Serological tests in a simple format, such as the HI and neuraminidase inhibition assays, would remain extremely useful as a high-throughput laboratory tool and together with the sequence analysis of HA and NA genes, they could provide the most practical framework for assigning existing or newly discovered HA and NA subtypes to influenza A virus isolates.
With the current intensified surveillance for influenza A virus in wild and domestic animals, it is probable that further novel HA and NA subtypes will be detected. This could either be due to the emergence of “new” influenza A virus subtypes, perhaps in part as the result of genetic mixing or adaptation to new hosts, or due to the circulation of unknown influenza A virus subtypes in specific ecological niches that were previously not explored. Gaining information on the full spectrum of influenza A viruses circulating in our environment and developing reagents for the specific detection of these viruses, will remain important tasks for influenza surveillance, outbreak control, and animal and public health.

Acknowledgements

We thank C. Eising and T. Groothuis for providing avian specimens, L. van der Kemp for technical assistance, J.C. de Jong for critically reading the manuscript and R.G. Webster for providing influenza A virus reference strains. This is contribution #198 from Ottenby Bird Observatory.

5

Multiple gene segment reassortment between Eurasian and American lineages of influenza A virus (H6N2) in Guillemot (*Uria aalge*)

Archives of Virology, 2005, 150: 1685-1692

Anders Wallensten*
Vincent Munster*
Johan Elmberg
Albert Osterhaus
Ron Fouchier
Björn Olsen

* These authors contributed equally to the results of this study

Guillemots banded in the northern Baltic Sea were screened for influenza A virus (IAV). Three out of 26 sampled birds tested positive by RT-PCR. Two of these were characterized as subtype H6N2. Phylogenetic analyses showed that five gene segments belonged to the American avian lineage of IAVs, whereas three gene segments belonged to the Eurasian lineage. Our findings indicate that avian IAVs may have a taxonomically wider reservoir spectrum than previously known and we present the first report of a chimeric avian IAV with genes of American and Eurasian origin in Europe.

Waterfowl and shorebirds are the main hosts for IAVs in nature[1]. In avian hosts IAVs replicate in the respiratory and intestinal tracts and are spread via the fecal-oral-route. Whereas avian IAVs are generally non-pathogenic in waterfowl, they may cause morbidity and mortality upon transmission to other species. To date, IAVs representing sixteen hemagglutinin (HA) and nine neuraminidase (NA) subtypes have been detected in wild birds and poultry worldwide[1,6].

The auks (family Alcidae) occupy much the same ecological niche as the penguins (family Spheniscidae) do in the southern oceans[122]. An important ecological characteristic shared by marine birds is their obligation to return to land to breed. Many species thus congregate in large colonies, numbering several thousands to millions of individuals[123]. Crowded conditions promote an exchange of endo- and ectoparasites[124]. The Guillemot (*Uria aalge*) is found along the coasts in the North Atlantic and the Northern Pacific Ocean. Banding and satellite telemetry has shown that some birds end up breeding in another colony than where they were born[125,126] and that non-breeding birds frequently visit non-natal colonies[127]. Until now there has only been one isolation of IAV in guillemots, a human-like H3N2 IAV isolated in 1974 (A/Common Murre/Sakhalin/1/74)[128].

IAVs are genetically distinguishable based on host (e.g. avian, human, swine and equine) and geographical origin (e.g. Eurasian and American lineages of avian IAVs)[129,130]. Reassortment of internal gene segments of multiple IAV genotypes have been described for some subtypes, but such reassortment is generally limited due to geographical constraints[131]. The HA gene (subtype H2) of Eurasian avian origin was detected in shorebirds in North America[31], and avian IAVs (subtype H2) with internal gene segments originating from both the Eurasian and American avian lineages were detected in Japan[32]. However, data on gene flow between the American and Eurasian IAV lineages remain limited.

One of the largest seabird colonies in the Baltic Sea is on Bonden island, a rocky outcrop located 12 km off the Swedish mainland (6326N, 2003E, surface area 0.45 km 2). During the breeding season this island holds 4,000 pairs of Razorbills (*Alca torda*), 800 pairs of Guillemots, 50 pairs of gulls (*Larus spp*), and 20 pairs of Arctic Terns (*Sterna paradisaea*). In July 2000 as part of our ongoing IAV surveillance study in wild birds[63], cloacal swab samples were collected from 26 randomly chosen guillemots (10 adults and 16 nestlings). RT-PCR analysis specific for the IAV matrix gene[80] revealed that all adult birds were IAV negative, but that three of the nestlings were positive for IAV. The RT-PCR positive samples were then inoculated into 11-day old embryonated chicken eggs for virus isolation. An IAV isolate was obtained from one of the samples that tested positive by RT-PCR (A/Guillemot/Sweden/3/00).

Repeated attempts to isolate IAVs from the other two samples were unsuccessful. The HA and NA gene segments of A/Guillemot/Sweden/3/00 were amplified by RT-PCR and sequenced[81]. Upon analysis with the basic local alignment search tool (BLAST) available from Genbank, the HA gene segment of A/Guillemot/Sweden/3/00 displayed the highest percentage nucleotide (97%) and amino acid (98%) sequence identity to HA of A/Shorebird/Delaware/194/98 (H6N4). Upon BLAST analysis of the NA gene segment of A/Guillemot/Sweden/3/00, the highest percentage nucleotide and amino acid (aa) sequence identity, 97 and 98% respectively, were observed with the NA gene segment of A/Pheasant/Ireland/PV18/97 (H9N2). The phylogenetic tree based on the HA_1 aa sequences ofA/Guillemot/Sweden/3/00 and all H6 sequences available from public databases revealed that the Guillemot HA gene was closely related to a group of sequences obtained from shorebirds in Delaware between 1994 and 1998 (Fig. 1A). A DNA maximum likelihood tree for this cluster of shorebird H6 IAVs further revealed the close genetic relationship between the HA genes of A/Guillemot/Sweden/3/00 and A/Shorebird/Delaware/194/98 (Fig. 1B)[108].

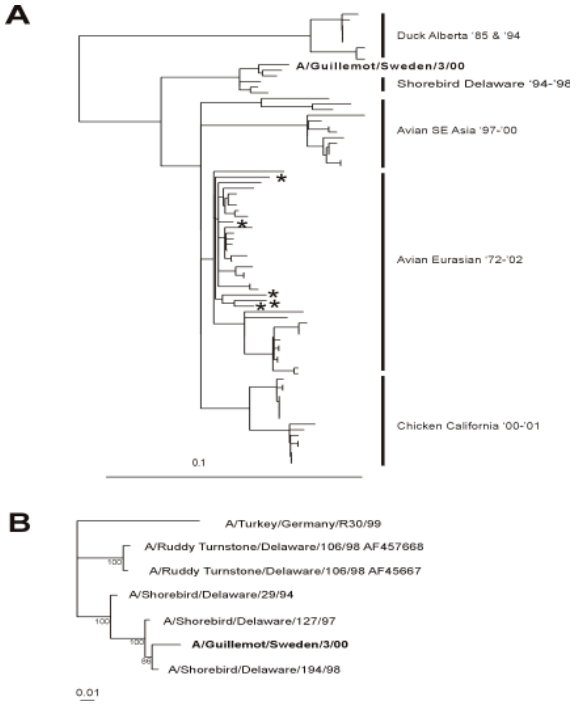


Figure 1. Phylogenetic analyses of the HA of A/Guillemot/Sweden/3/00. **A**) A phylogenetic tree based on the amino acid sequence distance matrix for the HA1 open reading frames of all H6 sequences available from public databases was generated. The scale bar represents ~10% of amino acid changes between close relatives. *Represent american avian IAV strains isolated from ducks in Alberta (1992-1999) within the Eurasian avian lineage. **B**) A DNA maximum likelihood tree for the cluster of shorebird H6 viruses and A/Guillemot/Sweden/3/00 was generated using A/Turkey/germany/R30/99 as outgroup. The scale bar represents ~1% of nucleotide changes between close relatives. The numbers in the three represent bootstrap values. The HA nucleotide sequence of A/Guillemot/Sweden/3/00 is available from GenBank (accession number AY703832).

The phylogenetic tree based on the NA gene of A/Guillemot/Sweden/3/00 and all avian N2 aa sequences available from public databases revealed that the NA ORF of A/Guillemot/Sweden/3/00 was closely related to Eurasian avian IAVs of subtype H9, isolated between 1995 and 2000 (Fig. 2A). A DNA maximum likelihood tree for this cluster of N2 IAVs confirmed that the NA gene of A/Guillemot/Sweden/3/00 was most closely related to NA of A/Pheasant/Ireland/ PV18/97 (H9N2) (Fig. 2B).

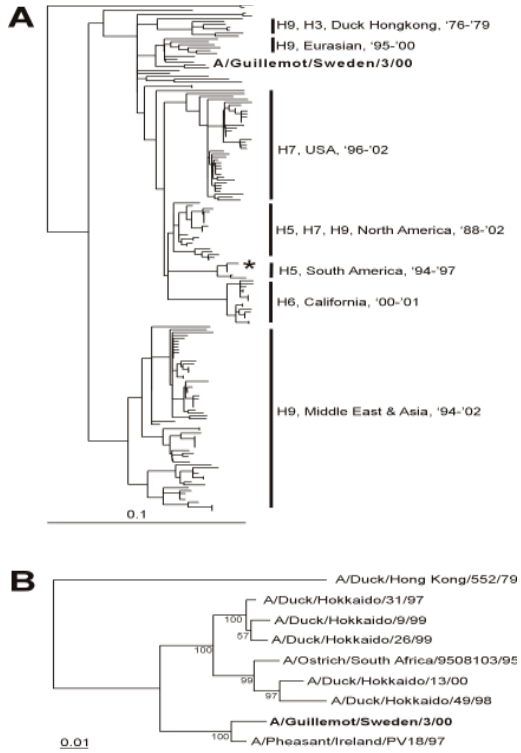


Figure 2. Phylogenetic analyses of the NA gene of A/Guillemot/Sweden/3/00. A) A phylogenetic tree based on the amino acid sequence distance matrix for the NA open reading frames of all N2 sequences available from public databases was generated. The scale bar represents ~10% of amino acid changes between close relatives. *Represents avian influenza A virus (H5N2) of South American lineage in Southeast Asia (A/chicken/Taiwan/1209/03). B) A DNA maximum likelihood tree for the cluster of Eurasian avian IAVs of the subtype H9N2, isolated between 1995-2000, and A/Guillemot/Sweden/3/00 was generated using A/duck/HongKong/552/79 as outgroup. The scale bar represents ~1% of nucleotide changes between close relatives. The NA nucleotide sequence of A/Guillemot/Sweden/3/00 is available from GenBank (accession number AY703834).

Since the phylogenetic trees revealed that the HA gene of A/Guillemot/ Sweden/3/00 belonged to the American avian IAV lineage, whereas the NA gene belonged to the Eurasian avian lineage, we next sequenced the remaining gene segments of this guillemot IAV. BLAST searches revealed the highest percentage nucleotide and amino acid sequence identity to strains of Eurasian origin for the PA (98% nt identity, 99% aa identity) and NP (97% nt identity, 99% aa identity) gene segments of A/Guillemot/Sweden/3/00. In contrast, the other internal gene segments of A/Guillemot/Sweden/3/00 revealed the highest identity to those of American avian origin (PB2;

97% nt identity, 98% aa identity, PB1, 96% nt identity, 98% aa identity, MA; 98% nt identity, 99% aa identity, NS; 98% nt identity, 96% aa identity). DNA maximum likelihood trees in which all known genetic lineages of IAV were represented confirmed that A/Guillemot/Sweden/3/00 is a chimera between viruses belonging to the American and Eurasian avian IAV lineages (Fig. 3). Although only one out of three RT-PCR positive samples could be cultured, one of the remaining two samples contained enough viral RNA to perform limited genotype analyses. These analyses revealed the same genetic makeup as A/Guillemot/Sweden/3/00, with the exception of single nucleotide substitutions (data not shown).

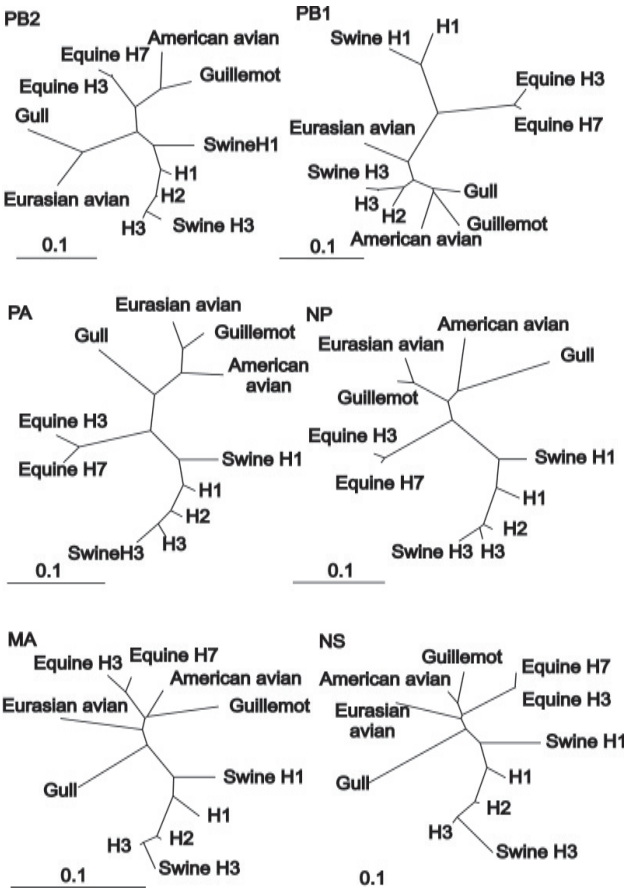


Figure 3. Phylogenetic trees representing the internal gene segments of a/Guillemot/Sweden/3/00 and prototypic IAVs. DNA maximum likelihood trees were based on sequences obtained from A/Guillemot/3/00 and reference strains representing the known genetic lineages of IAV[6]. The scalebar represents ~10% nucleotide changes between close relatives. Nucleotide sequences are available from GenBank (accession numbers AY703829-AY703836).

In conclusion, this is the first report on the isolation and characterization of an avian IAV from guillemots belonging to the Atlantic population. Sazonov and co-workers isolated a human like IAV of subtype H3N2 in Pacific guillemots, whereas we isolated a subtype (H6N2) that has not been found in humans[128]. Unlike most IAVs

described to date, A/Guillemot/Sweden/3/00 was found to contain 3 gene segments of Eurasian avian origin and 5 gene segments of the American avian lineage. Previous studies have shown that the H2 HA gene of Eurasian avian origin was present in shorebirds in North America[31], and the PB2 and PA genes of American avian origin were detected in Eurasian IAVs isolated from ducks in Japan[32]. Based on these studies, it was hypothesized that there is an interregional flow of IAV genes, due to overlapping migratory routes of ducks and shorebirds between Eurasia and the Americas. However, the detection of an American-Eurasian chimeric IAV in guillemots in Sweden implies yet another taxonomic and geographic pathway. Guillemots breeding in the Baltic Sea do not leave it, as shown by the limited number of recoveries in the Atlantic based on 43,000 ringed birds[132]. Thus, dispersal of IAV within the Baltic can be explained by guillemot movements, but less likely so a spread between the Atlantic and the Baltic Sea. Moreover, despite 6,700 recoveries of 240,000 Guillemots ringed in the British Isles and Norway, none has been recovered in North America[133,134]. Likewise, there are no recoveries in Europe of guillemots ringed in North America. Baltic, East Atlantic and West Atlantic guillemots thus never or very rarely interact. We believe that other species of birds with different migratory routes must be involved to explain the chimeric constitution of the present virus. Since we have not found the parental viruses we can only speculate that either the reassortment has taken place in a doubly infected auk, infected with at least one virus from another species, or that the chimeric virus has been introduced by another bird species. The Bonden guillemots do not frequently come in contact with waterfowl or shorebirds, as the latter are rarely found on this remote island during the nesting season of the auks. The only ducks on Bonden are the pelagic species Eider (*Somateria mollissima*) and Velvet Scoter (*Melanitta fusca*), none of which have been shown to be positive for IAV in preliminary studies[63]. One explanation could be that the guillemot nestlings that tested positive for IAV had acquired it from adult birds in the colony and that the virus is endemic in the guillemot population. Alternatively, terns or gulls breeding on Bonden could have transmitted the virus to the guillemots. The best candidate among the former is the Arctic Tern, which breeds within and close to the auk colony, on Bonden and elsewhere. Arctic Terns from North Europe and North America have migration routes and wintering areas in common in the southern Atlantic. Based on this dispersal pattern we hypothesize that the Arctic Tern may represent a vector and/or mixing vessel for American and Eurasian lineages of avian IAVs. Regardless of transmission route, we think that auks may constitute a natural reservoir and a part of a pelagic niche for avian IAVs. This study highlights the need to differentiate between avian reservoirs that occupy different ecological niches, e.g. auks vs. dabbling ducks, to fully understand the ecology of IAV. We also show that IAV can be found in many types of aquatic birds even in those breeding on isolated islands and cliffs. Further studies are needed to evaluate the role of auks and, indeed, terns, in the epidemiology and genetic diversification of IAV.

Acknowledgements
We thank S. Herfst and T. Bestebroer for excellent technical assistance.


923A

6

Hampered foraging and migratory performance in swans infected with low-pathogenic avian influenza A virus

Submitted

Jan van Gils
Vincent Munster
Reinder Radersma
Daan Liefhebber
Ron Fouchier
Marcel Klaassen

It is increasingly acknowledged that migratory birds, notably waterfowl, play a critical role in the maintenance and spread of influenza A viruses. In order to elucidate the epidemiology of influenza A viruses in their natural hosts, a better understanding of the pathological effects in these hosts is required. Here we report on the feeding and migratory performance of wild migratory Bewick's swans (*Cygnus columbianus bewickii* Yarrell) naturally infected with low-pathogenic avian influenza (LPAI) A viruses of subtypes H6N2 and H6N8. Using information on geolocation data collected from Global Positioning Systems fitted to neck-collars, we show that infected swans experienced delayed migration, leaving their final stopover site during southward migration more than a month after uninfected animals. This was correlated with infected birds travelling shorter distances and fuelling and feeding at reduced rates. The data suggest that LPAI virus infections in wild migratory birds may have higher clinical and ecological impacts than previously recognised.

Introduction

Influenza A viruses have been isolated from many species including humans, pigs, horses, mink, felids, marine mammals and a wide range of domestic birds, but wild birds belonging to the orders Anseriformes (particularly ducks, geese, and swans) and Charadriiformes (particularly gulls, terns, and waders) are thought to form the virus reservoir in nature[61]. In wild birds and poultry throughout the world, influenza viruses representing 16 hemagglutinin (H) and 9 neuraminidase (N) antigenic subtypes have been detected[6]. In contrast to other virus subtypes, influenza viruses of subtypes H5 and H7 may become highly pathogenic following introduction in poultry and cause outbreaks of highly pathogenic avian influenza (HPAI). Although LPAI viruses may also cause mild symptoms in poultry, it is generally thought that they do not cause disease in wild birds[61]. However, this information is based primarily on experimental infection of captive birds rather than information on natural infections of wild migrating birds. Subclinical or mild diseases observed in the laboratory may have significant ecological fitness consequences in the field.

Upon infection, migratory birds may reallocate some of their resources to boost their immune system[135], at the expense of other demanding processes such as the accumulation of body stores, which are required to fuel their migratory flights and to overcome periods of adverse weather and foraging conditions. Lower fuelling rates will presumably lead to slower migrations with more frequent stopovers along the route[136]. In the context of our studies on migration and feeding patterns of Bewick's swans (*Cygnus columbianus bewickii* Yarrell, breeding in NW Russia and wintering in NW Europe), 25 swans were caught in The Netherlands at one of their last stopover sites during autumn migration, and 12 were fitted with a coded GPS-collar. From the same group of birds, cloacal swabs and blood samples were collected to test for the presence of influenza A virus as part of ongoing wild bird influenza A virus surveillance[79]. Three out of 25 swans were infected with influenza A virus at the time of capture, two of which we could track in great detail due to them carrying a GPS-collar (Fig. 1). Here we compare their feeding and migratory performance with the performances of their healthy counterparts.

Figure 1. Bewick's swan 923A infected with an H6 influenza A virus. Photo by W. Tijssen.

Materials and Methods

Swan catching and processing

By means of canon netting, we caught 25 Bewick's swans on 18 December 2005 on a sugar beet field in Wieringerwerf, Wieringermeer, The Netherlands (52°48' N, 05°05' E). The birds were aged and sexed on the basis of plumage coloration and cloacal examination, respectively. We determined wing length (to nearest mm), total head size (to nearest mm) and body mass (to nearest 0.1 kg). Twelve GPS-collars were fitted to 9 adults (presumably 1 male and 8 females; 4 of which were paired of which 2 were accompanied by young), 2 yearlings (sex unknown; of which one still accompanied its parents) and 1 juvenile male (accompanying its parents).

GPS-collars

GPS-collars were manufactured by Microtes Wildlife Engineering (Arnhem, The Netherlands). Each collar was fitted with a miniature GPS-receiver and antenna, a Bluetooth transceiver and antenna, a flash storage device, and a time-scheduled microprocessor controlling data collection and transmission. Total weight of the collar devices was 80 g. The GPS automatically collected geographical positions during 2-4 prescheduled times a day (accuracy < 50 m). When within close range of the bird (< 300-400 m), GPS-data were downloaded via Bluetooth at prescheduled times. Each collar had an individual code engraved (readable up to 600 m; Fig. 1), which enabled us, through the help of many volunteer ring-readers, to trace the birds for data collection. Until our last day in the field (22 February 2006), we were able to collect GPS-data on 8 birds; the whereabouts of the other 4 birds were reconstructed using resightings.

Visual observations
Throughout winter, we were able to regularly observe six of the GPS-collared swans, the two virus-infected birds included. We performed so-called focal scans[137], which meant that we carefully observed one of the collared swans for a one-hour period, or less when disturbed. Through a 20-60 × spotting scope (Kowa Company Ltd., Chuo-Ku Tokyo, Japan) and a 60 × optolite (Meade Instruments Corporation, Irvine, USA), we counted the number of bites taken (when feeding on grass) and, when possible, the number of droppings produced (mean ± SE number of scans per bird = 9 ± 2, of which during 5 ± 2 scans the bird was near enough to enable dropping scans). At constant bite and dropping mass, the bite/dropping ratio is a measure of a bird's digestive performance (high at a high ratio). At the end of each scan we estimated the bird's abdominal profile index (API). This is a measure frequently used in waterfowl ecology to estimate a bird's abdominal fat storage[138,139,140] (on a scale of 1-6, in steps of ¼ unit). The change in API over time can be used to estimate a bird's rate of body store accumulation[140]; here we used the first and last API-estimate for this purpose (mean ± SE of time interval between first and last score per bird = 31 ± 6 days). All observations were performed by DL and RR, who were unaware of the identity of the infected birds, and therefore all measurements can be considered as blind with respect to infection-status. The design of the measurements was such that each observer observed all six birds, with no difference between observers in the number of observations per bird ($F_{1,10}$ = 1.61, p > 0.2; GLM). Moreover, observations were equally spread in time for infected (range = 22 Dec-21 Feb; mean ± SE = 27 Jan ± 3.4 days) and uninfected birds (29 Dec-22 Feb; 24 Jan ± 2.5 days).

Statistics
Generalized Linear Models were used for statistical comparison of infected and uninfected birds, with age and body size as covariates (using the GLM package in SYSTAT 10[141]). The number of birds varied between comparisons; 25 swans were caught for analysis of biometric data at the start of the study (3 infected, 22 uninfected), 12 of these were GPS collar-banded (2 infected, 10 uninfected), and 6 of these were regularly observed to perform focal scans (2 infected, 4 uninfected).

Specimens
Cloacal swabs were collected using sterile cotton swabs (Greiner, The Netherlands), stored in transport media (Hanks balanced salt solution containing 0.5% Lactalbumin, 10% glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulfate, 250 µg/ml gentamycin (ICN, The Netherlands)), and shipped to the laboratory where they were stored at –70ºC. Blood samples were obtained by using manual restraint from the ulnar vein, centrifugation for 10 minutes at 14,000 rpm and separation of serum.

RNA isolation and virus detection
RNA isolation and RT-PCR was performed as described previously[73]. In short, RNA was isolated using a MagnaPure LC system with the MagnaPure LC Total nucleic acid isolation kit (Roche Diagnostics, Almere, The Netherlands) and influenza A virus was detected using a real-time RT-PCR assay. Amplification and detection was performed on an ABI7700 with the TaqMan EZ RT-PCR Core Reagents kit (Applied

Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The samples were prepared and processed in parallel with several negative and positive control samples in each run.

Virus isolation and characterization
For influenza A virus RT-PCR positive samples, 200 µl of the original material was inoculated into the allantoic cavity of 11-day-old embryonated hens' eggs. The allantoic fluid was harvested two days after inoculation and influenza A virus was detected using hemagglutination assays with turkey erythrocytes. When no influenza A virus was detected upon the initial virus isolation attempt, the allantoic fluid was passaged once more in embryonated chicken eggs. Virus isolates were characterized using the hemagglutination inhibition (HI) assay with turkey erythrocytes and subtype-specific hyperimmune rabbit antisera raised against all HA subtypes[6].

Sequence analysis and phylogeny
All eight gene segments of the influenza A virus isolates were characterized by RT-PCR and sequencing, essentially as described by others[81]. PCR products were purified from agarose gels using the Qiaquick Gel Extraction kit (Qiagen, Leusden, The Netherlands) and sequenced using the Big Dye terminator sequencing kit version 3.0 (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) and an ABI PRISM 3100 genetic analyzer (Applied BioSystems, Nieuwerkerk a/d IJssel, The Netherlands), according to the instructions of the manufacturer. All primer sequences are available upon request. Trees were generated using full-length HA nucleotide sequences, including sequences from public databases. Sequences were aligned using the Clustal W program running within the BioEdit software package, version 5.0.9[107]. Alignments were bootstrapped 100 times using the Seqboot package of Phylip version 3.6 and trees were constructed with the Dnaml package, using 3 jumbles. The consensus tree was calculated using the Consense package of Phylip 3.6 and this tree was used as usertree in Dnaml to recalculate the branchlengths from the nucleotide sequences. Trees were visualized with the Treeview 1.6.6 program distributed with Bioedit version 5.0.9. All nucleotide sequences presented in this manuscript are available from GenBank under accession numbers DQ822187 - DQ822202.

Serology
Swan sera were analysed using a commercially available influenza A virus antibody ELISA kit (European Veterinary Laboratory, Woerden, The Netherlands) as previously described[142]. For the detection of specific anti-NP antibodies, we used horseradish peroxidase-conjugated goat anti-wild bird immunoglobulin (Ig) G (Bethyl Laboratories, Inc., TX, USA) instead of the supplied anti-bird conjugate.

Results

Influenza A virus isolates of subtypes H6N2 and H6N8 were obtained from two out of the three infected birds (the GPS-collared ones). Phylogenetic analyses based on full-length H6 nucleotide sequences and sequences available from public databases revealed that the two swan H6 gene segments were closely related to sequences obtained from an influenza A virus found in a free-ranging migrating duck in NW Europe (Fig. 2). BLAST analyses of the 7 other gene segments of the two swan viruses, indicated a 95 to 99 percent nucleotide sequence identity with avian influenza A viruses of Eurasian origin (data not shown; the split-up of the Eurasian Poultry Lineage into two groups is based on genetic distance, relatedness with the ancestor and putative date of isolation). The phylogenetic and BLAST analyses indicate that the two influenza A viruses obtained from the infected swans are ordinary LPAI viruses circulating in the natural wild bird reservoir. Serological analyses revealed that 5 out of 25 swans had detectable antibodies against the nucleoprotein (NP) of influenza A viruses. The highest antibody response was found in the two swans from which influenza A viruses were isolated.

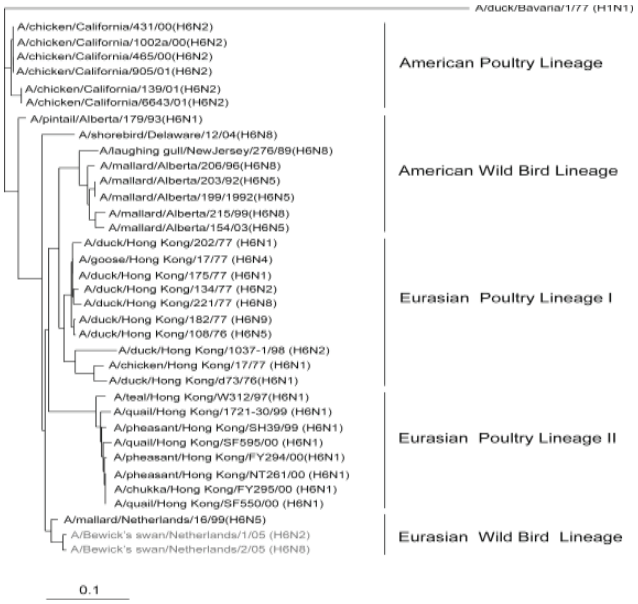


Figure 2. Phylogenetic tree for H6 influenza viruses isolated from swans. The DNA maximum liklihood tree was constructed using A/duck/Bavaria/1/77 as outgroup and includes sequences from public databases. The scalebar represents ~10% of nucleotide changes between close relatives.

Not knowing the infection status of the swans during processing immediately after capture, two of the three influenza A virus infected swans were fitted with a GPS collar. The 25 infected and uninfected swans did not differ in structural body size ($F_{1,21}$ = 0.62, p > 0.4; GLM with age ($F_{2,21}$ = 0.10, p > 0.9) as covariate; body size was calculated as the first principal component (PC1) from a PCA including wing length and total head size, a method often used in avian studies[143], e.g. see[145] for applications in swans), nor in body mass right after catch ($F_{1,20}$ = 0.17, p > 0.6;

GLM with age ($F_{2,20}$ = 1.60, p > 0.2) and body size ($F_{1,20}$ = 23.03, p < 0.0005) as covariates). This result was maintained when restricting the analyses to the 12 GPS-collared birds ($F_{1,8}$ = 0.81, p > 0.3 for body size; $F_{1,7}$ = 0.80, p > 0.4 for size-corrected body mass).

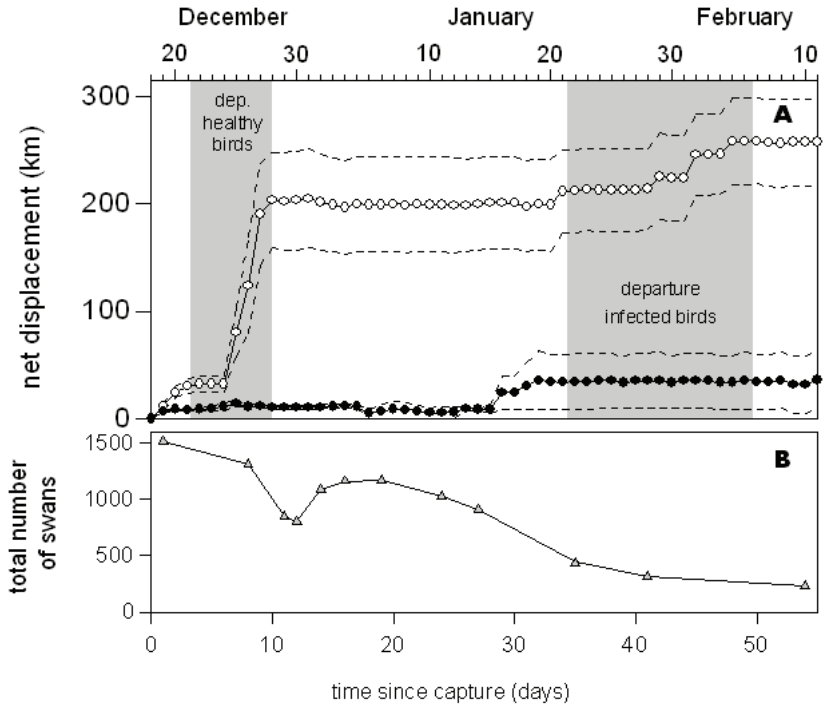


Figure 3. Timing of migration in healthy and infected swans. A) Infected swans left the study area more than a month after the uninfected birds, indicated by broad vertical grey bars ranging from mean-SE to mean+SE. Moreover, their mean net displacement after departure (losed dots ± dashed SE-lines) was shorter than for the uninfected birds (open dots ± dashed SE-lines). Sample sizes of displacements estimates n = 2 for infected birds (whole time span); sample size declined for uninfected birds due to birds that flew out of range: n = 10 (days 0-41), n = 9 (days 42-47), and n = 8 (days 48-55). B) Total number of swans in our study area declined more or less gradually over time (W. Tijssen et al. unplubished data), suggesting a continuous departure of about 30 birds per day (apart from the short dip around day 12 during a snowy cold spell).

While the biometric parameters of the GPS-collared birds did not differ at the time of capture, infected and uninfected birds differed in all ecological parameters collected after release. Date of departure from Wieringermeer, the agricultural area in NW Netherlands where the birds were caught, differed by more than a month, with the infected birds leaving the area later than the uninfected birds (Fig. 3A; $F_{1,7}$ = 10.53, p < 0.05; GLM with age ($F_{2,7}$ = 0.29, p > 0.7) and body size ($F_{1,7}$ = 0.61, p > 0.4) as covariates). Compared to the phenology of the population as a whole (Fig. 3B), the healthy birds were among the first to leave the study area, while the infected birds were among the last. Furthermore, the next (wintering/spring stopover) site visited was nearer by Wieringermeer for infected individuals than for uninfected individuals (Fig. 3A; $F_{1,6}$ = 11.03, p < 0.05; GLM with age ($F_{2,6}$ = 0.43, p > 0.6) and body size ($F_{1,6}$

= 0.58, $p > 0.4$) as covariates; note that we have one degree of freedom less since one bird was never seen after it left the study site). In spite of these differences in distance flown, a possible trigger to move on to the next site is the absolute amount of accumulated body stores[136]. Indeed, rate of fuelling, expressed as the rate of change in the visually scored abdominal-profile index (API)[138,139,140] , was lower in infected birds than in uninfected birds (Fig. 4A; $F_{1,2} = 22.96$, $p < 0.05$; GLM with age ($F_{1,2} = 4.95$, $p > 0.1$) and body size ($F_{1,2} = 0.42$, $p > 0.5$) as covariates), whereas APIs upon departure did not differ ($F_{1,2} = 1.90$, $p > 0.3$; GLM with age ($F_{1,2} = 0.15$, $p > 0.7$) and body size ($F_{1,2} = 2.09$, $p > 0.2$) as covariates). The observations on fuelling rates are consistent with the lower feeding or bite rates observed in the infected birds compared with the uninfected animals (Fig. 4B; $F_{1,2} = 47.55$, $p < 0.05$; GLM with age ($F_{1,2} = 0.94$, $p > 0.4$) and body size ($F_{1,2} = 23.34$, $p < 0.05$) as covariates). Possibly, infected swans cannot achieve high bite rates because they have impaired digestive functions as compared to their healthy counterparts (bite rates in herbivorous waterfowl are generally considered to be digestion- rather than encounter-limited[148]). This is supported by the finding that infected swans took fewer bites per produced faecal dropping than uninfected swans (Fig. 4C; $F_{1,2} = 19.28$, $p < 0.05$; GLM with age ($F_{1,2} = 19.19$, $p < 0.05$) and body size ($F_{1,2} = 29.35$, $p < 0.05$) as covariates).

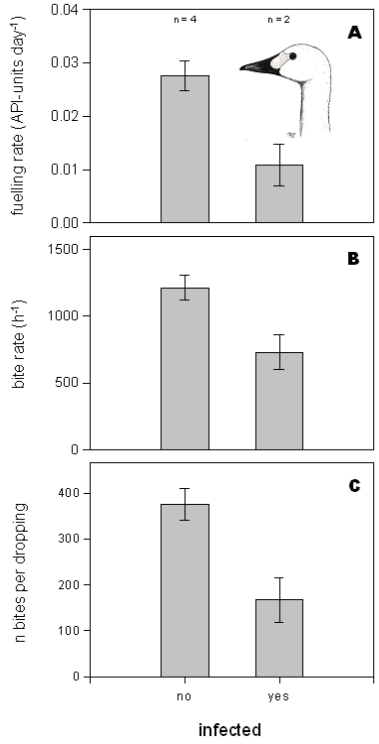


Figure 4. Feeding parameters as a function of health status. Compared to healthy birds, infected birds (A) fuelled slower, (B) took fewer bites per hour, and (C) took fewer bites per produced dropping. Because of a seasonal increase observed in bite rate ($p < 0.05$ when all observations pooled), bite rate plotted in (B) were standardized to 1 February. In all graphs, bars give least-square means, error bars are SE.

Discussion

Influenza A virus infected Bewick's swans thus showed reduced feeding and migratory performances. Fuelling in avian migrants is thought to be enhanced by the flexible enlargement of digestive organs[149], and indeed fuelling Bewick's swans increase the length of their intestine by 50% throughout winter[150]. In the infected birds the ability to make adjustments to the digestive system may have been impaired (as revealed by the lower bite-to-dropping ratio), leading to reduced bite rates, reduced fuel storage rates and a delayed migration schedule. That the infected birds ended up in a negative feedback loop *because* they traded off resources allocated towards "fighting the infection" with resources allocated towards fuelling and developing the digestive machinery can of course only be firmly established by experimental manipulation of infections in otherwise similar wild birds (which would ethically be impossible). An obvious alternative explanation is that the infected swans were poor foragers with such reduced energy allocation towards "infection prevention" that they could not *prohibit the control* of the infection. However, the fact that at capture there was no difference in body condition between infected birds and uninfected birds makes the former point of view more likely. Presumably the two swans were infected shortly before capture, which resulted in reduced migratory and foraging performance. As migratory (re)fuelling in Bewick's swans takes place from late October onwards[151], the "poor-forager idea" would predict the infected birds to have had lower body conditions already at the time of capture (mid December), which was not the case. Whatever cause and effect may be in this matter, the epidemiology of influenza A viruses is clearly affected by the fact that infected birds are migrating slower.

Tentatively accepting an LPAI virus infection to be the cause rather than the consequence, it is tempting to speculate about the knock-on effects of an infection throughout the rest of the annual cycle. Even if infected birds eventually overcome their disease during further migration, a delayed departure from one stopover site is likely to lead to delayed departures from all sites en route (strengthened by the fact that food stocks are largely depleted by the first birds using a stopover site[152]), thereby eventually delaying arrival on their arctic breeding grounds. If so, reproductive output will be reduced as the best territories may have been occupied[153] by the time infected birds arrive and eventual broods may hatch too late to take full advantage of the food peak[154]. If, on the other hand, they did manage to reach the breeding grounds in time, reproduction may still be affected because of the trade-off between reproductive effort and immunocompetence[76]. In case also survival is affected, migration will likely act as a filter, eliminating infected individuals along the way due to the heavy demands of migration. If so, this will temper the rate of spread of AI.

In domestic birds, the clinical signs and disease observed following infection with LPAI viruses vary with host species, age, the presence of other micro-organisms and environmental factors. In these hosts, LPAI viruses can cause a mild disease consisting primarily of respiratory symptoms, depression and egg production problems in laying birds[7]. Upon experimental infection of ducks, LPAI viruses replicate in the epithelial cells of the intestine of birds and virus may be shed in high concentrations in the faeces, without inducing apparent signs of disease[19]. It is generally believed that LPAI viruses are also non-pathogenic upon natural infection of

wild birds, although data is scarce[1]. Influenza A viruses of the H6 subtype are among the most commonly isolated viruses in wild ducks in North America and Europe and have been isolated frequently from other wild birds, including geese, gulls, waders, and auks[63]. Our observations that infection of swans with such “ordinary” LPAI H6 viruses can result in altered migratory and feeding patterns may indicate that LPAI virus infections in wild birds have a higher clinical, epidemiological, and ecological impact than previously recognised. Based on these observations, the implications of influenza virus infections, including those caused by HPAI H5N1 viruses, for bird health and ecology and virus epidemiology may require re-evaluation.

Acknowledgments
We thank T. Haitjema, M. Klaij and K. Oosterbeek for catching the birds and Family Verheijen for kindly hosting us on their farm and allowing us to catch ‘their’ swans. W. Tijsen and his large network of volunteer ring-readers located the GPS-collared birds and counted the numbers. GPS-collars were manufactured by T. Gerrits and K. van ’t Hoff (Microtes Wildlife Engineering), who continued to support us during occasional technical hiccups in the field. T. de Boer, O. Duriez and P. de Vries gave helping hands during field observations. We thank C. Baas for excellent technical assistance. Z. Barta, T. Piersma, E. Rees and J. Waldenström provided constructive comments on draft manuscripts.

7

Mallards and highly pathogenic avian influenza ancestral viruses, Northern Europe

Emerging Infectious Diseases, 2006, 11: 1545-1551

Vincent Munster
Anders Wallensten
Chantal Baas
Guus Rimmelzwaan
Martin Schutten
Björn Olsen
Albert Osterhaus
Ron Fouchier

Outbreaks of highly pathogenic avian influenza (HPAI), which originate in poultry upon transmission of low pathogenic viruses of wild birds, have occurred relatively frequently in the last decade. During our ongoing surveillance studies in wild birds, we have isolated several influenza A viruses of hemagglutinin subtype H5 and H7, that contain various neuraminidase subtypes. For each of the recorded H5 and H7 HPAI outbreaks in Europesince 1997, our collection contained closely related virus isolates recovered from wild birds, as determined by sequencing and phylogenetic analyses of the hemagglutinin gene and antigenic characterization of the hemagglutinin glycoprotein. The minor genetic and antigenic diversity between the viruses recovered from wild birds and those causing HPAI outbreaks indicates that influenza A virus surveillance studies in wild birds can help generate prototypic vaccine candidates and design and evaluate diagnostic tests, before outbreaks occur in animals and humans.

Introduction

Wild birds, predominantly ducks, geese and shorebirds, form the reservoir of influenza A viruses in nature[1,99]. Influenza A viruses are subtyped on the basis of the antigenic properties of the hemagglutinin (HA) and neuraminidase (NA) glycoproteins, expressed on the surface of virus particles. To date, 16 HA and 9 NA subtypes have been detected in wild birds and poultry throughout the world[100,6]. Viruses containing HA of subtypes H5 and H7 may become highly pathogenic after introduction in poultry and cause outbreaks of highly pathogenic avian influenza (HPAI, formerly termed fowl plague)[1,99]. The switch from a low pathogenic avian influenza (LPAI) phenotype, common in wild birds and poultry, to the HPAI phenotype is achieved by the introduction of basic amino acid residues into the HA_0 cleavage site. HPAI isolates have been obtained primarily from commercially raised birds such as chickens, turkeys, quail, guinea fowl and ostriches[7]. In the last decade, the frequency of detected HPAI outbreaks has increased, with outbreaks of avian influenza A viruses of subtype H5N2 in Mexico (1994); Italy (1997) and Texas (2004); H5N1 in Hong Kong (1997) and Southeast Asia (ongoing since 1997); H7N3 in Australia and Pakistan (1994); H7N4 in Australia (1997); H7N1 in Italy (1999); H7N3 in Chile (2002) and Canada (2003); and H7N7 in The Netherlands (2003) [43,55,156-162]. Influenza A viruses of subtypes H5 and H7 have been detected in mammals frequently[163]. H7N7 viruses were endemic in horses for a long time[164], were transmitted from seals to humans in the USA in 1980[48,165], and were isolated from humans in the UK in 1996[166] and The Netherlands in 2003[55,161]. H7N2 and H7N3 influenza A viruses were isolated from humans in the USA in 2003[167,168] and Canada in 2004[167,169,170] respectively. HPAI H5N1 viruses circulating in Southeast Asia since 2003 have been detected in at least 108 human cases of respiratory illness, of which 54 were fatal[170]. In addition, these H5N1 influenza A viruses have been detected in pigs[171], cats, leopards and tigers[68,172-174] in Southeast Asia. As a consequence of the relatively frequent zoonoses caused by influenza A viruses of subtypes H5 and H7, these virus subtypes are given high priority with respect to pandemic preparedness.

Wild birds harbor the low pathogenic avian influenza (LPAI) ancestral viruses of

HPAI strains of poultry (and mammals). In influenza A virus surveillance studies in wild birds in Northern Europe, we have detected numerous influenza A viruses of subtype H5 and H7 in Mallards (*Anas platyrhynchos*). We show that for each of the HPAI outbreaks that occurred in Europe in the last decade, we have found close LPAI relatives in Mallard ducks. Our observations indicate that Influenza A virus surveillance in wild birds provides opportunities for pandemic preparation; the prototype influenza A viruses obtained from wild birds may guide production of vaccines as well as reagents for the development and validation of diagnostic tests.

Materials and Methods

Specimens

In our ongoing influenza A virus surveillance studies in wild birds in Northern Europe[63], Mallards were trapped using duck traps in Lekkerkerk and Krimpen aan de Lek in The Netherlands and Ottenby Bird Observatory on the southernmost point of the island Öland in Sweden (Fig. 1). Cloacal samples were collected using cotton swabs (Greiner, The Netherlands) and stored in transport media consisting of Hanks' balanced salt solution, 10% v/v glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulfate, 250 µg/ml gentamycin (MP Biomedicals, Zoetermeer, The Netherlands) at -70ºC.

RNA isolation and virus detection

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously for samples obtained until 2002[80]. From 2003 onwards, RNA was isolated using a MagnaPure LC system with the MagnaPure LC Total Nucleic Acid Isolation kit (Roche Diagnostics, Almere, The Netherlands) and influenza A virus was detected using a real-time RT-PCR assay[70,71]. To ensure efficient influenza A virus detection, the published probe sequence was changed to 6-FAM-TTT-GTG-TTC-ACG-CTC-ACC-GTG-CC-TAMRA-3', based on the avian influenza A virus sequences available from public databases. Amplification and detection was performed on an ABI7700 machine with the TaqMan EZ RT-PCR Core Reagents kit (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) using 20 µl of eluate in an end volume of 50 µl. Pools of five individual samples were prepared and processed in parallel with several negative and positive control samples in each run. Upon identification of influenza A virus positive pools, the RNA isolation and RT-PCR procedures were repeated for the individual samples within each positive pool and individual RT-PCR positive samples were subsequently used for virus isolation.

Virus isolation and characterization

For influenza A virus RT-PCR positive samples, 200 µl of the original material was inoculated into the allantoic cavity of 11-day-old embryonated hens' eggs. The allantoic fluid was harvested two days after inoculation and influenza A virus was detected using hemagglutination assays with turkey erythrocytes. When no influenza A virus was detected upon the initial virus isolation attempt, the allantoic fluid was passaged once more in embryonated hens' eggs. Virus isolates were characterized using a hemagglutination inhibition (HI) assay with turkey erythrocytes and subtype-

specific hyperimmune rabbit antisera raised against all HA subtypes[155].

Sequence analysis and phylogenetic trees

NA subtypes of influenza A virus isolates were characterized by RT-PCR and sequencing. RT-PCR and sequencing of the HA and NA genes were performed essentially as described by others[81]. PCR products were purified from agarose gels using the Qiaquick Gel Extraction kit (Qiagen, Leusden, The Netherlands) and sequenced using the Big Dye terminator sequencing kit version 3.0 (Amersham Pharmacia Biotech, Roosendaal, the Netherlands) and an ABI PRISM 3100 genetic analyzer (Applied BioSystems, Nieuwerkerk aan den IJssel, The Netherlands), according to the instructions of the manufacturer. All primer sequences are available upon request. Nucleotide and amino acid sequences were aligned using the Clustal W program running within the Bioedit software package, version 5.0.9[107]. We first generated trees for H5 and H7 using all full-length HA_1 amino acid sequences available from public databases. Amino acid sequence alignments were bootstrapped 100 times and distance matrices were generated using Kimura parameters. The trees were generated using the UPGMA (unweighted pair-group method with arithmetic mean) clustering method of the Neighbor program of Phylip version 3.6. The consensus of 100 UPGMA trees was calculated, and the branch length of this consensus tree was recalculated using the Fitch program of Phylip 3.6[108].

For selected influenza A virus isolates of European origin, DNA maximum likelihood trees were generated using full-length HA nucleotide sequences from which the sequences encoding the HA cleavage site were excluded. Alignments were bootstrapped 100 times using the Seqboot package of Phylip version 3.6 and trees were constructed with the Dnaml package, using 3 jumbles. The consensus tree was calculated using the Consense package of Phylip 3.6 ; this tree was used as usertree in Dnaml to recalculate the branch lengths from the nucleotide sequences. Finally, the trees were rerooted at midpoint using the Retree software of Phylip 3.6. Trees were visualized with the Treeview 1.6.6 program distributed with Bioedit version 5.0.9. All nucleotide sequences presented in this manuscript are available from GenBank under accession numbers AY684894, AY338460, AY995883-AY995898 and AY999977-AY999991.

Serology

HI assays were performed to compare of the antigenic properties of influenza A virus strains by using post-infection ferret antisera and hyperimmune rabbit antiserum generated against the following influenza viruses: A/Tern/South Africa/61 (H5N3), A/Duck/Hong Kong/205/77 (H5N3), and A/Hong Kong/156/97 (H5N1), A/Equine/Prague/1/54 (H7N7), A/Seal/Massachusetts/1/80 (H7N7), A/Mallard/Netherlands/12/00 (H7N3), A/Netherlands/033/03 (H7N7) and A/Netherlands/219/03 (H7N7), as described previously[6,69]. HI assays were performed in duplicate. All serum samples were treated overnight with receptor-destroying enzyme at 37˚C and subsequently incubated at 56˚C for one hour. Twofold serial dilutions of each antiserum, starting at a 1:20 dilution were tested for their ability to inhibit the agglutination of horse red blood cells by 4 hemagglutinating units of influenza A virus. Serum dilutions were made in PBS containing 0.5% vol/vol bovine serum albumine (BSA, fraction V, Gibco, Breda, The Netherlands). Horse erythrocytes were stored

in PBS containing 0.5% vol/vol BSA. In the HI-assay, 50 µl of a 1% vol/vol horse red blood cell dilution was added to each well.

Results

Avian influenza A virus in wild birds in Europe

Of 172 virus isolates obtained within this study period, 33 contained HA genes of subtypes H5 or H7; 6 were of subtype H5N2, 2 were H5N3, 1 was H5N6, 8 were H5N9, 1 was H7N3, 14 were H7N7 and 1 was H7N9. All H5 and H7 influenza A viruses were isolated from samples collected from Mallards during fall migration at marshalling sites in The Netherlands (1 H5 isolate from October 1999, and 1 H7 isolate from December 2000) and Sweden (all other H5 and H7 isolates collected between September and January 2002) (Fig. 1).

Figure 1. Main fall migration route of wild waterfowl in Northern Europe[11,155]. The sample locations Öland (Sweden) and Lekkerkerk and Krimpen a/d Lek (The Netherlands) are marked with asterisks.

Characterization of H7 influenza A viruses

Sequence analyses of the HA open reading frames (ORFs) of the 16 H7 influenza A viruses isolated from Mallards revealed that the HA_0 cleavage site lacked basic amino acid (aa) residues, which is typical for LPAI viruses. We next determined the genetic relationship between the HA genes of our H7 influenza A viruses isolated from European Mallards and those available from public sequence databases. The phylogenetic tree, based on HA_1 aa sequences, showed the typical separation of H7 strains in the Eurasian and American genetic lineages. Within the Eurasian H7

HA lineage, the European Mallard influenza A viruses were found in different parts of the tree, clustering closely with influenza A viruses responsible for recent H7 HPAI outbreaks in Europe (Fig. 2A). We next generated a DNA maximum likelihood phylogenetic tree using prototypic European Mallard influenza A viruses and strains representing each of the H7 HPAI outbreaks that occurred in Europe (H7N1 in Italy 2000/2001 and H7N7 in the Netherlands 2003) in the last decade (Fig. 2B). This tree revealed the cocirculation of two genetically distinct lineages of H7 HA in European Mallards; one closely related to H7N7 and H7N1 HPAI strains causing outbreaks in The Netherlands (2003) and Italy (2000/2001), and one closely related to the H7N7 isolate obtained from a woman suffering from conjunctivitis in the UK in 1996[166]. The maximum nucleotide/amino acid (nt/aa) sequence identity between the Italian H7N1 HPAI virus A/Chicken/Italy/445/99 and the most closely related LPAI virus A/Mallard/Netherlands/12/00 was 98% nt and 98% aa respectively. The maximum nt/aa identity between the Dutch H7N7 HPAI virus A/Chicken/Netherlands/1/03 and the most closely related LPAI virus A/Mallard/Netherlands/12/00 is 98% nt and 99% aa respectively. The maximum nt/aa identity between influenza virus A/Turkey/Ireland/PV74/95 (H7N7), influenza virus A/England/268/96 (H7N7) and the most closely related LPAI virus A/Mallard/Sweden/56/02 was 95% nt, 96 % aa and 96% nt, 97% aa respectively.

Figure 2. Phylogenetic tree of hemagglutinin H7 sequences. A) Phylogenetic tree based on the amino acid sequence distance matrix for the HA_1 open reading frames of all H7 sequences available from public databases. The scale bar represents ~10% of amino acid changes between close relatives. *Represents the locations of the Mallard influenza A virus isolates. B) DNA maximum liklihood tree for the European highly pathogenic avian influenza viruses and the low pathogenic avian H7 influenza viruses isolated from migrating Mallards by using A/FPV/Dutch/27 as outgroup. The scale bar represents 10% of nucleotide changes between close relatives.

We next analyzed the antigenic relatedness of the H7 influenza A viruses obtained from wild Mallards in HI assays with postinfection ferret antisera and hyperimmune rabbit antisera. The hyperimmune rabbit antisera were chosen on the basis of their ability to provide a broad response, which would recognize a wide range of strains within 1 subtype, whereas the post-infection ferret antisera were chosen on the basis of high specificity. HI assays showed that the antigenic properties of the H7 influenza A viruses from Mallards were relatively conserved, and that the HI data for the Mallard influenza A viruses did not differ significantly (i.e., up to 4-fold) from those obtained with strains causing the HPAI outbreak in The Netherlands in 2003 (Table 1). The antigenic analyses therefore confirmed the genetic data, which showed little genetic diversity between the H7 strains isolated from wild Mallards and the strains causing the H7 HPAI outbreaks.

Table 1. Hemagglutination inhibition assays with postinfection ferret antisera and hyperimmune rabbit antisera raised against H7 influenza A viruses.

Virus	A/Eq/Prague/ 1/54[a]	A/Seal/ Mass/1/80[a]	A/NL/219/ 03[b]	A/Mallard/ NL/12/00[b]	A/NL/33/ 03[b]
A/Equine/Prague/1/54 H7N7	**1,280**[c]	1,280	20	20	20
A/Seal/Massachusetts/1/80 H7N7	160	**1,280**	20	20	20
A/Netherlands/219/03 H7N7	160	1,280	**40**	40	80
A/Mallard/Netherlands/12/00 H7N3	160	1,280	20	**80**	40
A/Netherlands/33/03 H7N7	320	1,280	80	160	**160**
A/Mallard/Sweden/56/02 H7N7	640	5,120	80	80	160
A/Mallard/Sweden/105/02 H7N7	320	2,560	80	80	80
A/Mallard/Sweden/85/02 H7N7	160	1,280	40	80	80

[a] Hyperimmune rabbir antisera
[b] Postinfection ferret antisera
[c] Homologous titers are represented boldface

H5 sequence analysis, phylogeny, and antigenic characterization

Sequence analyses of the HA ORFs of the 17 H5 influenza A viruses isolated from Mallards showed that the HA_0 cleavage site lacked basic amino acid residues, which is typical for LPAI viruses. To determine the genetic relationship between the H5 influenza A virus isolates obtained from wild birds and strains causing recent H5 HPAI outbreaks (H5N2 in Italy 1997), we generated a phylogenetic tree based on the aa sequences of the HA_1 domain of all H5 influenza A viruses currently available from public sequence databases (Figure 3A). As for H7, this tree showed the two clearly distinguishable Eurasian and American genetic lineages. The H5 HA sequences that we obtained from influenza A viruses isolated from European Mallards were closely related to the influenza A virus strains responsible for the H5 HPAI outbreak in Italy in 1997. The H5 HPAI influenza A strains isolated in Southeast Asia beginning in 1997 form a continuous genetic lineage, presumably evolving from a common LPAI wild bird ancestor around 1997[43]. Similarly, we did not detect close relatives of the recent HPAI Asian strains in Mallards in Europe (Figure 3A). The DNA maximum likelihood tree based on the full-length HA nucleotide sequences of the 17 H5 HA genes of Mallard influenza A viruses and those of the Italian H5 HPAI influenza A

viruses confirmed the close genetic relationship (Figure 3B). Maximum nucleotide/ amino acid identity between the Italian HPAI virus A/Chicken/Italy/312/97 H5N2 and the most closely related LPAI virus A/Netherlands/3/99 H5N2 is respectively 96% nt identity and 98% aa identity (Fig. 3B).

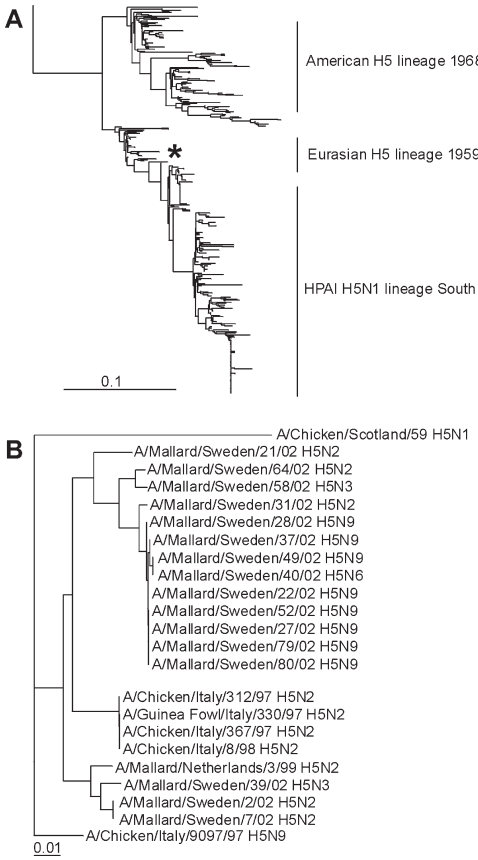


Figure 3. Phylogenetic tree of hemagglutinin H5 sequences. A) Phylogenetic tree based on the amino acid sequence distance matrix for the HA_1 open reading frames of all H5 sequences available from public databases. The scale bar represents ~10% of amino acid changes between close relatives. *Represents location of the H5 influenza A virus isolates isolated from Mallards. B) DNA maximum liklihood tree for the cluster of European H5 highly pathogenic avian influenza viruses and the low pathogenic avian H5 influenza viruses isolated from migrating Mallards A/chicken/Scotland/59 as outgroup. The scale bar represents 1% of nucleotide changes between close relatives.

In HI assays with post-infection ferret antisera and hyperimmune rabbit antisera raised against H5 influenza A viruses, the influenza A viruses obtained from wild Mallards were antigenically conserved, and did not differ significantly (up to 4-fold) from the prototypic strains used in the HI assay (Table 2). In agreement with the phylogenetic analysis of the current H5N1 HPAI influenza a viruses from Southeast Asia, the antigenic properties of H5N1 influenza virus A/Vietnam/1194/04 differ significantly from those of the LPAI strains isolated from Mallards used in this study when analysed with the highly specific post-infection ferret antisera. Hyperimmune rabbit antisera failed to discriminate the antigenic properties of all strains, due to the broader antigenic reactivity of these sera.

Table 2. Hemagglutination inhibition assays with postinfection ferret antisera and hyperimmune rabbit antisera raised against H5 influenza A viruses.

Virus	A/Tern/ SA/61[a]	A/Tern/ SA/61[b]	A/Dk/HK/ 205/77[a]	A/Dk/HK/ 205/97[b]	A/HK/ 156/97[a]	A/HK/ 156/97[b]
A/Tern/South Africa/61 H5N3	**640**[c]	**320**	80	640	80	20
A/Duck/Hong Kong/205/77 H5N3	1,280	640	**240**	**1,280**	160	80
A/Hong Kong/156/97 H5N1	1,280	640	320	1,280	**640**	**320**
A/Vietnam 1194/04 H5N1	1,280	40	640	80	640	<20
A/Mallard/Sweden/21/02 H5N2	640	320	160	640	160	20
A/Mallard/Sweden/49/02 H5N9	320	320	40	320	160	40
A/Mallard/Netherlands/3/99 H5N2	640	1,280	160	5,120	160	80
A/Mallard/Sweden/7/02 H5N2	1,280	640	320	1,280	320	40

[a] Hyperimmune rabbir antisera
[b] Postinfection ferret antisera
[c] Homologous titers are represented boldface

Discussion

Because HPAI outbreaks in poultry find their origin in the LPAI viruses present in waterfowl, influenza A virus surveillance in wild birds could function as an early warning system for HPAI outbreaks, and as a means to keep panels of reference reagents, required for diagnostic purposes and vaccine production, up-to-date. Wild bird surveillance would also be relevant for the HPAI viruses that represent pandemic threats. However, limited information on the prevalence of avian influenza A viruses in wild birds in Europe, and on the genetic and antigenic variability of the viruses in this part of the world, has made assessing the value of such surveillance studies difficult. We isolated influenza A viruses of subtypes H5 and H7 from Mallards in Northern Europe. During a 4-year surveillance period, we isolated influenza A viruses of subtypes H5N2, H5N3, H5N6, H5N9, H7N3, H7N7 and H7N9, among many other influenza A virus isolates. All of these H5 and H7 influenza A virus isolates were obtained from Mallards during fall migration at a Swedish location and at two Dutch wintering sites. Using this relatively limited setting, we have isolated influenza A viruses which possess H5 and H7 glycoproteins and gene segments that are closely related to those of influenza A viruses responsible for HPAI outbreaks in Europe, H5N2 in Italy (1997), H7N1 in Italy (1999-2000) and H7N7 in The Netherlands (2003). Thus, we conclude that influenza A virus surveillance in wild birds is indeed very useful to keep the panels of reference reagents up-to-date. Whether surveillance studies could be useful as sentinel system.

We observed only minor antigenic and genetic diversity between the HA genes of Mallard influenza A virus isolates and those of HPAI virus strains. This implies that the influenza A virus isolates obtained during wild bird surveillance studies may also be prototypic vaccine candidates for human or veterinary use. Limited numbers of prototype vaccine strains, representing both the American and Eurasian genetic lineages of influenza A virus, could be generated to cover a wide range

of HPAI strains. Such vaccine seed strains can be produced well ahead of times of outbreaks in poultry, other animals, or humans. The disadvantage of the minor antigenic differences between the vaccine strain and the epidemic strains will likely be compensated by the immediate availability of the vaccine. An additional advantage of the use of LPAI strains from wild birds as prototype vaccine strains is that they do not contain a basic cleavage site in the HA gene. Before HPAI strains can be used as vaccine candidates, the basic amino acid residues in the HA gene need to be removed using reverse genetics technology, this would result in an extra modification step, which would consuming precious time. Moreover, these vaccine strains can only be generated after an outbreak of HPAI has started.

We thus suggest that a thorough genetic and antigenic characterization of avian influenza A viruses isolated in the Americas, Asia and Europe would be useful to prepare for outbreaks. While this usefulness has been demonstrated in our study with influenza A viruses of the H5 and H7 subtypes, it should be applied also to other influenza A virus strains relevant to animal and public health, in particular of subtypes H1, H2, H3 and H9.

Acknowledgments

We thank the ornithologists for collecting bird samples, in particular Hans Zantinge and Bert Pellegrom, we thank Martin Stervander, Judith Guldemeester and Theo Bestebroer for excellent technical assistance and Jan de Jong for critically reading the manuscript. This is contribution no. 205 from Ottenby Bird Observatory.

8

Protection of mice against lethal infection with highly pathogenic H7N7 influenza A virus by using a recombinant low-pathogenicity vaccine strain

Journal of Virology, 2005, 79: 12401-12407

Emmie de Wit*
Vincent Munster*
Monique Spronken
Theo Bestebroer
Chantal Baas
Walter Beyer
Guus Rimmelzwaan
Albert Osterhaus
Ron Fouchier

* These authors contributed equally to the results of this study

In 2003, an outbreak of highly pathogenic avian influenza occurred in The Netherlands. The avian H7N7 virus causing the outbreak was also detected in 88 humans suffering from conjunctivitis or mild respiratory symptoms and one person who died of pneumonia and acute respiratory distress syndrome. Here, we describe a mouse model for lethal infection with A/Netherlands/219/03 isolated from the fatal case. Because of the zoonotic and pathogenic potential of the H7N7 virus, a candidate vaccine carrying the avian hemagglutinin and neuraminidase proteins produced in the context of the high-throughput vaccine strain A/PR/8/34 was generated by reverse genetics and tested in the mouse model. The hemagglutinin gene of the recombinant vaccine strain was derived from a low-pathogenicity virus obtained prior to the outbreak from a wild mallard. The efficacy of a classical non-adjuvanted subunit vaccine and an immune stimulatory complex-adjuvanted vaccine were compared. Mice receiving the nonadjuvanted vaccine revealed low antibody titers, lack of clinical protection, high virus titers in the lungs, and presence of virus in the spleen, liver, kidneys and brain. In contrast, mice receiving two doses of the immune stimulatory complex-adjuvanted vaccine revealed high antibody titers, clinical protection, ~1,000-fold reduction of virus titers in the lungs and rare detection of the virus in other organs. This is the first report of an H7 vaccine candidate tested in a mammalian model. The data presented suggest that vaccine candidates based on low-pathogenicity avian influenza A viruses, which can be prepared ahead of pandemic threats, can be efficacious if an effective adjuvant is used.

Introduction

In 2003, an outbreak of highly pathogenic avian influenza (HPAI) occurred in The Netherlands, caused by an influenza A virus of subtype H7N7[55,161]. The HA and NA genes of the H7N7 HPAI viruses displayed a high sequence identity to the HA and NA genes of low-pathogenicity viruses isolated from Dutch mallards in the framework of our ongoing surveillance studies in wild birds[55,63]. During the 2003 outbreak, 89 cases of human infection were detected, including 3 probable cases of human-to-human transmission. Most of these individuals suffered from conjunctivitis or mild respiratory disease, but a fatal case of acute respiratory distress syndrome also occurred. The virus that was isolated from the fatal case, A/Netherlands/219/03, was found to differ from a virus isolated from a case of conjunctivitis, A/Netherlands/33/03, and the chicken isolate A/Chicken/Netherlands/1/03, in 14 amino acid positions scattered throughout the genome[55].

Because of their zoonotic potential and pathogenicity, and because close relatives of the H7N7 viruses that caused the outbreak may still circulate in wild birds, the generation of a vaccine strain based on the H7N7 virus was considered to be desirable. Because the H7N7 viruses isolated from poultry and humans during the outbreak are highly pathogenic, they cannot be used directly for generating a vaccine strain. Recently, vaccine strains for H5 influenza A virus have been generated by removal of the basic cleavage site from the HA genes of highly pathogenic strains. The resulting genetically modified virus strains, lacking this important determinant

of high pathogenicity, were used as seed viruses for vaccine production[175-179]. Since the HA gene of the virus that caused the H7N7 outbreak in The Netherlands was closely related to the HA gene of influenza virus A/Mallard/Netherlands/12/00, we decided to produce a subunit vaccine containing the HA of this low-pathogenicity H7 virus. Previously, it was shown that vaccination of naive animals with the traditional, nonadjuvanted subunit vaccine did not induce sufficient antibody titers to protect against challenge with influenza A virus[180,181]. Therefore, we tested the use of immune stimulatory complexes (ISCOMs) as an adjuvant in parallel with the traditional nonadjuvanted subunit vaccine. ISCOMs are particles of approximately 40nm in diameter with incorporated antigens[182]. ISCOMs are known to induce both antibody and cell-mediated immunity and have been tested as an adjuvant for influenza vaccines in a number of animal models and in humans[180,181,183,184].
Instead of using laborious classical reassortment techniques to produce the vaccine seed virus strain, we used the recently developed reverse genetics technology[185-188]. Reverse genetics techniques enable the generation of a reassortant virus with a backbone of influenza virus A/PR/8/34 and H7 and N7 envelope proteins obtained from avian influenza viruses, that can be used as seed virus for a vaccine, within a relatively short period of time[175,177,179].
Here, we describe the development and evaluation of a heterologous vaccine against HPAI virus of the H7N7 subtype. First, a mouse model for lethal infection with HPAI H7N7 was developed. Subsequently, the protective efficacy of a classical influenza subunit vaccine preparation was compared to that of an ISCOM-adjuvanted vaccine preparation. The classical preparation did not elicit a sufficient immune response to protect mice from a lethal challenge with influenza virus A/Netherlands/219/03 (H7N7) even after two doses. In contrast, two doses of an ISCOM-adjuvanted vaccine preparation were sufficient to protect mice against the lethal challenge, although at day 4 after challenge infection virus could be detected in their lungs.

Materials and methods

Viruses

The molecular clone version of influenza virus A/PR/8/34 has been described previously[185]. Influenza virus A/Mallard/Netherlands/12/00 (H7N3) was isolated from a cloacal swab collected from a Mallard in The Netherlands in 2000 and subsequently passaged twice in embryonated chicken eggs. Influenza virus A/Netherlands/33/03 (H7N7) and A/Netherlands/219/03 (H7N7) were isolated from patients during the H7N7 outbreak in the Netherlands in 2003[55] and passaged twice in embryonated chicken eggs. Segment 4 (HA) of influenza virus A/Mallard/Netherlands/12/00 (H7N3) and segment 6 (NA) of influenza virus A/Netherlands/33/03 (H7N7) were amplified by RT-PCR and cloned in the BsmBI site of a modified version of plasmid pHW2000[185-188]. A recombinant influenza virus A/PR/8/34 containing subtype H7 and N7 surface glycoproteins (rPR8-H7N7) was generated by reverse genetics as described previously[185]. To this end, transient calcium phosphate-mediated transfections of 293T cells were performed as described previously[185] with plasmids encoding gene segments 1, 2, 3, 5, 7 and 8 of influenza virus A/PR/8/34, segment 4 of influenza virus A/Mallard/Netherlands/12/00 and segment 6 of influenza virus

A/Netherlands/33/03. The supernatant of the transfected cells was harvested 48 h after transfection and was used to inoculate embryonated chicken eggs.
Influenza virus rPR8-H7N7 was passaged 6 times in the allantoic cavity of 11-day old embryonated chicken eggs. The allantoic fluid was harvested and cleared by low speed centrifugation. The cleared supernatant was subsequently centrifuged 2 h at 85,000xg in a SW28 rotor at 4˚C. The virus pellet was resuspended in 2 ml PBS and loaded on a 20 to 60% (wt/wt) sucrose gradient and centrifuged overnight at 300,000xg in a SW41 rotor at 4˚C.

Vaccine preparation
Decanoyl-N-methylglucamide (MEGA-10, Sigma, Zwijndrecht, The Netherlands) was added to the concentrated and purified virus to a final concentration of 2% and ribonucleoprotein complexes were removed by centrifugation through a layer of 20% (wt/wt) sucrose at 150,000xg for 2 h in a SW41 rotor at 20˚C. The resulting HA/NA preparation on top of the sucrose layer was harvested and tested for purity by performing SDS-polyacrylamide gel electrophoresis. Western blotting using a monoclonal antibody directed against the influenza A virus nucleoprotein was performed to confirm the absence of nucleoprotein in the HA/NA preparations. The amount of protein in the HA/NA preparation was determined using a BCA Protein Assay Kit (Pierce, Rockford, USA). A part of the preparation was dialyzed against PBS and used without further modification as a traditional HA/NA subunit vaccine preparation. The other part was dialyzed against PBS after addition of cholesterol (1 mg/mg protein, Sigma), phosphatidyl ethanolamine (1 mg/mg protein, Sigma) and Quillaja glucosides (5 mg/mg protein ISCOPREP 703, Iscotec, Lulea, Sweden). The ISCOM preparation was analyzed by negative contrast electron microscopy, revealing the typical particles with a diameter of approximately 40nm. An ISCOM-measles virus control vaccine was kindly provided by Dr. K. Stittelaar[189].

Immunization and challenge of animals
To generate post-infection antisera, ferrets were inoculated intranasally with approximately 10^6 50-percent egg infectious dose (EID_{50}) of influenza virus A/Mallard/Netherlands/12/00, A/Netherlands/33/03 or A/Netherlands/219/03. Antisera were collected from these animals after 14 days for use in hemagglutination inhibition assays. A hyperimmune rabbit antiserum raised against A/Seal/Massachusetts/1/80 (H7N7), that has been described previously[6], was used in parallel.
We used 6-8 week old female Balb/c mice for vaccination-challenge experiments. First, four groups of six mice were infected intranasally with $1x10^2$, $3x10^3$, $1x10^5$ and $3x10^6$ EID_{50} respectively to determine the optimal challenge dose for mice. Six groups of six animals each were vaccinated once or twice intramuscularly at 3-week intervals. The following groups were tested: two doses of PBS; two doses of 1 µg of a control ISCOM-measles vaccine; two doses of 5 µg of the HA/NA preparation; a single dose of 5 µg of the ISCOM-H7N7 preparation; two doses of 1 µg of the ISCOM-H7N7 preparation; two doses of 5 µg of the ISCOM-H7N7 preparation. A blood sample was collected from the animals prior to the second vaccination and prior to virus challenge by orbital puncture. Three weeks after the second vaccination, all animals were challenged intranasally with $3x10^3$ EID_{50} of influenza virus A/Netherlands/219/03. Although we did not determine the 50% lethal dose (LD_{50}) for

mice, $3x10^3$ EID_{50} corresponds to >30 LD_{50} (Fig.1). After challenge, the animals were observed for clinical signs and weighed twice daily as an indicator of disease. At day 4 after infection, 3 animals from each group were sacrificed and analyzed for the presence of virus in the lungs, spleen, liver, kidneys and brain. The other 3 animals were sacrificed 14 days after challenge, or upon development of severe disease or discomfort in agreement with national animal welfare regulations.
All intranasal infections, orbital punctures and euthanasia were performed under anesthesia with inhaled isoflurane. All animal studies were approved by the Animal Ethics Committee of Erasmus Medical Center, Rotterdam, The Netherlands. All experiments were performed under BSL3(+) conditions.

Table 1. HI assay determining cross-reactivity between A/Mallard/Netherlands/12/00 and two H7N7 viruses isolated during the outbreak, A/Netherlands/33/03 and A/Netherlands/219/03.

strain	HI titer of sera raised against			
	A/Mallard/ NL/12/00[1]	A/NL/ 33/03[1]	A/NL/ 219/03[1]	A/Seal/ Mass/1/80[2]
A/Mallard/Nl/12/00 (H7N3)	80	40	30	1280
A/NL/33/03 (H7N7)	160	160	80	1920
A/NL/219/03 (H7N7)	40	80	40	2560

[1] Postinfection serum raised in a ferret
[2] Hyperimmune serum raised in a rabbit

Serology
Hemagglutination inhibition (HI) assays were performed for the comparison of the antigenic properties of influenza A virus strains using post-infection ferret antisera and a hyperimmune rabbit antiserum and for the determination of antibody levels in vaccinated mice essentially as described previously[98]. All serum samples were treated overnight with receptor-destroying enzyme and subsequently incubated at 56˚C for 1 hour. Twofold serial dilutions of each antiserum, starting at a 1:20 or 1:40 dilution were tested for their ability to inhibit the agglutination of turkey or horse erythrocytes by 4 hemagglutinating units of influenza A virus. When horse erythrocytes were used instead of turkey erythrocytes, serum dilutions were made in PBS containing 0.5% bovine serum albumine (BSA, fraction V, Gibco, Breda, The Netherlands). Horse erythrocytes were stored in PBS containing 0.5% BSA. In the HI-assay, 50 µl of a 1% horse erythrocyte dilution was added to each well.

Virus titrations
Virus titrations were performed by end-point titration in MDCK cells as described previously[185]. Lungs, spleen, liver, kidneys and brain were collected and homogenized in 3 ml transport medium, consisting of Hanks balanced salt solution containing 10% glycerol, 200 U/ml penicillin, 200 mg/ml streptomycin, 100 U/ml polymyxin B sulfate, and 250 mg/ml gentamycin (all from MP Biomedicals, Zoetermeer, The Netherlands), using a Polytron homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) and centrifuged briefly. The supernatant was used directly to inoculate MDCK cells. MDCK cells were inoculated with tenfold serial dilutions of tissue homogenates. One

hour after inoculation, cells were washed once with PBS and grown in 200 µl of infection media, consisting of EMEM (Cambrex, Heerhugowaard, The Netherlands) supplemented with 4% BSA, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodiumbicarbonate (Cambrex), 10 mM Hepes (Cambrex), non-essential amino acids (MP Biomedicals) and 20 µg/ml trypsin (Cambrex). Three days after inoculation, the supernatants of infected cell cultures were tested for agglutinating activity using turkey erythrocytes as an indicator of infection of the cells. Infectious titers were calculated from five replicates by the method of Spearman-Karber[104].

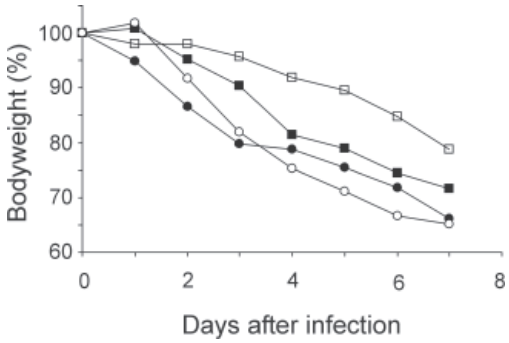


Figure 1. Loss of bodyweight as the result of infection with influenza virus A/Netherlands/219/03. Mice were infected intranasally with four different infectious doses: $1x10^2$ (□), $3x10^3$ (○), $1x10^5$ (■) and $3x10^6$ (●) EID_{50}. Following infection, mice were weighed daily. Percentage bodyweight was calculated as compared to bodyweight at time of infection.

Results

Analysis of antigenic properties of H7 influenza A virus isolates

Sequence analyses of the strains circulating during the H7N7 outbreak in The Netherlands in 2003 showed that the HA genes of the viruses isolated from chickens and humans were 98% identical to the HA gene of a low-pathogenicity avian influenza (LPAI) virus A/Mallard/Netherlands/12/00 (H7N3) isolated within the framework of a surveillance study for influenza A virus in wild birds[55,63]. Because the mallard virus lacked the basic cleavage site in the HA gene characteristic for HPAI strains, it was considered as a candidate vaccine strain, without the need of genetic modification of the HA gene. We first compared the antigenic properties of the HA genes of influenza viruses A/Mallard/Netherlands/12/00, A/Netherlands/33/03, and A/Netherlands/219/03. As shown in Table 1, the ferret antisera raised against influenza viruses A/Netherlands/33/03 and A/Netherlands/219/03 had similar homologous and heterologous titers against these viruses. A hyperimmune rabbit antiserum raised against A/Seal/Massachussetts/1/80 (H7N7) was included as a positive control and reacted with all H7 viruses with high titers. Of special interest was the reactivity of the ferret serum directed against influenza virus A/Mallard/Netherlands/12/00 with the other two strains. The titers against A/Netherlands/33/03 and A/Netherlands/219/03 were within two-fold of the homologous titer, indicating that these three viruses are

antigenically similar. Both horse and turkey erythrocytes were used in the HI assays, but the source of erythrocytes did not have a major influence on the outcome of the experiments. Based on these data it was anticipated that antibodies raised against the HA of the LPAI A/Mallard/Netherlands/12/00 would provide protection against the HPAI. Therefore, the HA of the LPAI H7N3 virus was selected for use in the candidate vaccine against HPAI H7N7 strains.

A lethal challenge mouse model for H7N7 influenza A virus

We wished to develop an animal model for lethal infection with the virus isolated from the fatal human case, A/Netherlands/219/03. To this end, groups of six Balb/C mice were infected with 30-fold serial dilutions of infectious virus stocks, ranging in dose from $1x10^2$ to $3x10^6$ EID_{50}. All mice became severely ill, indicated by loss of bodyweight (Fig. 1), ruffled fur and lethargy. Three animals out of each group were sacrificed at day 4 after infection to analyze virus infection of the lungs of these mice. The three remaning mice in each group were sacrificed because of the severity of the disease signs at day 7 after infection, in accordance with national ethical guidelines for experiments with laboratory animals.

In mice infected with $3x10^3$, $1x10^5$ and $3x10^6$ EID_{50} the same rate of loss of bodyweight was observed. The mice infected with $1x10^2$ EID_{50} lost bodyweight slightly more slowly than mice in the other groups, but also became severely ill, necessitating euthanasia at day 7 after infection. In accordance with the relatively low rate of bodyweight loss, mice infected with a virus dose of $1x10^2$ EID_{50} had ~10-fold lower virus load in the lungs at 4 and 7 days after inoculation as compared to the mice infected with $3x10^3$, $1x10^5$ and $3x10^6$ EID_{50} (data not shown). We decided to use 3 x 10^3 EID_{50} as a reproducible minimal challenge dose that resulted in lethal infection of mice for our vaccination experiments.

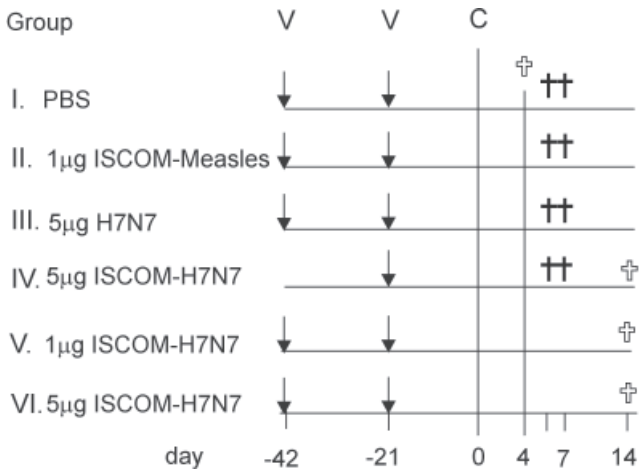


Figure 2. An overview of the vaccination strategies used to test H7N7 vaccine preparations. Six groups of six mice were vaccinated once or twice intramuscularly with the different vaccine preparations as indicated (↓). On day 4 after challenge, 3 animals from each group were sacrificed (open crosses). On day 6 and 7 after challenge animals from groups I, II, III and IV were sacrificed due to severity of signs of disease (cross). On day 14 all remaining animals were sacrificed (open crosses). V: vaccination; C: challenge.

HI antibody titers in vaccinated mice

Mice were immunized according to the schedule depicted in figure 2. Three weeks after the first vaccination and 3 weeks after the booster vaccination, blood samples were collected from the vaccinated mice. The sera were used in HI assays to determine the antibody titers against the heterologous challenge virus A/Netherlands/219/03. After the first vaccination with 1 or 5 µg of ISCOM-H7N7, mice developed antibody titers ranging from 160 to 960. In contrast, only three out of six mice vaccinated with the classical subunit influenza vaccine developed detectable antibody titers up to 40 (Fig. 3). After the second vaccination, antibody titers in the mice vaccinated with the classical H7N7 subunit vaccine increased, but were still significantly lower than the antibody levels in mice vaccinated with either a single dose or two doses of the ISCOM-H7N7 vaccine. Mice receiving two doses of ISCOM-H7N7 vaccine developed high antibody responses after two vaccinations, ranging from 960 to 7680 (Fig. 3). There was no significant difference in antibody production between the mice vaccinated with either 1 or 5 µg of the ISCOM-H7N7 vaccine. None of the mice that received PBS or ISCOM-measles vaccine produced detectable HI antibodies.

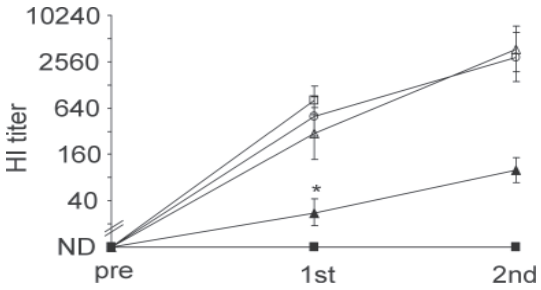


Figure 3. Serum HI antibody titers against A/Netherlands/219/03 after the first and second vaccination. Blood samples were taken before the start of the experiment and at three weeks after the first and second vaccination. Serum was used in a HI test against A/Netherlands/219/03. Indicated are geometric mean titers and 95% confidence interval. The asterisk indicates that in this group three mice had an HI titer below detection limit and three mice had a titer of 40. ND: not detected. PBS and ISCOM-measles controls (■); two 5 µg H7N7 vaccinations (▲); one 5 µg ISCOM-H7N7 vaccination (□); two 1 µg ISCOM-H7N7 vaccinations (○); two 5 µg ISCOM-H7N7 vaccinations (∆).

Protection from lethal infection

Three weeks after the booster vaccination, all animals were challenged with $3x10^3$ EID_{50} of influenza virus A/Netherlands/219/03. Following challenge, the bodyweight of the animals was determined twice daily as an indicator for disease. From 3 days after the challenge onwards, the mice vaccinated once with the ISCOM-H7N7 preparation or twice with the classical influenza vaccine preparation, the control ISCOM-measles, or PBS started to develop signs of disease such as loss of bodyweight, a ruffled fur and lethargy. At days 6 and 7 post infection, all the mice from these groups, except for one mouse in the group vaccinated once with 5 µg ISCOM-H7N7 vaccine, were sacrificed for ethical reasons, since they all became very ill and lost 20% of their original bodyweight (Fig. 4A). One mouse in the group that was vaccinated once with ISCOM-H7N7 became only slightly ill and recovered

completely. The mice vaccinated twice with either 1 or 5 µg of ISCOM-H7N7 vaccine appeared to remain healthy after the H7N7 influenza A virus challenge despite a temporal small loss of bodyweight. Figure 4B summarizes the survival of the mice upon the lethal challenge with A/Netherlands/219/03.

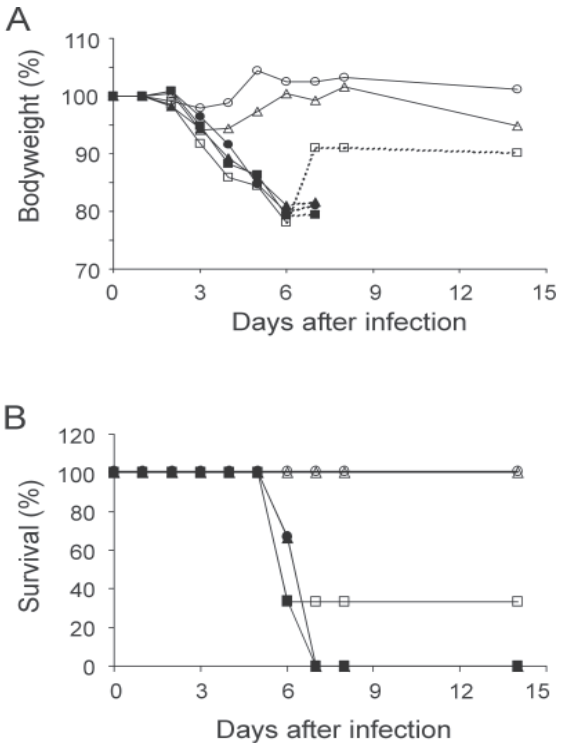


Figure 4. Loss of bodyweight and survival of vaccinated mice after challenge with influenza virus A/Netherlands/219/03. Vaccinated mice were challenged intranasally with $3x10^3$ EID_{50} of influenza virus A/Netherlands/219/03. After challenge, mice were weighed daily. Percentage bodyweight per group was calculated as compared to bodyweight at time of challenge (A). Mice were either sacrificed due to the severity of their disease signs at day 6 or 7, or at the end of the experiment on day 14 after challenge. The dotted line indicates that one or two mice out of the group were already sacrificed and data are thus based on bodyweight of the remaining mice. The percentage of mice surviving the lethal challenge as a function of time is also shown (B). Since three mice out of each group were sacrificed at day 4 after infection, this graph is based on the survival of the remaining three mice from day 5 onwards. Groups: PBS (■) and ISCOM-measles (●) ; two 5 µg H7N7 vaccinations (▲); one 5 µg ISCOM-H7N7 vaccination (□); two 1 µg ISCOM-H7N7 vaccinations (○); two 5 µg ISCOM-H7N7 vaccinations (Δ).

Virus titers in the organs of vaccinated animals

On day 4 after challenge, three mice from each vaccinated group were sacrificed and their lungs, spleens, livers, kidneys and brains were collected for analysis of virus replication (Fig. 5). High virus titers were detected in the lungs of all mice on day 4 after challenge. Virus titers in the lungs of mice vaccinated twice with ISCOM-H7N7 had a geometric mean titer (GMT) of $10^{4.44}$ $TCID_{50}$/gram tissue (95% confidence interval (CI): $10^{2.33}$ to $10^{6.54}$), approximately 1000-fold lower than those of the mice

in the other groups (GMT $10^{7.41}$ $TCID_{50}$/gram tissue, 95% CI: $10^{6.94}$ to $10^{7.87}$, P<0.05, one-way analysis of variance test).
In the two groups of mice vaccinated twice with ISCOM-H7N7, virus was not detectable outside the lungs, except for marginal virus titers in the spleen and kidney of one mouse vaccinated twice with 1 µg of ISCOM-H7N7 and in the brains of one mouse in each of the groups vaccinated with 1 and 5 µg of ISCOM-H7N7. In contrast, in all other groups of mice virus titers were detected in the spleen, liver, kidney and brain at 4 days after infection (Fig. 5). Because the number of animals with detectable virus titers in organs other than the lung varied between groups, no statistical differences could be calculated for these organs.

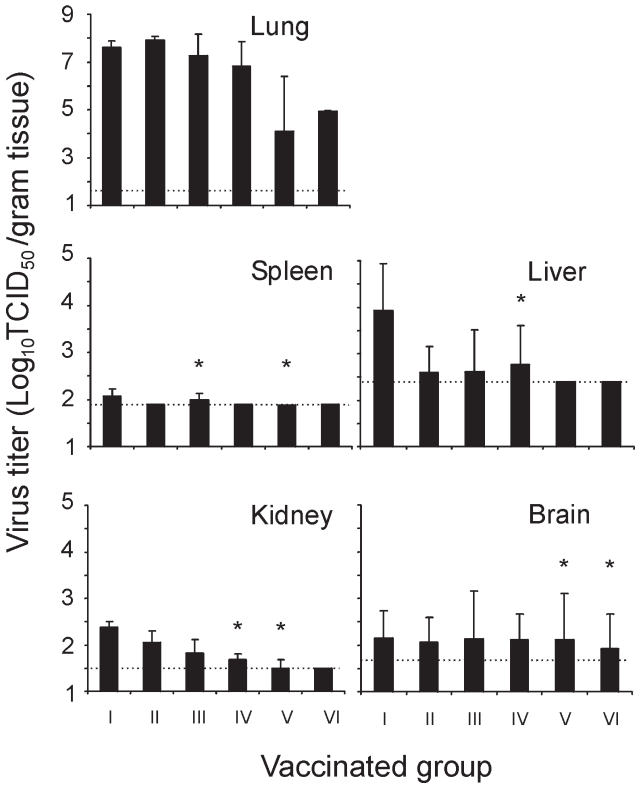


Figure 5. Virus titers in the mice after challenge with influenza virus A/Netherlands/219/03. Vaccinated mice were challenged intranasally with $3x10^3$ EID_{50} of influenza A virus A/Netherlands/219/03. On day four after challenge three mice from each group were sacrificed, tissues were collected and virus titers in lungs, spleen, liver, kidney and brain were determined in MDCK cells. The geometric mean virus titer per group was calculated. To calculate the geometric mean, the cutoff value was used for negatives. Error bars indicate standard deviation. The dotted line indicates the cutoff value of the assay for each of the organs. Asterisks indicate that only one of the tested animals in the group was positive. Roman numbers refer to the vaccination status as explained in figure 2.

Discussion

In 2003 there was an outbreak of HPAI (H7N7) in The Netherlands. Because of the zoonotic and pandemic potential of the viruses that caused the outbreak the availability of an effective vaccine was highly desirable. The HA of a virus isolated from a mallard in 2000 had a high sequence identity to the HA gene of the viruses that caused the outbreak. This offered the opportunity to use this LPAI virus obtained through routine influenza surveillance in wild birds as the basis of a vaccine directed against HPAI H7N7 virus. Using reverse genetics, we generated a reassortant seed strain that was used for vaccine preparation. We compared a classical, nonadjuvanted influenza vaccine preparation containing HA and NA, with an ISCOM-adjuvanted preparation.

Our experiments clearly showed that, although an antibody response was mounted, the classical influenza vaccine did not protect against lethal infection with influenza virus A/Netherlands/219/03, not even when two doses were administered. One dose of the ISCOM-adjuvanted vaccine preparation did not protect either. Two doses of the ISCOM-adjuvanted vaccine were required for clinical protection against influenza virus A/Netherlands/219/03. Although two doses of the ISCOM-adjuvanted preparation did not lead to sterile immunity against HPAI H7N7, virus titers in the lungs of vaccinated mice were reduced considerably. We speculate that this ~1000-fold reduction of pulmonary virus titers was responsible for the survival of the animals receiving two doses of the ISCOM-vaccine. In the event of a pandemic outbreak, a vaccine offering clinical protection could be sufficient to reduce the clinical impact of infection with the pandemic virus.

Our experiments also showed that it was possible to use a heterologous LPAI strain as donor of the HA protein for vaccines directed against HPAI strains. It should be noted, that the HA amino acid sequence of the LPAI mallard virus and the HPAI viruses differed very little; only the HA cleavage site and single amino acid substitutions in the signal peptide, HA1 and HA2 were different. LPAI strains with such high amino acid sequence identity and such small antigenic differences from HPAI strains may not always be available.

An important question that remains is why infected animals were not protected from infection or death despite high HI antibody titers upon repeated vaccination with adjuvanted or nonadjuvanted vaccines respectively. We performed virus neutralization assays with the sera from vaccinated mice and influenza viruses A/Mallard/Netherlands/12/00 (H7N3) and A/Netherlands/219/03 (H7N7). These experiments revealed that despite the high titers of HI antibodies in these sera (HI titers ranging up to 7680), virus-neutralizing antibodies were not detectable (titers <40). In contrast, rabbits hyperimmunized with HA and NA of A/Seal/Massachusetts/1/80 (H7N7), which had HI antibody titers of 1280 and 2560 against A/Mallard/Netherlands/12/00 (H7N3) and A/Netherlands/219/03 (H7N7) (Table 1), had virus-neutralizing antibody titers against these viruses of 1280 and 5120 respectively. Since the Dutch H7 viruses are thus not resistant to neutralization, the lack of virus neutralization with the mouse sera must be related to the poor induction of neutralizing antibodies upon vaccination. It is of interest to note that the post-infection ferret sera raised in this study also lacked detectable neutralizing antibody titers despite the presence of HI antibodies (Table 1 and data not shown). Furthermore, the H7N7-infected humans

in The Netherlands who developed HI antibodies during the outbreak in 2003 did not develop detectable virus neutralizing antibodies (unpublished). Thus, it may be that the Dutch H7 viruses are poor inducers of neutralizing antibodies. Hence it is possible, that cell-mediated immunity or antibodies with poor virus-neutralizing capacity were responsible for the clinical protection of mice vaccinated with the ISCOM-based vaccine.

Experiments like the one described here have been conducted with highly pathogenic strains of the H5N1 subtype, although it is difficult to compare these studies to our own because of the use of different virus subtypes, animal models, adjuvants, vaccine types, timing and dosage. Using the LPAI A/Duck/Singapore/Q/F119-3/97 (H5N3)[179,190], the LPAI A/Duck/Hokkaido/67/96 (H5N4) or a H5N1 reassortant thereof[191], inactivated virus preparations were tested in mice for their possible use in a pandemic situation. In these studies, vaccinated mice survived challenge with the HPAI H5 strains A/Hong Kong/483/97 or A/Hong Kong/156/97, even when no adjuvant was used in the vaccine preparations. When alum was used as an adjuvant, mice were even protected from infection[190]. However promising these studies may seem compared to ours, our results are more in agreement with vaccination studies in a chicken[181] and monkey[180] model and with experimental vaccination in humans[192]. When surface-antigen of the same A/Duck/Singapore/Q/F119-3/97 strain used in the mouse studies was used as a vaccine in a phase I randomised trial in human volunteers, two doses of a preparation with the adjuvant MF59 were required to reach antibody levels that are considered to be protective[192].

Although ISCOMs are not registered for use in humans, the immune response to an ISCOM-adjuvanted influenza vaccine has been tested in humans. When ISCOMs were tested in humans in a randomized, double blind study, antibody responses to the ISCOM-adjuvanted preparation were improved as compared to antibody responses to the conventional influenza vaccine preparation[184]. Although in this study individuals were vaccinated with an ISCOM preparation containing influenza A virus antigens against which there was pre-existing immunity, the results in a range of animal models suggest that the ISCOM-adjuvant could also work very well to induce immune responses in naive individuals. In addition, ISCOMs are used as an adjuvant in registered equine influenza vaccines[183,193].

In case of a pandemic, a two-dose vaccine will be unpractical. Therefore, there is an urgent need for new adjuvants or improved vaccine-delivery approaches, yielding better immune responses and improved protection against lethal infection, preferably after administration of a single dose.

Although vaccination experiments using highly pathogenic H5 viruses with a deleted basic cleavage site have been successful in animals[176,178,194], these vaccine strains can only be produced as soon as a pandemic threat arises. Furthermore, removal of the basic cleavage site requires an extra modification step and thus more time, in the production of a reassortant seed virus. Since we show here that a heterologous vaccine could work well, prototypic envelope proteins of potentially pandemic viruses could be cloned in advance, to speed up seed virus production further. Alternatively, vaccine seed viruses could be generated by classical reassortment, thus eliminating the need of reverse genetics technology. This would not only bypass the patent-related costs of influenza vaccines, but would also enable vaccine manufacturers to produce vaccines without the need of facilities equipped for working with genetically

modified organisms.
An important conclusion that can be drawn from the experiments described above is that prototypic LPAI strains obtained through routine surveillance of wild birds could be used effectively to generate vaccines directed against HPAI viruses long before outbreaks in poultry or pandemic threats emerge. A repository of seed virus containing the HAs of viruses with known zoonotic or pandemic potential (H1, H2, H3, H5, H7, H9) could be prepared in advance. High-growth strains could be generated, in order to respond quickly to outbreaks and pandemic threats. Based on the antigenic properties of the virus strains, the most suitable vaccine candidate could then be selected from the repository using new methods for antigenic characterization[56].

Acknowledgements
We thank Koert Stittelaar, Bé Niemeyer, Robert Dias d'Ullois and Geert van Amerongen for technical assistance and helpful comments. This work was supported by framework 5 grant "Novaflu", QLRT-2001-01034, from the European Union.

9

Highly pathogenic avian influenza (H7N7): vaccination of zoo birds and transmission to non-poultry species

Vaccine, 2006, 23: 5743-5750

Joost Philippa
Vincent Munster
Hester van Bolhuis
Theo Bestebroer
Willem Schaftenaar
Walter Beyer
Ron Fouchier
Thijs Kuiken
Albert Osterhaus

In 2003 an outbreak of highly pathogenic avian influenza virus (H7N7) struck poultry in The Netherlands. A European Commission directive made vaccination of valuable species in zoo collections possible under strict conditions. We determined pre- and post-vaccination antibody titres in 211 birds by haemagglutination inhibition test as a measure of vaccine efficacy. After booster vaccination, 81.5% of vaccinated birds developed a titre of ≥40, while overall geometric mean titre (GMT) was 190 (95% CI: 144–251). Birds of the orders Anseriformes, Galliformes and Phoenicopteriformes showed higher GMT, and larger percentages developed titres ≥40 than those of the other orders. Antibody response decreased with increasing mean body weight in birds ≥1.5 kg body weight. In the vicinity of the outbreak, H7N7 was detected by RT-PCR in wild species (mallards and mute swans) kept in captivity together with infected poultry, illustrating the potential threat of transmission from poultry into other avian species, and the importance of protecting valuable avian species by means of vaccination.

Introduction

Avian influenza A virus (AIV) is a member of the *Orthomyxoviridae* family and can be classified according to the antigenicity of its surface proteins hemagglutinin (H) and neuraminidase (N), and on the basis of its pathogenicity in chickens. Highly pathogenic avian influenza (HPAI), an acute generalised disease in which mortality may be as high as 100%, is restricted to subtypes H5 and H7, although not all viruses of these subtypes necessarily cause HPAI. All other AIV strains are low pathogenic avian influenza (LPAI) virus strains and cause a much milder, primarily respiratory disease with loss of egg production[195].

The host range of AIV includes a large number of avian species[196-199], with a worldwide distribution and variable morbidity per virus isolate and avian species[198-204]. Free-ranging bird species of the order Anseriformes (ducks, geese, swans and screamers) and Charadriiformes (shorebirds), are considered natural reservoirs in which morbidity is low[1,198,205]. HPAI outbreaks have generally been restricted to poultry (turkeys and chickens), and farmed ostriches[206,207]. Exceptions, where HPAI outbreaks have been reported in free-ranging birds, have been in terns in 1961[23], several species of wild migratory birds as well as captive Phoenicopteriformes (flamingos) in 2002[36,40], and in wild migratory birds in 2005[37].

The Netherlands did not have an outbreak since 1927, but in March 2003 the diagnosis HPAI virus (H7N7) infection was made by RT-PCR[55,208]. HPAI is categorised as an Office International des Epizooties (OIE) list A disease, for which the European Union (EU) has a non-vaccination policy. Eradication measures during an outbreak include confinement, stamping out of animals on the infected farm, pre-emptive culling of animals on neighbouring farms, and emergency vaccinations (EU Directive 92/40/EEC). The area of the outbreak expanded[209], and threatened to encroach on Dutch zoos. The Dutch Zoo Federation (Nederlandse vereniging van dierentuinen, NVD) proposed vaccination to avoid not only pre-emptive culling of the valuable and sometimes endangered species kept in zoo collections, but also confinement (of unknown duration) of birds, which they considered to be detrimental to welfare.

Commission Decision 2003/291/EC of 25 April 2003 replaced the implementation of pre-emptive culling and confinement in zoos with the preventive vaccination of zoo bird species listed as susceptible to avian influenza: Galliformes (fowl, quail, pheasants), Anseriformes, Struthioniformes (emus, nandus and ostriches) and Rock Doves (*Columba livia*) kept for consumption, provided that far-reaching requirements were met.

Inactivated monovalent and polyvalent AIV water-in-oil emulsion vaccines have been demonstrated to be effective in reducing mortality and/or preventing morbidity, as well as reducing viral shedding[210] after HPAI virus challenge in chickens and turkeys[211]. The use of a heterologous vaccination (in which the N protein differs from the field strain) makes it possible to distinguish vaccinated birds from infected birds, while maintaining acceptable efficacy[212].

The goals of this study were to determine whether transmission of HPAI H7N7 virus from poultry to free-ranging birds during an outbreak occurred, to evaluate the efficacy and safety of an inactivated H7 vaccine in a large number of exotic avian species, and whether vaccination would be a suitable alternative to pre-emptive culling and confinement during future outbreaks of H7 HPAI virus.

Materials and methods

Serology

An inactivated H7N1 vaccine, strain A/CK/Italy/473/99, inducing at least 4 log2 hemagglutination inhibition (HI) units according to potency test, with liquid paraffin adjuvant, (Nobilis influenza®, Intervet International, Boxmeer, the Netherlands) was used. The vaccine strain had a homology of 97.4% to the field strain on the basis of nucleotide sequence (1174 base pairs, excluding basic cleavage site), and 98.7% on the basis of amino acids. The vaccine was produced and assayed according to the requirements made in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Chapter 2.7.12 (further details may be obtained from the manufacturer). The birds were vaccinated twice with 6 weeks interval. Mode of vaccination [subcutaneous (s.c.) or intramuscular (i.m.)] differed according to zoo. Birds with a body weight smaller than 1.5 kg (n = 74) were given 0.25 ml, those with a body weight of 1.5 kg or more (n = 137) were given 0.5 ml. Bodyweights were not assessed individually, but published mean weights of the species were used[11,213]. Blood was collected from 211 birds from 13 orders (Apendix A), approximately 10% of the total number vaccinated in 10 participating zoos from the Netherlands. Blood was collected using manual restraint from the right jugular vein or the ulnar vein (left or right) on the day of vaccination and between 30 and 60 days following the last vaccination. Blood collection on the second vaccination date was not mandatory, and was only done in three out of 10 zoos.

To evaluate humoral immunologic response to vaccination, H7N1 (A/CK/Italy/473/99) specific antibody titres were determined by HI test, following standard procedures, using turkey erythrocytes[214,215.] Undetectable titres (<10) were regarded as 5 for calculation of geometric mean titres (GMT). Vaccine-induced HI titres of ≥40 were considered protective, as in humans[216]. GMT and 95% confidence intervals (CI) were then calculated per order. For orders Chariidiformes, Ciconiiformes (storks, herons,

egrets), Columbiformes (doves, pigeons), Coraciiformes (hornbills), Gruiformes (cranes), Pelicaniformes (pelicans, cormorants), Psittaciiformes (parrots, cockatoos), Sphenisciformes (penguins), and Strigiformes (owls), it was not meaningful to calculate 95% CI of the GMT because of small numbers (<10). Birds in these orders were combined in two groups: “other orders <1.5 kg”, and “other orders ≥1.5 kg”.
To evaluate vaccine-induced immunity against the HPAI H7N7 strain, a virus neutralisation (VN) assay was performed on post-vaccination serum from 48 randomly selected birds with different HI titres. VN assays were performed essentially as described previously[180]. Briefly, serial dilutions of the respective serum samples, which had been heat-inactivated for 30 min at 56°C, were incubated 1 h at 37°C with 100$TCID_{50}$ of A/Chicken/Netherlands/1/03 (H7N7). The mixture was then transferred to MDCK monolayers in microtitre plates. After incubation for 1 h, the cells were washed with PBS and incubated in infection medium for 5 days. The supernatants of the cultures were then tested for HA activity.

Virus detection
A cloacal swab was taken from 108 birds from one zoo on the first day of vaccination, and stored in 1 ml transport medium (Hank’s balanced salt solution containing 10% glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulphate, 250 µg/ml gentamycin (ICN, Zoetermeer, the Netherlands) to detect active shedding of H7 AIV. RNA was isolated from 200 µl supernatant using the MagnaPure LC system (Roche Diagnostics, Almere, The Netherlands) and influenza A virus was detected by a Taqman assay, based on influenza A virus H7 gene sequences of A/Chicken/Netherlands/1/03, as described[55,80].

Detection of AIV transmission from poultry to other species
Free-ranging domestic and wild birds (n = 109), mainly road casualties but also birds, found dead, or euthanized or shot, were collected either directly in the vicinity of depopulated HPAI virus infected poultry farms, or arbitrarily throughout the infected area. Species, sex and age category were determined, and the location and date found were recorded. Cloacal swabs were taken from all birds, with additional tracheal swabs from dead birds, and stored in transport medium. Full necropsies were performed on four mallards kept at a camping near an infected poultry farm, which had cloacal swabs positive for H7 AIV. Their carcasses were stored frozen prior to necropsy. Tissue samples of lung, brain, kidney, liver, spleen, duodenum, jejunum, ileum, caecum, colon, pancreas, heart, skin, and proventriculus were homogenized in 3 ml transport medium using a Polytron PT2100 (Kinematica, Lucerne, Switzerland) and subsequently centrifuged (1590 × g). Virus detection was performed as described above, and a sequence analysis was performed on H7 AIV positive specimens.

Sequence analysis
RT-PCR specific for the non-coding regions of H7 AIV was performed on the original material as described[81]. PCR products were run on a 1% agarose gel 1 × TBE and purified by using the QIAquick gel extraction kit (Qiagen, Leusden, The Netherlands) and sequenced directly. The entire H and N gene segments of the 7 H7

AIV positive birds were sequenced. Sequencing was performed using the Big Dye Terminator sequencing kit, version 3.1 (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands) and an ABI Prism 3100 genetic analyser (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). Primer sequences are available on request. Nucleotide sequences were aligned using BIOEDIT 5.0.9.

Results

Serology

For 116 birds, GMT after first and second (booster) vaccination could be assessed. Overall, GMT after second vaccination [166 (95% CI: 117–237)] and percentage of birds with titre ≥40 (80.2%) were much higher than after first vaccination [20 (95% CI: 14–30) and 36.2%], demonstrating the need for booster vaccination. All but three birds (a domestic Chicken, an Emu and an Egyptian Goose) had a pre-vaccination GMT <10. In the following text, titres after one vaccination are ignored, and only titres after two vaccinations of all 211 birds in this study are used. The post-vaccination GMT was 190 (95% CI 144–251) and 81.5% of all tested birds produced an antibody titre ≥40. GMT was significantly different between orders (Fig. 1). No significant differences were found when Anseriformes, Galliformes, and Phoenicopteriformes were analysed for species differences within the orders.

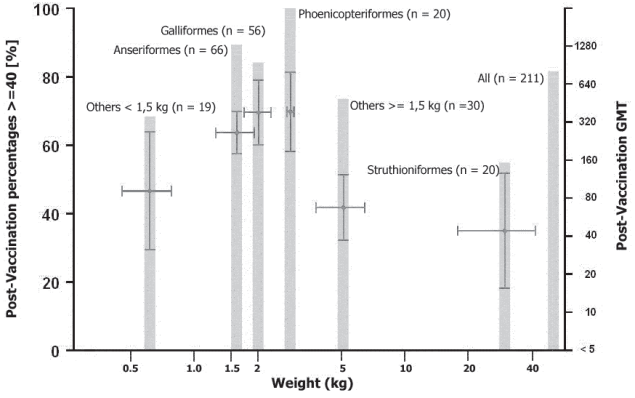


Figure 1: Post-vaccination percentages titre ≥40, and GMT versus mean body weight per order. (lines: 95% CI's). The humoral response of different avian orders to vaccination against avian influenza (H7) using an inactivated vaccine administered twice with 6 weeks interval. Titres shown were measured 30 to 60 days following the second vaccination. The bars represent the percentage of birds with titre ≥40. The points represent the geometric mean titre (GMT) and mean body weight per order, with the 95% confidence intervals (CI) represented by vertical (GMT) and horizontal lines (mean body weight), respectively.

There was an inverse correlation between weight and antibody response (Fig. 1) in birds ≥1.5 kg (regression coefficient: −1.543, $P < 0.001$), but not for those <1.5 kg (regression coefficient: −0.076, $P < 0.913$). However, it should be noted that body weights used were published species averages, and not from the individual birds, therefore these results should be treated cautiously, and may not be regarded significant.

Generally, i.m. administration (n = 109) resulted in both a higher GMT [214 (95% CI: 149–306)]versus [168 (95% CI 110–257)] and higher % of animals with a titre ≥40 (84.4 versus 78.4) than s.c. administration (n = 102). However, a statistical comparison between s.c. and i.m. administration with regard to antibody response was not possible (no random administration). Mode of administration was nonetheless not regarded as a factor influencing antibody response in the statistical analysis of the results, as no large differences were observed. The degree of agreement (within a four-fold margin) between the post-vaccination results of HI and VN test against the HPAI virus strain was 79.2% (Fig. 2). Necropsies of birds that died during this period did not reveal any HPAI virus- or vaccine-related cause of death.

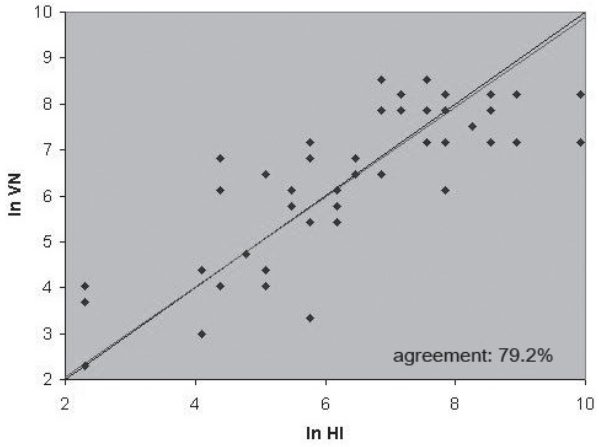


Figure 2. Post-vaccination natural logarithms of titres measured by virus neutralisation (ln VN) and by hemagglutination inhibition (ln HI). The Natural logarithms of titres shown are 30 to 60 days following the second vaccination with an inactivated H7N1 vaccine in 48 birds chosen at random. Titres measured by hemagglutination inhibition are against the vaccine strain, those measured by virus neutralisation are against the highly pathogenic H7N7 strain. The grey bar represents the regression line, the black bar represents the diagonal.

Virus detection

H7 AIV could not be detected by RT-PCR carried out on the cloacal swabs collected in the zoo. H7 AIV virus was detected by RT-PCR from seven captive birds (four Mallards and three Mute Swans (*Cygnus olor*). All seven H and N gene segments were identical to the A/Chicken/Netherlands/1/03 H7N7 AIV (GenBank H AY338458, and N AY340077). All positive animals had been kept at a camp site situated about 100 metres from three of the first HPAI-positive poultry farms. These birds had been confined since the beginning of the HPAI outbreak, in the same shed as eight domestic Chickens (*Gallus gallus*), one Emu (*Dromaius novaehollandiae*), one Guinea Fowl (*Numida meleagris*), two Greylag Geese (*Anser anser*), two domestic Turkeys (*Meleagris gallopavo*), one other Mallard, and one other Mute Swan. The Guinea Fowl and one chicken died and tested serologically positive to H7 AIV (Koch, 2005, pers. comm.). The rest of the birds, which showed no clinical signs of AIV infection, were euthanized the next day. The turkeys, chickens and the Emu tested serologically positive. From the remaining birds, tracheal and cloacal swabs

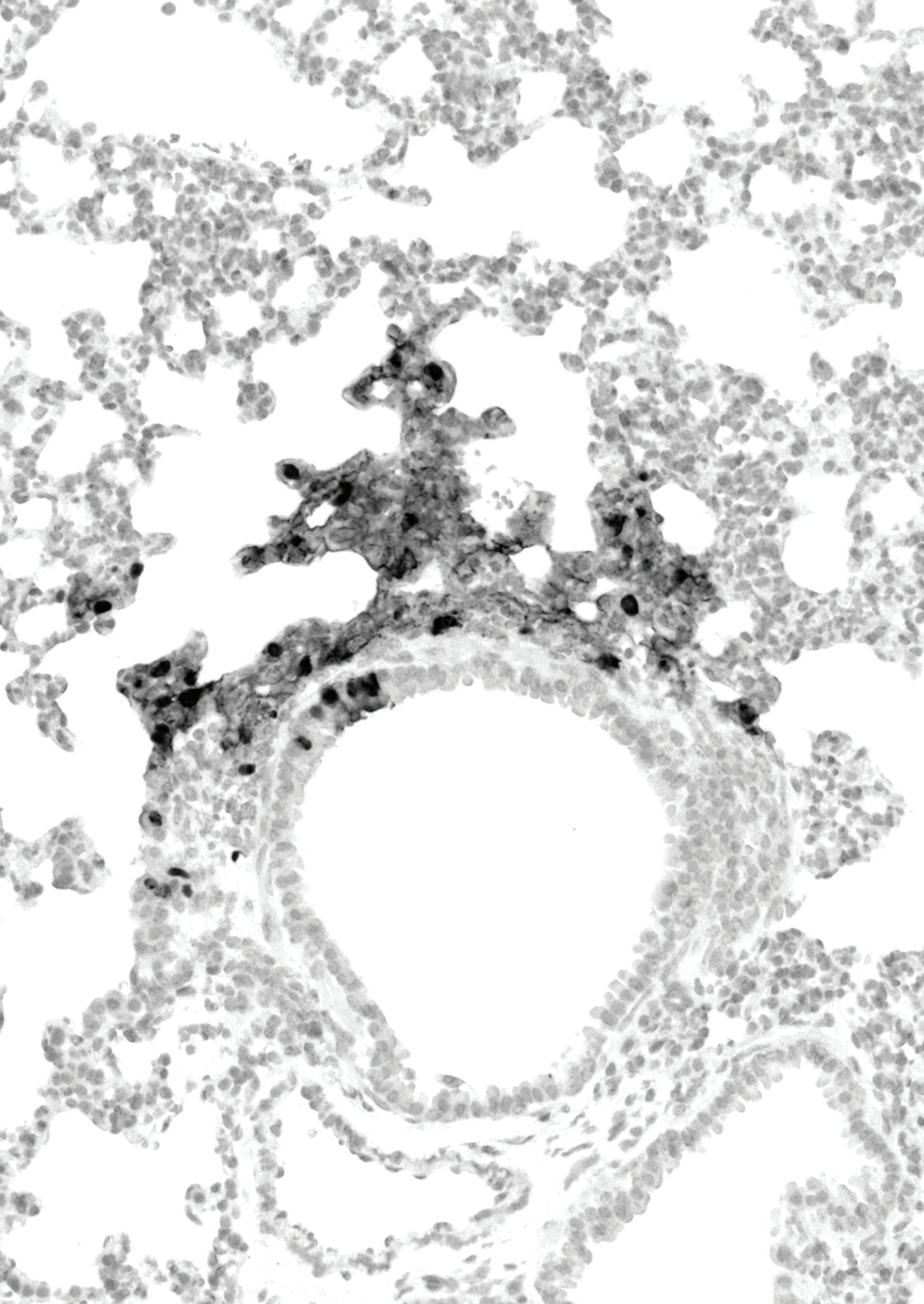
were tested for the presence of H7 AIV, but were negative, except from the four mallards and three mute swans described above. Free-ranging wild birds shot on the premises [one greylag goose, one Mallard and one Pheasant (*Phasianus colchicus*)] also tested negative for H7 AIV.
Full necropsies on the 4 RT-PCR positive Mallards showed the presence of H7N7 virus in the jejunum (three birds), ileum (four birds), caecum (three birds), colon (one bird), lung (one bird), spleen (two birds) and heart (one bird).

Discussion

In the present paper, we have shown that H7 AIV vaccination of exotic birds with Nobilis influenza® is safe and is expected to be efficacious. We consider it to be useful and necessary in case of an outbreak, as transmission of H7N7 from poultry into non-poultry species did occur. Overall 81.5% of the birds tested developed to a titre ≥40, 30–60 days after the second vaccination (Fig. 1). Antibodies produced against the surface protein H act as major determinants for protection against infection and disease in poultry[211,217,218]. Antibody titres to H7 were therefore used as a measure of immunogenicity, especially as efficacy could not be tested by challenge infections of these valuable or endangered species. Post-vaccination titres ≥32 in chickens have prevented shedding after challenge infection with the vaccine strain[219], and proven to be protective in chickens during an outbreak of HPAI virus (H5N1)[220]. On the basis of the antibody titres induced and the high degree of agreement (79.2%) between post-vaccination HI titres against the vaccine strain (H7N1) and VN titres against the HPAI virus strain (H7N7), vaccination may be expected to be efficacious during an outbreak. In analogy with vaccination of poultry, vaccination of exotic species may be expected to prevent morbidity and mortality, reduce environmental contamination with AI virus[221-223], and thus reduce or prevent subsequent bird-to-bird transmission. However, because clinically healthy birds can shed HPAI virus, enhanced bio-security measures and regular virologic monitoring of vaccinated and non-vaccinated birds in the infected area will remain necessary[195].
A booster vaccination was required to provide efficacy, using the criterion for efficacy as used by the Committee for Proprietary Medicinal Products (CPMP) for validation of human influenza vaccines (antibody titre ≥40 in ≥70% of the vaccinated population[224]); whether this criterion is valid for use in these avian species is unknown. In the present study only 36.2% of 116 birds developed a titre ≥40 after one vaccination, compared to a total percentage of 80.2% after the second vaccination. This booster effect has previously been shown in chickens and turkeys after AIV vaccination with 2 weeks interval[225], and is expected to be long-lasting[226]. However, the necessity for a booster requires vaccination to occur soon after detection of an outbreak, as some time is required to build up immunity.
\The GMT after two vaccinations were significantly different between orders (Fig. 1). Galliformes, the order for which the vaccine had been specifically produced, but also Phoenicopteriformes, reacted to vaccination with the highest GMT. Differences in neutralising antibody response to vaccination have been demonstrated between domestic avian species, with chickens producing highest titres[227]. More than 70% of birds from the orders Anseriformes, Galliformes, and Phoenicopteriformes developed

titres ≥40 in response to vaccination (Fig. 1), and the vaccine may therefore be considered efficacious in these orders.
The vaccine dose given to Struthioniformes, which are considered to be very susceptible to AIV infection[206], and have significantly higher mean weight than the other orders [Fig. 1: 29.7 kg (95% CI: 17.7–41.6)], was likely too low. The humoral immune response to AIV infection in chickens is known to be associated with the virus doses administered[209,228]. Therefore, based on the low % seroprotection and low GMT, which is inversely correlated with published mean body weight (Fig. 1), Struthioniformes are expected to respond better to a higher vaccine dose.
Route of vaccine administration (s.c. or i.m.) had no large effect on antibody response, therefore mode of administration was not regarded as a factor influencing antibody response in the statistical analysis of the results. However, formal statistical comparison between s.c. and i.m. administration with regard to antibody response was not possible, as administration was not at random, but differed per zoo, and distribution of orders among zoos was not similar.
The detection of HPAI H7N7 virus infection in mallards and swans kept in the same enclosure as infected chickens and guinea fowl demonstrates that this strain of HPAI H7N7 virus can be transmitted from poultry to other avian species. This illustrates the potential threat of transmission of HPAI H7N7 from poultry to other avian species.
In conclusion, vaccination is expected to be a useful and necessary tool for the protection of valuable exotic birds in zoos against HPAI H7 virus infection, and is a suitable alternative for confinement and pre-emptive culling when implemented together with virologic monitoring and strict bio-security measures at zoos within, or close to the infected area. The administration of twice 0.25 ml to birds <1.5 kg, and twice 0.5 ml to birds ≥1.5 kg, either by i.m. or s.c. route, is efficacious for Anseriformes, Galliformes, and Phoenicopteriformes, but possibly not for the other orders. Because of a negative correlation between antibody response and mean body weight, a higher dose may be required in Struthioniformes. A booster vaccination is essential for efficacy, therefore vaccination of zoo birds should commence soon after detection of an outbreak in poultry in the area. We recommend further research into the efficacy of vaccinating all orders of exotic birds against H7 and H5 AIV with a shorter interval before booster vaccination, and with higher doses for birds with high mean body weight.

Acknowledgements
We would like to thank Intervet for supplying the vaccine free of charge, and the veterinarians and all other people involved in catching and handling the birds in the participating zoos: Apenheul Primate Park Apeldoorn, Artis Amsterdam Zoo, Beekse Bergen, Burger's Zoo, Dierenpark Amersfoort, Ouwehands dierenpark, Plaswijck park, Overloon Zoo Parc, Rotterdam Zoo, Wisselzoo Epe. We are very grateful to the people from RVV Stroe, Woudenberg, municipalities in the infected area, Camping 'de Lucht', Groenservice Zuid Holland, and Ger van de Water for assisting with collection of free-ranging birds.



10

The molecular basis of pathogenicity of the Dutch HPAI H7N7 human influenza viruses

Submitted

Vincent Munster*
Emmie de Wit*
Debby van Riel
Walter Beyer
Guus Rimmelzwaan
Albert Osterhaus
Thijs Kuiken
Ron Fouchier

* These authors contributed equally to the results of this study

During the H7N7 highly pathogenic avian influenza (HPAI) outbreak in The Netherlands in 2003, 88 infected persons suffered from mild illnesses and one died of pneumonia. Here, we studied which of the 14 amino acid substitutions observed between the fatal case (FC) virus and a conjunctivitis case (CC) virus determined differences in virus pathogenicity. Using virus attachment studies, the CC and FC viruses revealed marked differences in binding to the lower respiratory tract of humans. In a mouse model, the HA gene of the FC virus was a determinant of virus tissue distribution. The lysine at position 627 of PB2 of the FC virus was the major determinant of pathogenicity and tissue distribution. Thus, remarkable similarities were revealed between recent HPAI H5N1 and H7N7 viruses. We conclude that the influenza virus HA and PB2 genes should be the prime targets for molecular surveillance during outbreaks of zoonotic HPAI viruses.

Introduction

The last decade has seen a marked increase in the number of outbreaks caused by highly pathogenic avian influenza (HPAI) viruses in domestic birds, some of which were subsequently transmitted to wild birds and a wide variety of mammalian species including humans[37,38,52,68,229,230]. Low pathogenic avian influenza viruses of the H5 and H7 subtypes that are circulating in the wild bird reservoir are the progenitors of HPAI viruses[73,231]. The switch from a low to high pathogenic phenotype in domestic birds is predominantly determined by the introduction of basic amino acids in the cleavage site of the hemagglutinin (HA). The molecular basis of the pathogenicity of HPAI viruses in humans is still poorly understood. Recent studies suggested that the viral ability to bind to sialic acid (SA) receptors present on the cells of the new host[232,233], efficient replication in these cells[234-236] and evasion of the hosts' immune response[237,238] contribute to pathogenicity.

During an H7N7 HPAI outbreak in The Netherlands in 2003, viruses were transmitted to humans who came in close contact with infected poultry. Of 89 laboratory-confirmed cases of human H7N7 infection, most suffered from conjunctivitis and a few from mild influenza-like illness. One veterinarian died as the result of pneumonia followed by acute respiratory distress syndrome and related complications[55,161]. Infection of the human eye, resulting in conjunctivitis, had been described previously for H7 influenza A viruses[166]. It was suggested that this may be due to the α2,3-linked SA binding preference of avian influenza A viruses and the presence of α2,3-linked SA on epithelial cells in the human cornea and conjunctiva, in contrast to the predominant presence of α2,6-linked SA in the human upper respiratory tract[239].

Sequence analyses of the virus isolated from the fatal H7N7 case (A/Netherlands/219/03, FC) revealed 14 amino acid substitutions as compared to viruses isolated from chickens and human cases of conjunctivitis (such as A/Netherlands/33/03, CC), in 5 different gene segments, PB2 (5), PA (1), HA (3), NA (4) and NS (1)[55]. One or more of these amino acid substitutions might have been responsible for higher virus pathogenicity and the fatal outcome of the infection.

Here, we describe the patterns of attachment of two prototype HPAI H7N7 viruses from the Dutch HPAI outbreak to human ocular and respiratory tissues and study

the molecular determinants of pathogenicity of these viruses in a mouse model. The increased understanding of the molecular determinants of efficient replication and spread of avian viruses in humans may lead to a targeted monitoring of HPAI viruses during future zoonotic influenza outbreaks.

Methods

Viruses

Influenza virus A/Netherlands/33/03 (H7N7) and A/Netherlands/219/03 (H7N7) were isolated from a patient suffering from conjunctivitis and the fatal case respectively, during the Dutch H7N7 outbreak[55]. The gene segments of the FC and CC viruses were amplified by RT-PCR and cloned and recombinant H7N7 influenza viruses were generated by reverse genetics as described previously and propagated in embryonated chicken eggs[185]. We generated recombinant FC and CC viruses and reassortants consisting of seven gene segments of the CC virus and one of the FC virus (CC-FC PB2, CC-FC PA, CC-FC HA, CC-FC NA and CC-FC NS). Mutant viruses containing an E627K mutation in PB2 of the CC virus (CC-PB2 E627K) or a K627E substitution in PB2 of the FC virus (FC-PB2K627E) were also produced. The genotypes of all recombinant viruses were confirmed by sequencing.

Virus histochemistry

Virus histochemistry using FC and CC viruses was performed as described previously[233,240]. After concentration and purification of virus stocks using sucrose gradients, viruses were inactivated by dialyses against 0.1% formalin and labeled with an equal volume of 0.1 mg/ml of fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Zwijndrecht, the Netherlands).

Formalin-fixed paraffin-embedded tissues were deparaffinized with xylene and rehydrated with alcohol. FITC-labeled influenza viruses (50-100 hemagglutination units) were incubated with the tissues overnight at 4°C. The FITC label was detected with a peroxidase-labeled rabbit-anti-FITC (DAKO, Heverlee, Belgium). Tissues were counterstained with hematoxylin and embedded in glycerol-gelatin (Merck, Amsterdam, the Netherlands).

To validate the method, we incubated labeled FC virus and H3N2 virus (A/Netherlands/213/03) with human trachea and mallard intestine. The pattern of attachment of both viruses to these tissues was as expected. FC virus bound abundantly to epithelial cells in duck intestine and bound rarely to human trachea. In contrast, H3N2 virus, bound abundantly to ciliated epithelial cells in human trachea and bound poorly to duck intestine.

Mouse model

Groups of six 6-8 week old female Balb/c mice (Harlan, Horst, The Netherlands) were inoculated intranasally with H7N7 viruses. The animals were observed for clinical signs and weighed twice daily as an indicator of disease. At day 3 after inoculation, 3 animals from each group were euthanized and virus titers in the lungs, spleen, liver, kidneys and brain were determined. The other 3 animals were euthanized 7 days after inoculation or upon development of severe disease or discomfort in agreement

with national animal welfare regulations. All intranasal inoculations and euthanasia were performed under anesthesia with inhaled isoflurane. All animal studies were approved by an independent Animal Ethics Committee. All experiments were performed under BSL3+ conditions.

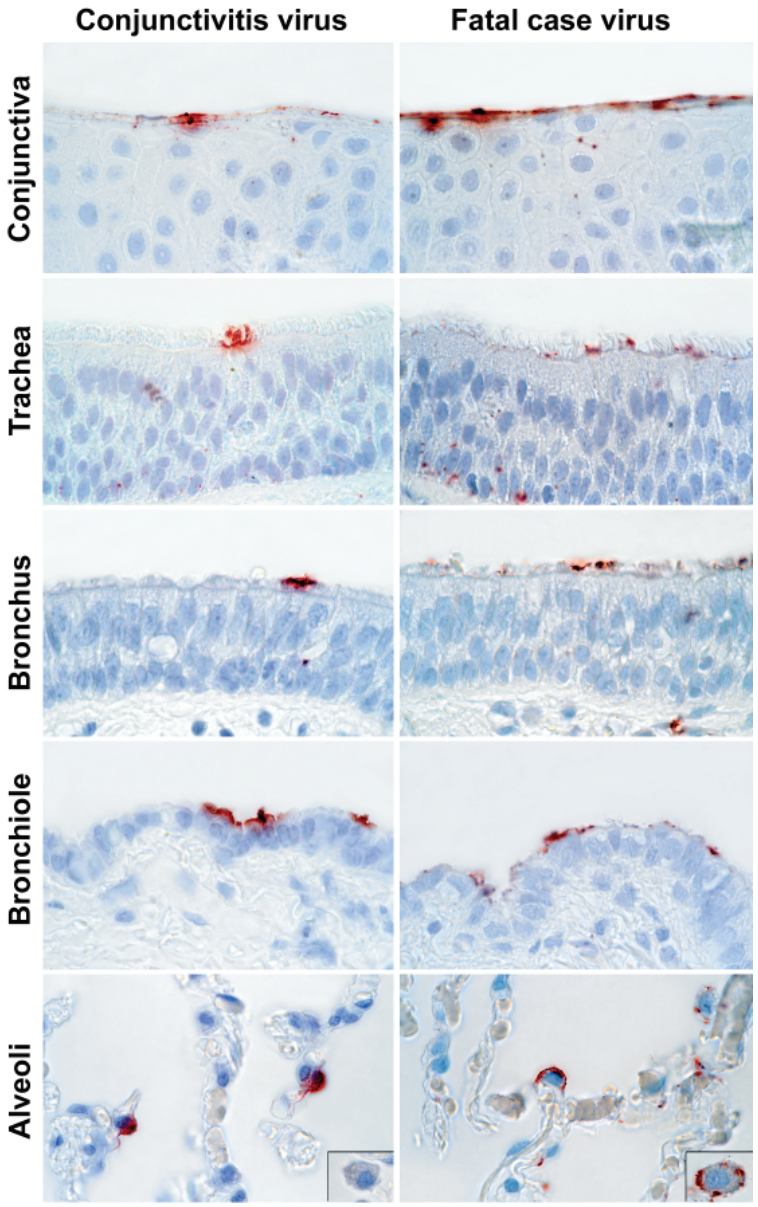


Figure 1. Attachment pattern of the CC virus and FC virus to human ocular tissues, including cornea and bulbar conjunctiva and tissues of the human lower respiratory tract, bronchus, bronchiolus and alveoli, and trachea. Virus attachment is shown in red. Inset in lower panel shows attachment to alveolar macrophages. (original magnification: 100x)

Virus titrations

Viruses were titrated by end-point dilution in MDCK cells as described previously[185]. Lungs, spleen, liver, kidneys and brain were collected and homogenized as described previously[241]. Tenfold serial dilutions of tissue homogenates were used to inoculate MDCK cells. Three days after inoculation, the supernatants of infected cell cultures were tested for agglutinating activity using turkey erythrocytes as an indicator of infection. Infectious titers were calculated from 5 replicates by the method of Spearman-Karber[242].

Histopathology and immunohistochemistry

Histopathological and immunohistochemical studies were performed for mice inoculated with the FC, CC, CC-FC PB2, CC-FC HA, CC-PB2 E627K and FC-PB2 K627E viruses. 4 mice were euthanized 3 days after inoculation by exsanguination under general anesthesia with isoflurane. Necropsies and tissue sampling were performed according to a standard protocol. After fixation in 10 % neutral-buffered formalin and embedding in paraffin, tissue sections were stained with hematoxylin and eosin for histological evaluation or with an immunohistochemical method using a monoclonal antibody against the nucleoprotein of influenza A virus[243]. Trachea, lung (after inflation with 10% neutral-buffered formalin in situ; cross-sections of left lobe and right cranial, medial, and caudal lobes), brain, liver, kidney, and spleen were examined. For semi-quantitative assessment of influenza virus-associated inflammation in the respiratory tract, each hematoxylin and eosin stained section of trachea and lung was examined for the presence of epithelial necrosis and infiltration by inflammatory cells and scored as mild (0-10 % of tracheal mucosa, bronchiolar cross-sections, or alveolar area affected), moderate (10-50 % affected), or severe (50-100 % affected).

Results

H7N7 virus attachment

We investigated the role of human conjunctiva as potential *porte d'entrée* for H7N7 influenza A viruses. Using virus histochemistry[233,240], we studied the attachment of the FC and CC viruses to human ocular tissues including cornea and the bulbar conjunctiva. Both viruses attached to the epithelium of the cornea and conjunctiva (Fig. 1). The human ocular tissues may thus represent a site of entry for H7N7 viruses. This was shown for both the CC and the FC virus, despite the fact that the FC virus was isolated from the lower respiratory tract (LRT) and no virus was detected in conjunctiva swabs from this patient[55].

To determine whether differences in pattern of viral attachment could help explain the difference in outcome of disease for the CC and FC viruses, we compared their attachment patterns to human trachea and tissues of the LRT (bronchus, bronchiole and alveoli). There was no difference in attachment to the trachea or bronchus between the CC and FC viruses (Fig. 1). Attachment was observed to only few ciliated epithelial cells, with more abundant binding to the epithelial cells and excreted mucus of the submucosal glands. In the bronchioles, both viruses attached to ciliated cells and non-ciliated cuboidal cells, but the FC virus attached more abundantly to non-

ciliated cuboidal cells. In the alveoli, both viruses predominantly attached to type II pneumocytes, but the FC virus showed more abundant attachment to alveolar macrophages (Fig. 1), while the CC virus showed more abundant attachment to type I pneumocytes. Thus, the attachment pattern of the FC virus differed from that of the CC virus in the alveoli, which was the site of the primary lesion -diffuse alveolar damage- in the person who died from this infection[30]. Furthermore, the attachment pattern of the FC virus showed marked similarities to the attachment pattern of a recent HPAI H5N1 virus[233].

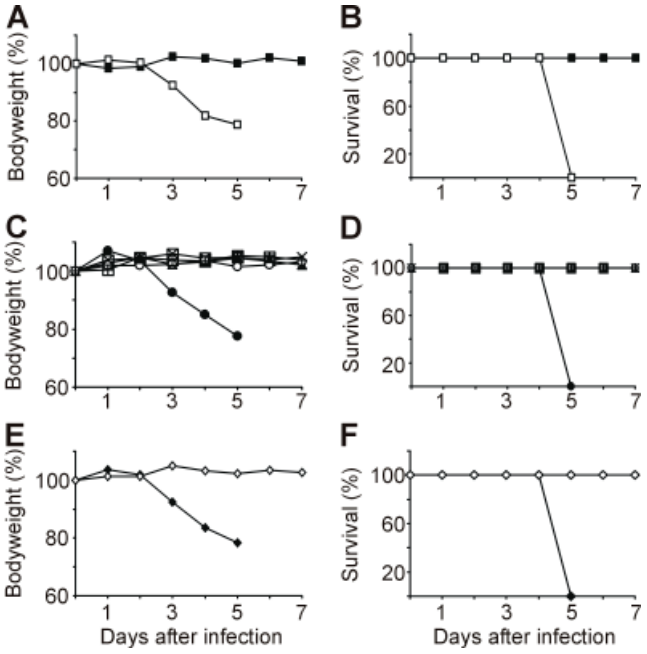


Figure 2. Loss of bodyweight and survival after intranasal infection of mice with HPAI H7N7 viruses. Groups of six mice were infected intranasally with $5x10^2$ $TCID_{50}$ of the CC (■), FC (□), CC-FC PB2 (●), CC-FC PA (x), CC-FC HA (▲), CC-FC NA (○), CC-FC NS (+), CC PB2 E627K (♦) or FC PB2 K627E (◊) virus. Following infection, mice were weighed daily. Percentage bodyweight was calculated as compared to bodyweight at time of infection (A, C, E). Mice were either sacrificed due to the severity of their disease signs at day 5 or at the end of the experiment on day 7 after infection. The percentage of mice surviving the infection as a function of time is shown (B, D, F).

A mouse model for infection with the CC and FC virus

We developed a mouse model in which we could distinguish the pathogenicity of the CC and FC virus. Groups of six female Balb/C mice were inoculated with the CC and FC viruses generated by reverse genetics, and mice were weighed twice daily and observed for clinical signs of disease. When inoculated with high virus doses, mice became severely ill and required euthanasia due to the severity of symptoms, irrespective of whether they were infected with the CC or FC virus (data not shown). Mice inoculated with $5x10^2$ $TCID_{50}$ of the CC virus showed no signs of disease and lost no bodyweight during a 7-day observation period. Mice inoculated with the same dose of the FC virus showed signs of disease such as loss of bodyweight (fig. 2A),

ruffled fur, lethargy, and respiratory problems from 2 days after inoculation onwards. These mice had to be euthanized due to the severity of symptoms 5 days after inoculation whereas mice inoculated with the CC virus survived the infection (Fig. 2B). We used a dose of $5x10^2$ $TCID_{50}$ to inoculate mice in all further experiments.
On day 3 after inoculation, 3 mice from each group were euthanized and virus titers in the lungs, spleens, livers, kidneys and brains were determined. In the lungs of mice inoculated with the FC virus, virus titers were >1000-fold higher than in the lungs of mice inoculated with the CC virus (Fig.3). In all mice inoculated with the FC virus, virus could be detected in spleen, liver, kidneys and brain, whereas virus could not be detected outside the lung in mice inoculated with the CC virus, except for one mouse where virus was detected in the brain (Fig. 3, black bars, viruses A and B).

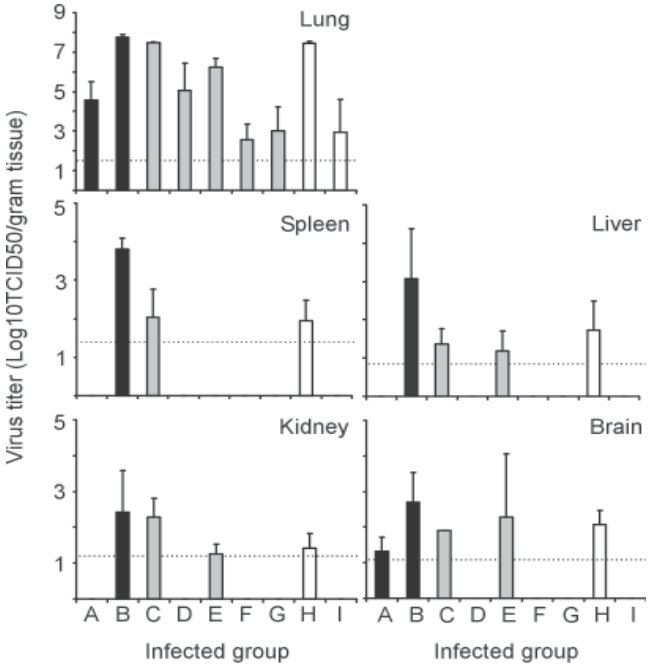


Figure 3. Virus titers in the mice after infection with HPAI H7N7 virus. Mice were infected intranasally with $5x10^2$ $TCID_{50}$ of the CC (A), FC (B), CC-FC PB2 (C), CC-FC PA (D), CC-FC HA (E), CC-FC NA (F), CC-FC NS (G), CC PB2 E627K (H) or FC PB2 K627E (I) virus. On day 3 after challenge three mice from each group were sacrificed, tissues were collected and virus titers in lungs, spleen, liver, kidney and brain were determined in MDCK cells. The geometric mean virus titer per group was calculated. To calculate the geometric mean, the cut off value was used for negatives. The dotted line indicates the cut off value of the assay for each of the organs; error bars indicate standard deviation.

Histopathological examinations performed on 4 mice at day 3 after inoculation revealed that in mice inoculated with the FC virus, lesions occurred throughout the respiratory tract and consisted of necrosis and inflammation (Table 1, Fig. 4). These lesions were most pronounced in the trachea, and were progressively milder in the bronchi, bronchioles, and alveoli. Lesions in the trachea, bronchi, and bronchioles were characterized by necrosis or loss of epithelial cells, infiltration of the epithelium

and subepithelial connective tissue with neutrophils and lymphocytes, and presence of cell debris mixed with erythrocytes and neutrophils in the lumen (Fig. 4).

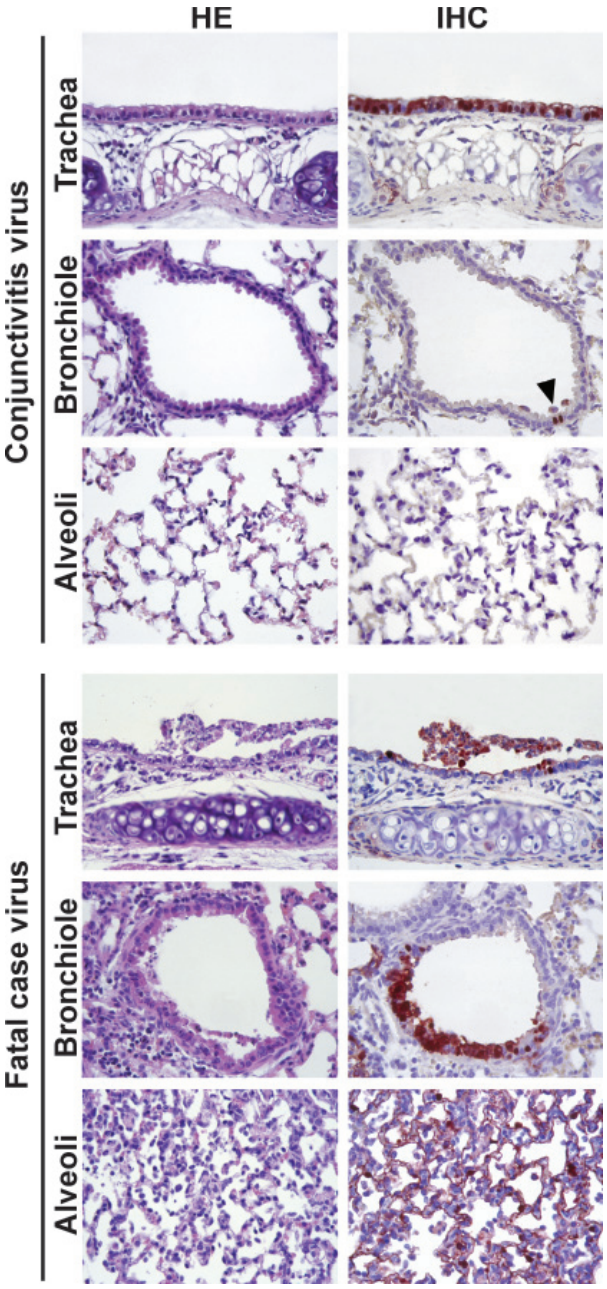


Figure 4. Histopathological analysis of respiratory tract tissue of mice infected with the CC or FC virus. Mice were infected with $5x10^2$ $TCID_{50}$ of the CC and FC virus and sacrificed at day three after infection. Tissue sections were stained with hematoxylin and eosin (HE) or with a monoclonal antibody against NP (IHC). (Original magnification 40x)

Lesions in the alveoli were centered around bronchioles, and were characterized by thickening and hypercellularity of the alveolar walls, and flooding of the alveolar lumina with variable proportions of cell debris, fibrin, edema fluid, erythrocytes, neutrophils, and mononuclear cells (Fig. 4). No lesions were detected in brain, heart, spleen, liver or kidney despite virus recovery from these organs. In mice inoculated with the CC virus, lesions were restricted to the respiratory tract of only one mouse (Table 1) and consisted of mild to moderate epithelial cell necrosis and infiltration by neutrophils in trachea, bronchi, and bronchioles. No lesions were detected in any of the tissues of the sham-inoculated mice.
By immunohistochemistry using an α-NP monoclonal antibody, expression of influenza virus antigen was limited to respiratory tract tissues. The more abundant presence of NP positive cells in the lesions of mice inoculated with the FC virus corresponded to the higher virus titers in the lungs of these mice (Table 1, Fig. 4). Influenza virus antigen expression was seen in ciliated and non-ciliated epithelial cells in trachea, bronchi, and bronchioles, and in type I and type II pneumocytes in the alveoli. Influenza virus antigen expression was associated with the presence of histological lesions and was generally strongest at the transition of normal and necrotic tissue.

Mapping the determinants of pathogenicity of the FC virus
To determine which gene segment was responsible for the increased pathogenicity of the FC virus, we constructed 5 reassortant viruses consisting of 7 gene segments of the CC virus and each of the gene segments of the FC virus harboring amino acid substitutions. Mice were inoculated and weighed twice daily and observed for clinical signs of disease. Only mice inoculated with CC-FC PB2 lost bodyweight and showed other symptoms of disease (Fig. 2C), while mice inoculated with CC-FC PA, CC-FC HA, CC-FC NA and CC-FC NS did not display any symptoms. Mice inoculated with CC-FC PB2 required euthanasia due to the severity of symptoms on day 5 (Fig. 2D). Like with the FC virus, mice inoculated with CC-FC PB2 had high virus titers in the lungs (Fig.3, grey bar, virus C). In mice inoculated with CC-FC PA, CC-FC NA and CC-FC NS, virus titers in the lungs were comparable to those in mice inoculated with the CC virus. The CC-FC HA virus displayed intermediate virus titers in the lungs. Virus was detected in spleen, liver, kidney and brain of mice inoculated with CC-FC PB2, but not in mice inoculated with CC-FC PA, CC-FC NA and CC-FC NS (Fig. 3, grey bars, viruses C-H). With the CC-FC HA virus, virus was detected in liver, kidney and brain, although not all organs were positive in all mice from this group. By histopathology and immunohistochemistry, the nature and severity of the lesions caused by the CC-FC PB2 virus did not differ from the lesions caused by the FC virus. Lesions caused by the CC-FC HA virus were comparable to those caused by the CC virus (Table 1).

Mapping the determinants of pathogenicity in PB2
Studies with HPAI H5N1 viruses have shown that an E627K substitution in PB2 was the main determinant of virulence[234,235]. Since this substitution is present in the FC virus, we determined whether it is also important for pathogenicity of HPAI H7N7 viruses. We constructed the CC virus with the E627K substitution in PB2 (CC-PB2 E627K) and the FC virus with a K627E substitution in PB2 (FC-PB2 K627E) and

inoculated groups of six mice. This E627K substitution reversed the phenotype of the FC and CC viruses. Mice inoculated with CC-PB2 E627K virus showed signs of disease, including loss of bodyweight from 2 days after inoculation onwards, whereas mice inoculated with FC-PB2 K627E virus did not (Fig. 2E). Consequently, mice inoculated with CC-PB2 E627K virus required euthanasia due to severity of symptoms at day 5, while mice inoculated with FC-PB2 K627E virus survived (Fig. 2F). Lung titers at day 3 in mice inoculated with CC-PB2 E627K virus were comparable to those in mice inoculated with wild type FC virus, while those in mice inoculated with FC-PB2 K627E virus were comparable to those in the CC virus inoculated mice (Fig. 3, white bars, viruses I and J). CC-PB2 E627K virus was also detected in spleen, liver, kidney and brain whereas FC-PB2 K627E was not. By histopathology and immunohistochemistry, the FC-PB2 K627E virus caused lesions comparable to the CC virus, whereas the CC-PB2 E627K virus caused lesions comparable to the FC virus (Table 1).

Table 1. Pathological analyses of respiratory tract tissues of mice infected with recombinant H7N7 viruses.

	trachea			bronchioles			alveoli		
	# of mice with		severity of	# of mice with		severity of	# of mice with		severity of
	antigen	lesion	lesion	antigen	lesion	lesion	antigen	lesion	lesion
mock	0	0	n.a.	0	0	n.a.	0	0	n.a.
CC	2	1	moderate	4[2]	1	mild	1[2]	0	n.a.
FC	4	4	severe	4	4	moderate/severe	4	4	mild/moderate
CC-FC PB2[1]	3[2]	3	severe	3	3	moderate/severe	3	3	mild/moderate
CC-FC HA	2	0	n.a.	1	1	mild	0	0	n.a.
CC-PB2 E627K	4	4	severe	4	4	mild/severe	4	4	mild/moderate
FC-PB2 K627E	3[2]	1	mild	4[2]	2	mild	3[2]	0	n.a.

[1] Only three mice were infected with this virus

[2] Antigen could be detected in only a few cells

Discussion

The closely related FC and CC viruses from the 2003 Dutch H7N7 outbreak offered a unique opportunity to study the determinants of pathogenicity of H7 viruses in *in vitro* and *in vivo* model systems. In mice, we could distinguish between the pathogenicity of the CC and FC virus. Although both viruses were lethal to mice at high doses, the FC virus was more pathogenic at a low dose. At a dose of $5x10^2$ $TCID_{50}$, the CC virus did not induce disease, whereas infection with the FC virus was lethal. Moreover, the FC virus was detected in spleen, liver, kidney and brain whereas the CC virus was confined to the lungs. Histopathologically, clear differences were observed in the respiratory tract of infected mice. Mild to moderate lesions could be detected in only 1 out of 4 mice at day 3 after inoculation with the CC virus, while with the FC virus severe lesions were detected in all mice. These data indicate that the FC virus is intrinsically more pathogenic to mice than the CC virus, and could explain why the FC presented with such severe symptoms and died.

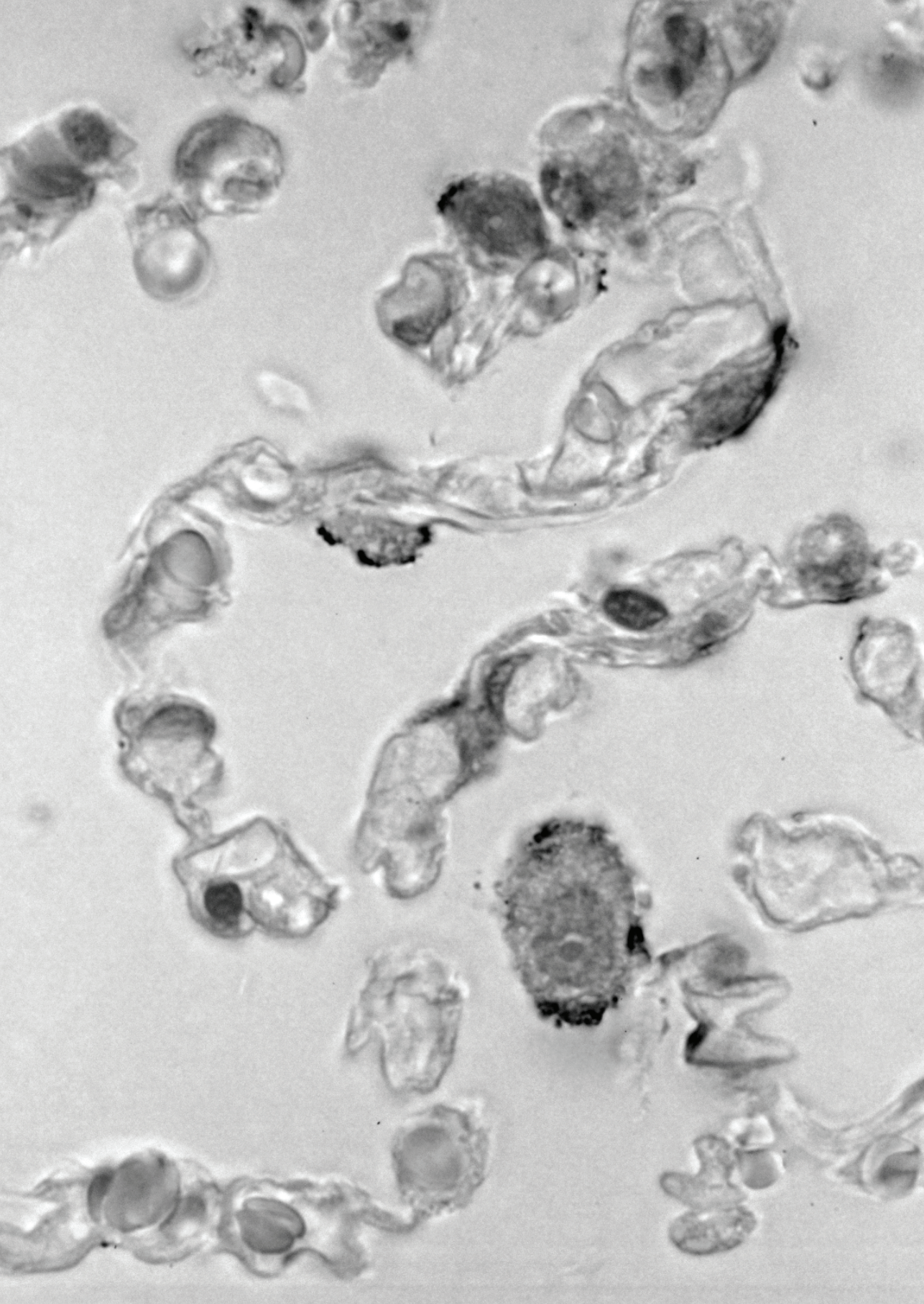
By producing single-gene reassortant and mutant viruses between the CC and FC virus and testing these in the mouse model, we showed that the pathogenicity of the FC virus in mice was not determined by the PA, NA or NS genes. Virus titers in lungs of mice inoculated with CC-FC HA were higher than in mice inoculated with the CC virus, and the virus was detected outside the lung, indicating that the FC HA gene contributed to enhanced virus replication and tissue distribution. From the mouse studies, we could not determine whether the effect of HA on spread of virus to different organs was due to altered receptor specificity/affinity or merely resulted from higher virus titers in the lung in a receptor-independent mechanism. By virus histochemistry, the FC virus showed a pattern of attachment to human respiratory tissues that could partly explain its pathogenicity. Both the CC and the FC virus showed only limited binding to tracheal epithelium, in contrast to a human H3N2 virus, which bound abundantly to this tissue[233]. The FC virus attached more abundantly than the CC virus to non-ciliated cuboidal cells in the bronchioles and to alveolar macrophages. In contrast, the CC virus showed more abundant binding to type I pneumocytes. These subtle differences in attachment between the CC and FC virus in the LRT of humans could indicate differences in specificity or affinity for the SA-residues present in the human host. The attachment pattern of the FC virus to the human respiratory tract showed great similarity with the patterns observed with A/Vietnam/1194/04, a recent H5N1 HPAI isolate[233], and is in agreement with the distribution of α2,3-linked SA in the human respiratory tract[232]. The viral attachment patterns of both A/Vietnam/1194/04 and the FC virus correspond to the pathological findings in fatal cases of H5N1 and H7N7 infection[52,55], which show diffuse alveolar damage in the lower pulmonary lobes. It has been shown that HPAI H5N1 viruses are potent inducers of the production of proinflammatory cytokines by macrophages[244] and it was suggested that this induction of cytokines may relate to the unusual severity of disease caused by HPAI H5N1 virus in humans[244]. The abundant attachment of the FC virus to alveolar macrophages may imply that similar mechanisms increase the pathogenicity of the FC virus.

It has been shown previously that mutations in the globular head of HA can have an effect on pathogenicity. Perdue *et al.* showed that addition of a glycosylation site in a H7N7 HA resulted in increased pathogenicity of this virus in chickens[245]. Furthermore, it was shown that A/HongKong/483/97 (H5N1) with a serine at position 227 of HA induced higher lung titers in mice than a virus with isoleucine at this position. The virus with serine could be detected outside the lung, whereas the virus with isoleucine could not[234]. Moreover, the addition of a glycosylation site near the receptor binding site was found to alter receptor binding affinity and/or specificity of an H5N1 virus[246].

The main determinant of pathogenicity of the FC virus in mice was PB2, more specifically the lysine at position 627 of this protein. Inoculation of mice with a double reassortant virus, CC-FC PB2 HA, did not result in increased pathogenicity as compared to the single reassortant CC-FC PB2 (data not shown). Pathogenicity studies using HPAI H5N1 viruses have also shown a prominent role for the lysine at position 627 of PB2 in mice[234,235], but not in a ferret model[235]. Since this lysine is present in the 1918 Spanish influenza virus and all subsequent human lineages of influenza A viruses and in many recent mammalian H5 HPAI isolates, but is absent in all but one of the published avian virus PB2 sequences, it is likely an important

determinant of efficient virus replication in humans[53,247].
Taken together, the data presented here lead us to suggest that the virus isolated from the fatal case either entered via the ocular epithelium and gained access to the LRT, e.g. via the lacrimal duct, or that the virus accessed the LRT directly. During the infection, the PB2 E627K substitution and one or more substitutions in HA allowed efficient replication of this virus in the LRT, leading to pneumonia, acute respiratory distress syndrome and eventually death. Given the similarities between pathogenesis studies on zoonotic H5N1 and H7N7 viruses, and the recorded changes in the pandemic influenza viruses that emerged in the last century, the HA and PB2 genes should be considered prime targets for genetic characterization during HPAI outbreaks.

Acknowledgements
We thank the Cornea Bank Netherlands Ophtalmic Research Institute and George Verjans for providing ocular tissues and Fiona Read, Frank van der Panne, Robert Dias d'Ullois and Geert van Amerongen for excellent technical assistance.



11

Determinants of enhanced replication of human H7N7 influenza A virus isolates

In preparation

Emmie de Wit*
Vincent Munster*
Debby van Riel
Brigitte van Uden
Walter Beyer
Guus Rimmelzwaan
Thijs Kuiken
Albert Osterhaus
Ron Fouchier

* These authors contributed equally to the results of this study

In 2003, an outbreak of highly pathogenic avian influenza (HPAI) virus (H7N7) occurred in The Netherlands, during which 89 humans were infected. Most persons suffered from conjunctivitis and, occasionally, mild respiratory disease. There was one fatal case of pneumonia. The virus isolated from the fatal case (FC) differed in 14 amino acid positions in five gene segments from a prototype conjunctivitis case (CC) virus. Here we show that each of the five gene segments that harbored amino acid substitutions contributed to increased virus replication efficiency in mammalian and avian cells *in vitro*. In minigenome assays, PB2 of the FC virus caused a large increase in reporter expression levels. This increase was caused by the E627K substitution in PB2 of the FC virus and was independent of temperature and host cell. The HA gene of the FC virus resulted in increased virus replication in reassortant viruses. This effect was caused by a single amino acid substitution, A143T, that also affected the attachment pattern of the FC virus to tissues of the human respiratory tract. The inefficient rescue of viruses with NA of the CC virus indicated that NA had a large effect on virus replication *in vitro*. Site-directed mutagenesis indicated that multiple substitutions were responsible for the NA phenotype. We did not observe differences in interferon-antagonism function of FC and CC NS1 genes.

We conclude that the pathogenic properties of the FC virus could be determined by genetic changes in at least four genes that affect virus replication *in vitro*. Detailed analyses of mutations that facilitate replication of avian viruses in mammalian cells remains important to assess the risks posed by such zoonotic viruses.

Introduction

Aquatic birds represent the natural reservoir for avian influenza A virus[1]. When low pathogenic avian influenza A (LPAI) viruses of the H5 or H7 subtypes are transmitted from wild birds to poultry, they may mutate into highly pathogenic avian influenza (HPAI) viruses[231]. The switch from a LPAI virus phenotype into a HPAI virus phenotype is determined by the introduction of multiple basic amino acid residues at the hemagglutinin (HA) cleavage site[8,231]. HPAI viruses of both the H5 and H7 subtypes have been transmitted directly to humans on several occasions. The direct transmission of HPAI viruses into humans leading to fatality was first described in 1997 during an outbreak of HPAI H5N1 in poultry in Hong Kong, when 18 people became infected and 6 died[35]. Since 2003, more than 200 humans were infected with HPAI H5N1 with a mortality rate of >50%[248].

In 2003, there was an outbreak of HPAI virus of the H7N7 subtype in The Netherlands. In order to control the outbreak that lasted approximately 3 months, 30 million chickens were culled. 86 people in close contact with infected poultry (cullers, farmers and veterinarians) became infected with H7N7 and, in addition, there were three probable cases of human-to-human transmission[55,161]. Most of these 89 persons suffered from conjunctivitis and/or mild influenza-like illness, but there was also one case of severe pneumonia leading to acute respiratory distress syndrome and ultimately death. Sequencing analysis revealed that conjunctivitis

viruses such as A/Netherlands/33/03 (CC) were almost identical to the prototype chicken virus, A/chicken/Netherlands/1/03. The virus isolated from the fatal case, A/Netherlands/219/03 (FC), harbored 14 amino acid substitutions in five different gene segments: PB2 (amino acid (a.a.) positions 79, 297, 355, 563, 627), PA (a.a. position 666), HA (a.a. positions 13, 143, 416), NA (a.a. positions 308, 346, 442, 458) and NS1 (a.a. position 137) when compared with the CC virus[55], suggesting rapid evolution within the fatal case of a virus originating from poultry. The closely related human H7N7 influenza A viruses from the Dutch outbreak offered an excellent opportunity to study the molecular determinants of pathogenicity of human HPAI viruses. Most of the gene segments of the FC virus that harbor amino acid substitutions have been implicated as determinants of pathogenicity in other influenza viruses. Substitutions in the PB2 gene have been described that increased the virulence of H5N1 HPAI viruses in mice and that enabled efficient replication of avian viruses in mammalian cells[234,235,249,250]. The HA of avian influenza viruses preferentially binds to α2,3-linked sialic acids (SA), whereas the HA of human influenza viruses preferentially binds to α2,6-linked SA[251]. Together with the predominant distribution of α2,6-linked SA in the upper respiratory tract of humans, this indicates that the replication of avian influenza A viruses in humans is usually restricted at the level of virus attachment[232,233]. NA is involved in facilitating the release of virus particles from the virus-infected cell surface by cleaving SA from the cell membrane and from virus particles. To warrant a proper balance between virus entry and release from the host cells, the HA-mediated receptor binding and fusion activity should be functionally compatible with the cleavage specificity and activity of NA of the virus[252]. Finally, the NS1 protein was described to be a determinant of pathogenicity by playing a key role in interference with the innate immune response of the host[237,238].

With numerous amino acid substitutions in the PB2, PA, HA, NA and NS genes of the FC virus as compared to the CC virus, there were thus multiple candidate genes that could explain the difference in pathogenicity of the two viruses and differences in the course of disease in the infected persons. In a mouse model, we have shown previously that the E627K substitution in PB2 of the FC virus is the main determinant of virus pathogenicity and tissue distribution (Munster et al., submitted for publication). Although data generated in a mouse models are very useful, they can not necessarily be directly extrapolated to humans. Using recombinant viruses with reassorted genomes and viruses with single amino acid replacements, we investigated the molecular basis of the differences in virus replication kinetics and specific steps of the virus replication cycle in *in vitro* model systems. Our data show that the determinants of efficient virus replication were distributed among different gene segments, indicating that H7N7 influenza A virus pathogenicity is a polygenic trait.

Materials and methods

Cells

Madin-Darby Canine kidney (MDCK) cells were cultured in EMEM (Cambrex, Heerhugowaard, The Netherlands) supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodiumbicarbonate, 10 mM

Hepes and non-essential amino acids. 293T cells were cultured in DMEM (Cambrex) supplemented with 10 % FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1 mM sodiumpyruvate and non-essential amino acids. QT6 cells were cultured in Medium 199 (Cambrex) supplemented with 5% FCS, 1% chicken serum, 5% triptose phosphate broth, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 0.75 mg/ml sodiumbicarbonate and non-essential amino acids.

Viruses

H7N7 influenza viruses A/Netherlands/33/03 (CC) and A/Netherlands/219/03 (FC) were isolated from patients during the H7N7 outbreak in the Netherlands in 2003[55] and passaged twice in embryonated chicken eggs. Recombinant H7N7 influenza viruses were generated by reverse genetics as described previously[185]. To this end, transient calcium phosphate-mediated transfections of 293T cells were performed as described previously[185] with plasmids encoding gene segments of the CC and FC virus. The supernatant of the transfected cells was harvested 48h after transfection and was used to inoculate embryonated chicken eggs. Wild type recombinant FC and CC virus were produced and all 60 possible reassortants thereof. Mutant viruses were produced containing a E627K mutation in PB2 of the CC virus (CC-PB2 E627K), a K627E substitution in PB2 of the FC virus (FC-PB2 K627E), a A143T mutation in HA of the CC virus (CC-HA 143T) or a T143A (FC-HA 143A) mutation in HA of the FC virus. Reassortant viruses consisting of seven gene segments encoding influenza virus A/PR/8/34 and HA of the CC or FC virus or mutants of the CC virus (PR8-CC HA, PR8-FC HA, PR8-CC HA I13S, PR8-CC HA A143T and PR8-CC HA K416R) were produced using a previously described reverse genetics system for influenza virus A/PR/8/34[185]. Viruses CC-NA N308S, CC-NA A346V, CC-NA T442A and CC-NA P458S were produced using segment 1 of the FC virus and the other segments of the CC virus. The genotypes of all recombinant viruses were confirmed by sequencing prior to use.

Plasmids

The gene segments of the CC and FC virus were amplified by RT-PCR and cloned in the BsmBI site of a modified version of plasmid pHW2000[185,188]. Gene segments 1, 2, 3 and 5 of the CC and FC virus were also cloned in the KpnI NheI sites of the eukaryotic expression plasmid pCAGGS. Plasmids pCAGGS and pISRE-CAT were kindly provided by dr. A. Garcia-Sastre (Mount Sinai School of Medicine, New York, USA).

For the construction of plasmids containing single nucleotide substitutions in PB2, HA and NA, a QuickChange Multi Site-directed mutagenesis kit (Qiagen, Venlo, The Netherlands) was used according to instructions of the manufacturer.

For construction of a vRNA-like reporter plasmid, a firefly luciferase open reading frame was PCR-amplified using primers extended with the non-coding regions (NCRs) of segment 8 of the CC virus. This insert was cloned in negative sense orientation under the control of a bacteriophage T7 RNA polymerase promoter, upstream of T7 RNA polymerase terminator.

All plasmids were sequenced using a Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands) and a 3130xl Genetic Analyser (Applied Biosystems), according to the instructions of the manufacturer.

Replication kinetics
Multi step replication kinetics were determined by infecting MDCK, QT6 or 293T cells with a multiplicity of infection (moi) of 0.01 $TCID_{50}$ per cell. Supernatants were sampled at 6, 12, 24 and 48 hours after infection and virus titers in these supernatants were determined as described below.

Virus titrations
Virus titrations were performed by end-point titration in MDCK cells as described previously[185]. MDCK cells were inoculated with tenfold serial dilutions of culture supernatants. One hour after inoculation, cells were washed once with PBS and grown in 200µl of infection media, consisting of EMEM (Cambrex, Heerhugowaard, The Netherlands) supplemented with 4% BSA, 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM glutamine, 1.5mg/ml sodiumbicarbonate (Cambrex), 10mM Hepes (Cambrex), non-essential amino acids (MP Biomedicals) and 20 µg/ml trypsin (Cambrex). Three days after inoculation, the supernatants of infected cell cultures were tested for agglutinating activity using turkey erythrocytes as an indicator of infection of the cells. Infectious titers were calculated from 5 replicates by the method of Spearman-Karber[242].

Virus histochemistry
Virus histochemistry using the CC-HA 143T and FC-HA 143A viruses was performed as described previously[233,240]. After concentration and purification of virus stocks using sucrose gradients, viruses were inactivated by dialyses against 0.1% formalin and labeled with an equal volume of 0.1 mg/ml of fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Zwijndrecht, the Netherlands).
Formalin-fixed paraffin-embedded tissues were deparaffinized with xylene and rehydrated with alcohol. FITC-labeled influenza viruses (50-100 hemagglutination units) were incubated with the tissues overnight at 4°C. The FITC label was detected with a peroxidase-labeled rabbit-anti-FITC (DAKO, Heverlee, Belgium). Tissues were counterstained with hematoxylin and embedded in glycerol-gelatin (Merck, Amsterdam, the Netherlands).

Minigenome assay
Minigenome assays were performed by transfecting 293T or QT6 cells with 5µg of pCAGGS expression plasmids encoding PB2, PB1, PA and NP, 5µg of the firefly luciferase reporter, 10µg of pAR3132 expressing T7 RNA polymerase[253] and 0.2µg of a renilla luciferase expression plasmid (pRL, Promega, Leiden, The Netherlands) as an internal control. 24h after transfection, luminescence was assayed using a Dual-Glo Luciferase Assay System (Promega) according to the instructions of the manufacturer. Luminescence was measured in a Lumistar Galaxy apparatus (BMG LABTECH GmbH, Offenburg, Germany). Relative Light Units (RLU) were calculated as the ratio of firefly and renilla luciferase luminescence. The relative luminescence was calculated as a percentage of the maximum RLU in each experiment.

Interferon assay
To measure induction or inhibition of the interferon pathways by NS1 of the CC and

FC virus, 293T cells were transfected in a 24-wells plate with 2μg of pISRE-CAT and 0.2 μg of a β-GAL construct as an internal control, or pISRE-LUC and pRL as an internal control, respectively. After transfection, cells were infected with a moi of 0.01$TCID_{50}$/cell of the CC or FC virus or with Sendai virus. 20 hours after infection cells were harvested and assayed for CAT activity using a CAT ELISA (Roche, Almere, The Netherlands), for β-GAL activity using a β-GAL ELISA (Roche), and for luciferase using a Dual-Glo Luciferase Assay System, according to the instructions of the manufacturer.

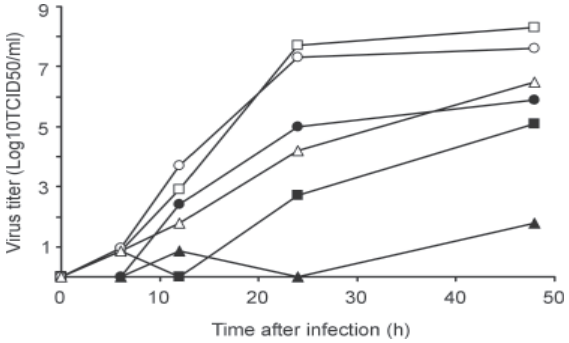


Figure 1. Replication kinetics of the CC and FC virus in MDCK, QT6 and 293T cells. Supernatants of MDCK (□), QT6 (○) and 293T (Δ) cells infected with 0.01 $TCID_{50}$/cell were harvested at 6, 12, 24 and 48 hours after infection. Filled symbols: CC virus; open symbols: FC virus. Geometric mean titers were calculated from two independent experiments.

Results

Replication kinetics of the CC and FC virus

The replication kinetics of the CC and FC viruses were studied in cells of different hosts: quail QT6 cells, canine MDCK cells and human 293T cells. In all three cell lines, the FC virus replicated to higher titers than the CC virus (Fig.1). This difference was most pronounced in human 293T cells in which the CC virus replicated very poorly; the maximum virus titers after 48 hours were $6.3x10^1$ $TCID_{50}$/ml, $5x10^4$-fold lower than for the FC virus. In quail QT6 cells, the differences in maximum virus titers after 48 hours were more modest, with 25-fold higher titers for the FC virus than the CC virus. The differences in replication kinetics between the FC and CC virus in canine MDCK cells were 792-fold, intermediate to 293T and QT6 cells. Thus, although some differences were observed in the susceptibility of the three cell lines to the two influenza A viruses, with QT6 and MDCK allowing more efficient virus replication than 293T cells, the higher replication kinetics of the FC virus as compared to the CC virus was consistent among different cell lines.

Replication kinetics of reassortant viruses

To determine which of the gene segments containing amino acid substitutions affect the replication kinetics of the FC virus, we produced five reassortant viruses consisting of seven gene segments originating from the CC virus and one gene segment with

substitutions originating from the FC virus: CC-FC PB2, CC-FC PA, CC-FC HA, CC-FC NA and CC-FC NS. Replication kinetics of these viruses were determined in MDCK cells. Each of the reassortant viruses replicated more efficiently than the CC virus, but none of the reassortants replicated as efficiently as the FC virus, indicating that multiple gene segments contributed to the efficient replication of the FC virus (Fig. 2). This same effect was observed in QT6 cells, where all reassortant viruses except CC-FC NS showed replication kinetics intermediate between the CC and the FC virus (data not shown).

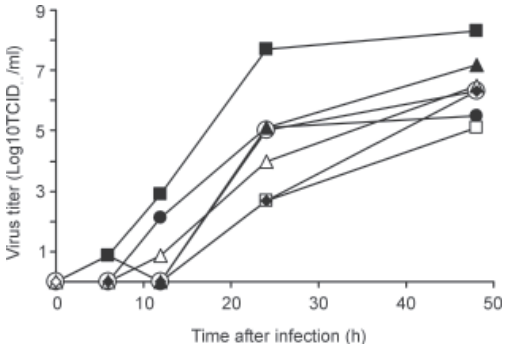


Figure 2. Replication kinetics of H7N7 viruses with reassorted genomes in MDCK cells. Supernatants of cells infected with 0.01 $TCID_{50}$/cell of the CC (□), FC (■), CC-FC PB2(●), CC-FC PA (○), CC-FC HA (▲), CC-FC NA (Δ) and CC-FC NS (♦) virus were harvested at 6, 12, 24 and 48 hours after infection. Geometric mean titers were calculated from two independent experiments.

Contribution of the polymerase genes

Comparing the FC and CC viruses, there were 5 amino acid substitutions in the PB2 gene (S79I, V297I, R355K, Q563R, E627K) and one in PA (F666L). We used a minigenome assay to study the effect of these substitutions on polymerase activity. The use of a T7 RNA polymerase promoter-driven construct for the production of the vRNA-like reporter minigenome enabled the study of polymerase activity in cells of both mammalian and avian origin. The reporter minigenome consisted of the firefly luciferase open reading frame flanked by the NCRs of gene segment 8 of the CC virus. Of note, the NCRs of gene segment 8 of the CC and FC virus were identical. Furthermore, we cotransfected a plasmid that constitutively expressed renilla luciferase, which was used as an internal control to standardize transfection efficiency and overall protein expression levels.

293T cells were transfected with plasmids encoding the polymerase proteins and NP of the CC or FC virus, a plasmid expressing T7 RNA polymerase, the firefly luciferase reporter minigenome and the renilla luciferase internal control; luminescence of the two luciferase proteins was measured 24 hrs after transfection. When comparing the FC and CC viruses, the polymerase complex of the FC virus induced higher levels of expression of the luciferase reporter than the CC virus (Fig. 3A). This difference was caused by PB2 of the FC virus; a CC virus polymerase complex with the PB2 gene of the FC virus yielded expression levels similar to a polymerase complex of the FC virus and vice versa (Fig. 3A). Although exchanging PA alone had no effect on the levels of luciferase reporter expressed by the polymerase complexes of the CC and

FC virus (data not shown), there was a significant difference between the expression of the luciferase reporter with FC-PB2 in the context of the FC polymerase complex or in the context of the CC virus (Fig. 3A). Since the only difference at amino acid level between the CC and FC virus other than in PB2 is in PA, PA could have an effect on expression levels in the context of the FC virus polymerase complex, but not in the context of the CC virus polymerase complex. Alternatively, silent nucleotide changes in PB1 (2), PA (4) or NP (1) could explain the difference in the activity of the CC and FC polymerase complexes in the presence of FC-PB2.

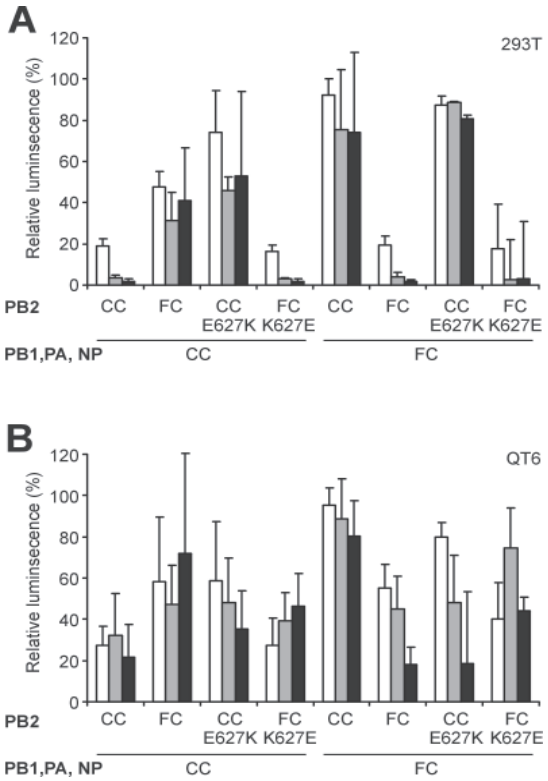


Figure 3. Polymerase activity of H7N7 virus polymerase complexes in a minigenome assay. Minigenome assays were performed by transfecting 293T (A) or QT6 (B) cells with plasmids encoding the polymerase proteins and NP of the CC and FC virus or mutants thereof. After transfection, cells were incubated 24 h at 33˚C (white bars), 37˚C (grey bars) or 41˚C (black bars). Luminescence of a firefly luciferase reporter was standardized using a plasmid constitutively expressing renilla luciferase protein. Relative luminescence was calculated as the percentage relative light units (firefly luciferase:renilla luciferase) of the maximum in each experiment. Average and standard deviation from three independent experiments are shown.

PB2 of the FC virus differs at 5 amino acid positions from PB2 of the CC virus. It has been described previously that one of these substitutions, glutamic acid to lysine at position 627 (E627K), enabled an avian influenza virus to replicate in mammalian cells and it was thus concluded to be a determinant of host range[249]. This same substitution was also described to enable replication of an avian influenza virus

polymerase complex at low temperatures in minigenome assays[250]. Therefore, we next tested the effect of the E627K substitution in PB2 on the activity of the polymerase complex in minigenome assays. To this end, two mutant PB2 genes were generated: the CC PB2 gene with a lysine at position 627 (CC E627K) and the FC PB2 gene with a glutamic acid at position 627 (FC K627E). The contribution of these amino acid substitutions to the expression levels of the luciferase minigenome reporter was evaluated. The expression levels of the luciferase minigenome reporter induced by a polymerase complex with the CC PB2 protein with E627K was comparable to the wild type FC PB2 protein, independent whether the rest of the polymerase complex originated from the CC or FC virus. When the polymerase complexes contained the FC PB2 protein with the E627K substitution, the reverse pattern was observed and the expression levels of the luciferase minigenome reporter was comparable to that of the wild type CC PB2 protein (fig. 3A, grey bars). Thus, a K at position 627 of PB2 had a large positive effect on the polymerase activity in 293T cells.
To further determine the effect of the substitution at position 627 of PB2, the minigenome assay was performed at two additional temperatures. Minigenome assays were performed at 33ºC, representing the temperature in the upper respiratory tract of humans, and at 41ºC, the temperature of the intestinal tract of birds. At 33ºC, the overall expression levels of the luciferase minigenome reporter were higher than those at 37ºC, irrespective of the polymerase complex used. At 41ºC, expression levels of the luciferase minigenome reporter were comparable to those at 37ºC (data not shown). No temperature specificity of the different polymerase complexes was observed, since the expression levels of the luciferase minigenome reporter induced by a polymerase complex with a lysine at position 627 of PB2 were higher than those of a polymerase complex with a glutamic acid at this position, regardless of the temperature at which the assay was performed.
In QT6 cells, differences in expression of the luciferase reporter by the CC or FC virus polymerase complex were relatively small as compared to 293T cells. The expression level of the reporter minigenome was slightly, but significantly, higher with the FC virus polymerase complex than with the CC virus polymerase complex. Exchanging PB2 of the CC and FC virus showed that this was due to PB2 of the FC virus, as was the case in 293T cells. However, there was no difference in expression levels with PB2 with either a glutamic acid or lysine at position 627 (Fig. 3B).This was in agreement with previous data that showed that avian and human polymerase complexes replicate equally well in cells of avian origin[250]. Like in 293T cells, there was no significant effect of temperature on expression levels of the minigenome reporter (Fig. 3B).

Effect of HA on replication

There were three amino acid differences between HA of the CC and FC virus: I13S, A143T and K416R. The A143T substitution introduces a potential glycosylation site at position 141, near the receptor binding site.
As shown in figure 2, a virus consisting of the CC virus with HA of the FC virus enhanced replication as compared to the wild type CC virus. To confirm these data, reassortant viruses consisting of seven gene segments of influenza virus A/PR/8/34 with HA of the CC or FC virus were produced (PR8-CC HA and PR8-FC HA), and replication kinetics of these two viruses in MDCK cells were studied (Fig. 4A). The

PR8-FC HA virus replicated to higher titers in MDCK cells than the PR8-CC HA virus. To determine which of the three substitutions in HA was responsible for the effect of HA on replication, three mutant HA genes were generated, consisting of the CC HA gene with either the I13S, A143T or K416R substitution. We produced reassortant A/PR/8/34 viruses with each of these three HAs (PR8-CC HA I13S, PR8-CC HA A143T and PR8-K416R) and studied the replication kinetics of these mutant viruses. As can be seen in figure 4B, the A143T substitution was solely responsible for the moderately enhanced replication kinetics observed with HA of the FC virus. As mentioned above, this substitution introduces a potential glycosylation site near the receptor binding site and this A143T substitution may affect the receptor binding specificity or affinity of HA.

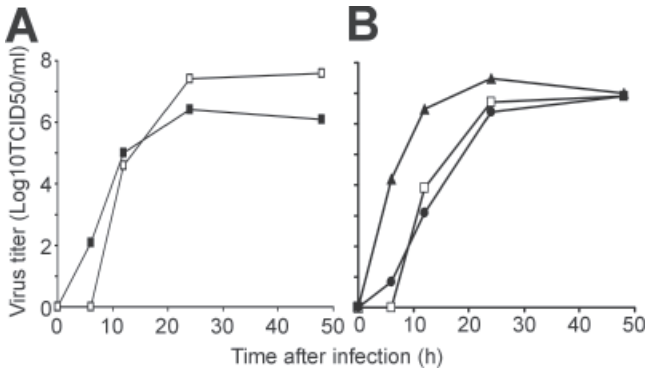


Figure 4. Replication kinetics of a A/PR/8/34 virus with the H7 HAs in MDCK cells. Supernatants of MDCK cells infected with 0.01 $TCID_{50}$/cell of (A) the PR8-CC HA (■) and PR8-FC HA (□)or (B) PR8-CC HA I13S (□), PR8-CC HA A143T (▲) and PR8-CC HA K416R (●) were harvested at 6, 12, 24 and 48 h after infection. Geometric mean titers were calculated from two independent experiments.

To determine whether the A143T substitution indeed could affect receptor binding of HA, two viruses with mutant HAs were generated. One consisted of the CC virus with a T at position 143 (CC-HA143T), the other of the FC virus with an A at position 143 (FC-HA143T). The attachment pattern of the CC-HA143T and FC-HA143A virus to human lung tissues was studied and compared to the attachment pattern of the CC and FC virus using virus histochemistry methods described earlier[233,240]. In the bronchioles, the CC-HA143T virus attached abundantly to non-ciliated cuboidal epithelial cells, whereas the FC-HA143A did to a lesser extent. In the alveoli, both the FC-HA143A and CC-HA143T virus attached to type II pneumocytes, but only the CC-HA143T virus attached to alveolar macrophages (Fig. 5). The FC-HA143A virus attached abundantly to type I pneumocytes, whereas the CC-HA143T virus hardly did (data not shown). The attachment pattern of the FC-HA143A virus was similar to that of the CC virus and vice versa, the attachment pattern of the CC-HA143T virus is in accordance with that of the FC virus (data not shown). Thus, differences in virus attachment existed between the CC and FC viruses that were caused by the amino acid substitution at position 143, that also had an effect on replication of these viruses *in vitro*.

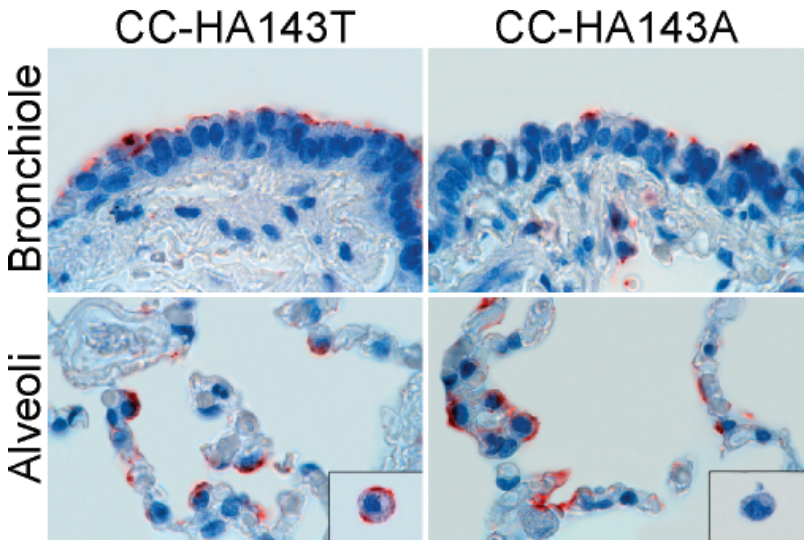


Figure 5. Attachment pattern of the CC-HA143T and FC-HA143A viruses to tissues of the human lower respiratory tract. The upper and lower left panel show attachment of CC-HA143T to bronchiole and alveoli, respectively; the upper and lower right panel show attachment of FC-HA143A to bronchiole and alveoli, respectively. Virus attachment is shown in red. Inset in lower panel shows virus attachment to alveolar macrophages. Tissues were counterstained with hematoxylin.

Role of NA in replication

When the reassortant H7N7 viruses with one gene segment of the FC virus and seven gene segments of the CC virus were generated, we found that the CC-FC PA, CC-FC HA and CC-FC NS viruses could not be rescued easily. In order to determine the cause of this inefficient rescue, all 60 possible reassortant viruses between the CC and FC virus were produced. By doing so, we found that all viruses containing either PB2 or NA of the FC virus (or both) could be rescued without problems, whereas viruses containing PB2 and NA of the CC virus could not be rescued easily, irrespective of the origin of the other 6 gene segments (data not shown). The rescue efficiency increased upon addition of an extra plasmid expressing PB2 (pCAGGS-PB2) to the transfection and addition of *Vibrio cholera* neuraminidase to the culture medium. From these data we concluded that besides PB2, NA also had a large effect on virus production from human cells *in vitro*. This was confirmed by the increased replication of the CC virus with NA of the FC virus as compared to the wild type CC virus (Fig. 2). We generated mutant NA genes consisting of the CC NA gene with single nucleotide substitutions leading to amino acid replacements, and produced reassortant viruses: CC-NA N308S, CC-NA A346V, CC-NA T442A and CC-NA P458S. Rescue of these mutant NAs in the background of the CC virus was very inefficient, indicating that none of the single amino acid substitutions was responsible for the increased replication of viruses with the FC NA gene (data not shown).

Role of NS in replication

It could be concluded from figure 2, that the FC-NS gene had an effect on the replication efficiency in the context of the CC virus. There was one amino acid

difference between NS1 of the CC and the FC virus. One of the functions of NS1 is to evade the host immune response. To test whether there was a difference between NS1 of the CC and FC virus in their ability to counteract stimulation of the interferon (IFN) pathways, we used reporter constructs under control of an Interferon Stimulated Response Element (ISRE). 293T cells transfected with this reporter and superinfected with either the CC or the FC virus, expressed lower levels of the CAT reporter than cells superinfected with Sendai virus as a positive control, but there was no difference in reporter expression between the CC and FC virus. There was thus no difference in induction of the IFN pathways. To test whether there was a difference in the ability of NS1 of the CC or FC virus to inhibit the IFN pathways, 293T cells were transfected with a plasmid expressing the NS gene of the CC or FC virus and superinfected with Sendai virus, that induces the IFN pathways. Again, no differences were observed between the CC and FC virus in their capacity to inhibit the IFN pathway.

Discussion

In 2003, an outbreak of HPAI of the H7N7 subtype occurred in The Netherlands. During this outbreak, 89 humans became infected, most of whom suffered from conjunctivitis and, occasionally, mild respiratory disease. There was one case of pneumonia, leading to respiratory distress syndrome and related complications, and death. The virus isolated from the fatal case differed in 14 amino acid positions from a prototype conjunctivitis virus[55]. Analysis of the replication kinetics of recombinant viruses with reassorted genomes revealed that most of the five gene segments harboring amino acid substitutions, could individually lead to increased replication of the reassortant viruses in mammalian and avian cells (Fig. 1 and 2).

The potential contribution of the polymerase proteins PB2 and PA to the enhanced virus replication was studied in minigenome assays. In these minigenome assays, PB2 of the FC virus caused a large increase in expression levels of the luciferase minigenome reporter. By creating mutant PB2 genes, it was shown that this increase was caused by the E627K substitution in PB2. This was in line with previous observations[235,250] and may explain why this substitution has a large effect on virulence of H5N1 and H7N7 HPAI viruses in mice[234,235] (Munster *et al*., submitted for publication). It was described previously that avian viruses, with a glutamic acid at position 627, do not express a reporter in a minigenome assay at 33ºC[235]. It was suggested that this may explain why avian influenza viruses do not replicate well in humans. In our hands, minigenome assays with the CC and FC virus both were functional at 33ºC. In fact, polymerase complexes with either a glutamic acid or lysine at position 627 of PB2 resulted in more luciferase protein expression at 33ºC than at 37ºC or 41ºC (Fig. 3A). The differences between the described temperature dependence and the absence thereof in H7N7 minigenome assays may in part be explained by the different experimental setups, such as the use of different cell types (COS-1 vs. 293T), different viruses and expression vectors used. The CC virus was isolated from the eye of an infected person and was thus able to replicate at approximately 35ºC[254], considerably lower than the temperature at its original site of replication, the intestinal tract of birds (41ºC). However, the virus isolates from

cases of conjunctivitis that were sequenced all had a glutamic acid at position 627, indicating that replication at a temperature of 35ºC was not restricted by this amino acid residue. The FC virus was isolated from the human lower respiratory tract (LRT), where temperature would be similar to the human eye. Thus, if adaptation was not necessary for replication in the eye, it should also not be necessary to enable replication in the human LRT based on temperature alone. We speculate that the E627K substitution was merely a determinant of overall increased virus replication and not of temperature sensitivity in the HPAI H7N7 virus described here.

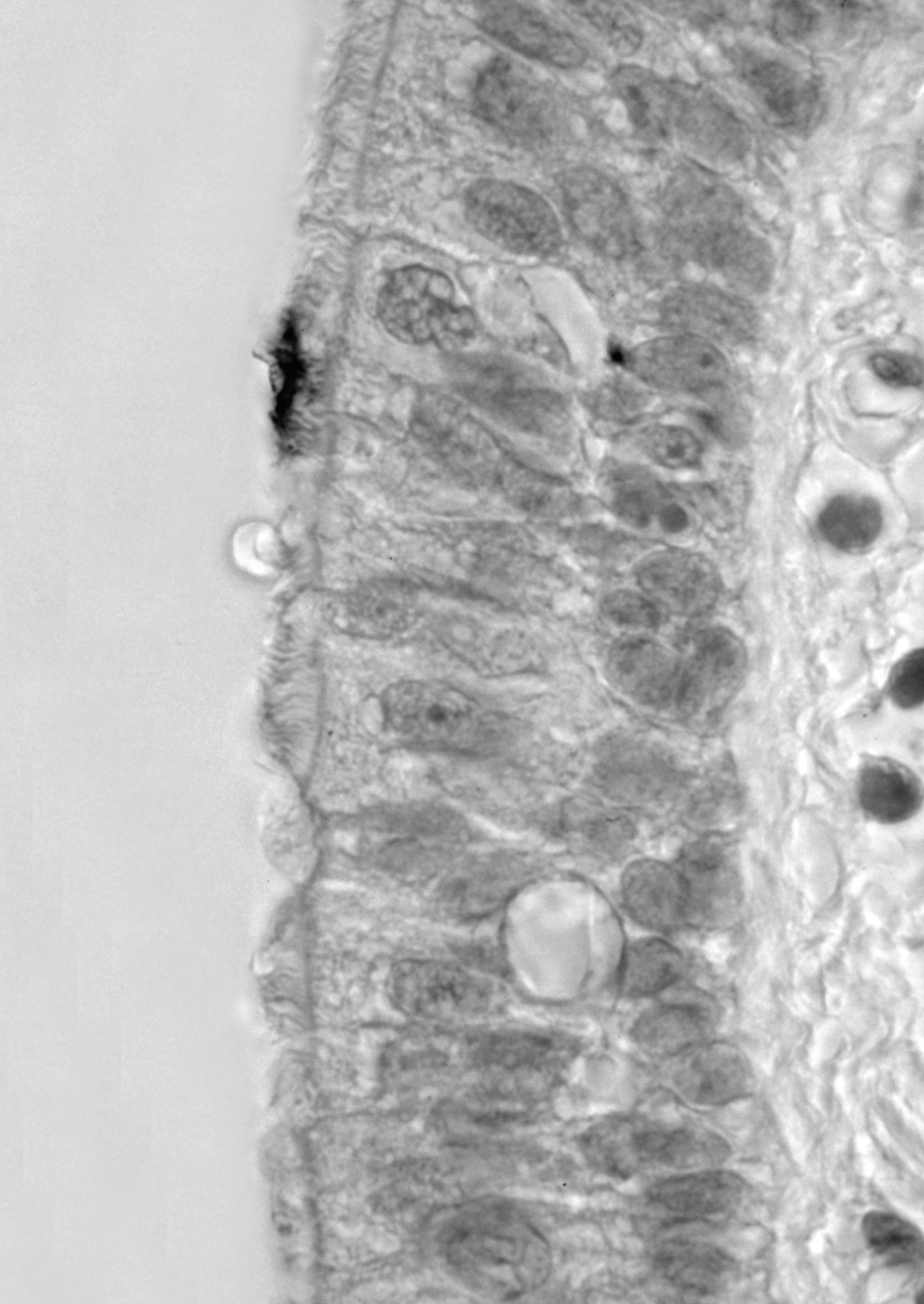
In QT6 cells, the differences in expression levels of a luciferase reporter driven by polymerase complexes with either a glutamic acid or lysine at position 627 of PB2 were only small. It seems that in HPAI H7N7 viruses this substitution was not an absolute determinant of host range restriction, in contrast to what was described previously[249]. The fact that polymerase complexes with the 'human-like' lysine at position 627 still function efficiently in cells of avian origin may explain why contemporary HPAI H5N1 viruses have been isolated from wild birds with a lysine at this position[255]. In minigenome assays where PA of the CC and FC virus was exchanged, no difference in expression levels of the luciferase minigenome reporter could be observed. However, when PB2 was exchanged, expression of the minigenome reporter was higher in the context of the FC virus than in the context of the CC virus. The only difference in the polymerase complex of the CC and FC virus at the amino acid level, besides the substitutions in PB2, was in PA and thus this difference in reporter expression was likely due to PA. It is also possible that this effect was caused by silent changes present in PB1, PA and NP of the FC virus polymerase complex. The effect of these silent changes was not studied here, but may be of interest to study in the future.

The HA gene of the FC virus also resulted in increased replication efficiency in reassortant viruses. This effect was caused by a single amino acid substitution from alanine to threonine at position 143 that introduced a potential glycosylation site at position 141 of HA. This substitution also affected the attachment pattern of the FC virus; a virus with a threonine at position 143 attached more abundantly to non-ciliated cuboidal epithelial cells than a virus with an alanine at this position. Moreover, the virus with a threonine at position 143 attached to alveolar macrophages, while the virus with alanine did not. On the other hand, the virus with alanine attached abundantly to type I pneumocytes, whereas the virus with threonine hardly did. Thus, the A143T substitution resulted in a difference in binding affinity or binding specificity. The observation that there was no difference between the CC and FC virus in hemagglutination of turkey erythrocytes, expressing α2,3- and α2,6-linked SA, or horse erythrocytes, expressing predominantly α2,3-linked SA[256], indicated that differences in receptor binding specificity or affinity are subtle (data not shown).

The inefficient rescue of viruses with NA of the CC virus indicated that this NA must have a large effect on replication *in vitro*. From the inefficient rescue of NA genes with single amino acid substitutions in the context of the CC virus, we concluded that multiple substitutions were responsible for this effect. Since a balance of HA and NA activity is required for efficient virus replication[257], the substitutions in NA may be related to the alterations in binding specificity or affinity in HA of the FC virus. By performing assays to determine the neuraminidase activity of the wild type and mutant NA viruses, it should be possible to determine whether the inefficient rescue

is indeed due to differences in neuraminidase activity.
Finally, the substitution in NS1 of the FC virus had an effect on the replication kinetics of the FC virus at the latest time point (Fig. 2). In experiments with a reporter gene under the control of the ISRE, we observed no differences in the interferon-antagonist properties of this protein (data not shown). NS1 has several other functions in the replication cycle of influenza A viruses, such as enhancing the synthesis of viral proteins and it is possible that through one of these functions NS1 of the FC virus can exert a moderate enhancing effect on virus replication.
Only recently, an H7N7 virus isolated from a chicken on the single farm that was visited by the deceased veterinarian was sequenced. Sequence analysis revealed that 12 out of 14 amino acid substitutions in the FC virus were already present in this chicken isolate, except for the E627K substitution in PB2 and the K416R substitution in HA (A/Chicken/Netherlands/03010132/03, GenBank accession numbers EF015551 to EF015558). Thus, most of the mutations observed in the FC virus as compared to the CC virus did not occur within the respiratory tract of the deceased veterinarian. Apparently, genetically diverse viruses were circulating in poultry in The Netherlands. It has been suggested that quail may act as an intermediate host in the zoonotic spread of avian influenza viruses from aquatic birds to other species[258]. Due to the extensive circulation of the H7N7 HPAI virus during the 2003 outbreak similar changes may have occurred in poultry in The Netherlands. These replication-enhancing mutations potentially paved the way for the virus to enter the human lower respiratory tract, where it subsequently acquired the PB2 E627K (and HA K416R) substitution. The sequence information from the chicken H7N7 isolate implies that mutations that facilitate replication in human cells could already occur upon virus replication in poultry. The current circulation in poultry and wild birds of HPAI H5N1 Qinghai lineage viruses, in Asia, Europe, the Middle East and Africa, with the E627K substitution already present are a reason for great concern. The identification of mutations that enhance replication in chickens as well as in mammalian species underline the need for continuous virus surveillance and virus sequencing, and assessment of the pathogenic properties of these viruses in mammalian species.

Acknowledgements
We thank Guus Koch and Sylvia Pritz-Verschuren at CIDC, Lelystad for providing us with A/Chicken/Netherlands/03010132/03. We thank Frank van den Panne, Bernadette van den Hoogen, Sabrina Benton, Frank Pistoor and Viroclinics B.V. for excellent technical assistance.



12

H5N1 virus attachment to lower respiratory tract

Science, 2006, 312: 399

Debby van Riel
Vincent Munster
Emmie de Wit
Guus Rimmelzwaan
Ron Fouchier
Ab Osterhaus
Thijs Kuiken

Highly pathogenic avian influenza virus (H5N1) may cause severe lower respiratory tract (LRT) disease in humans. However, the LRT cells to which the virus attaches are unknown for both humans and other mammals. We show here that H5N1 virus attached predominantly to type II pneumocytes, alveolar macrophages, and nonciliated bronchiolar cells in the human LRT, and this pattern was most closely mirrored in cat and ferret tissues. These findings may explain, at least in part, the localization and severity of H5N1 viral pneumonia in humans. They also identify the cat and the ferret as suitable experimental animals based on this criterion.

Highly pathogenic avian influenza virus of the subtype H5N1 may cause infection of the lower respiratory tract (LRT) and severe pneumonia in humans[52]. However, the cell types in the LRT to which the virus attaches are unknown for both humans and experimental animals. Although attachment is not the only factor required for virus replication, this information is important both to better understand the pathogenesis of H5N1 influenza and to assess the suitability of animal models. Therefore, we compared the pattern of H5N1 virus attachment to the LRT of humans and four animal species.

Influenza viruses attach to host cells by binding of the hemagglutinin to sialosaccharides on the host cell surface. Human influenza viruses prefer sialic acid (SA)-α-2,6-Gal-terminated saccharides, whereas avian influenza viruses prefer those terminating in SA-α-2,3-Gal[259]. The use of lectins that specifically detect α-2,6-and α-2,3–linked sialosaccharides is an indirect measure of influenza virus attachment to host tissues and does not account for other variables that influence the binding avidity, such as type of SA, and glycosylation and sialylation of the hemagglutinin close to the receptor binding site[259]. For a more direct method, which was modified from a previously used technique[240], we incubated formalin-fixed, paraffin-embedded tissue sections with formalin-inactivated fluorescein isothiocyanate (FITC)–labeled H5N1 virus (A/Vietnam/1194/04) and detected virus with a peroxidase-labeled rabbit antibody to FITC that was amplified with a tyramide signal amplification system. Tissues comprised histologically normal LRT (including alveolus, bronchiole, and bronchus), as well as trachea from three individuals of each of the following species: human, mouse (C57BL/6), ferret, cynomolgus macaque, and domestic cat[260].

In the human LRT, H5N1 virus attached predominantly to type II pneumocytes, alveolar macrophages, and nonciliated cuboidal epithelial cells in terminal bronchioles. Attachment became progressively rarer toward the trachea (Fig. 1 and table S1[260]). The identity of type II pneumocytes was confirmed by double staining with antibody to human surfactant apoprotein A (fig. S1[260]). These findings fit with the limited pathology data for H5N1 virus infection in humans, which show diffuse alveolar damage[52] and the presence of H5N1 virus antigen in type II pneumocytes[261]. However, they contrast with the idea that avian influenza viruses generally have little affinity for human respiratory tissues[259].

The predilection of H5N1 virus for type II pneumocytes and alveolar macrophages may contribute to the severity of the pulmonary lesion. Because type II pneumocytes are metabolically active and are the most numerous cell type lining the alveoli, targeting of this cell type may lead to abundant virus production. Damage to type II pneumocytes may impair their functions, including re-epithelialization after alveolar

damage, ion transport, and surfactant production, and so may inhibit tissue repair. Targeting of alveolar macrophages may be important because of their role in limiting viral replication and in the immune response to viral infection.

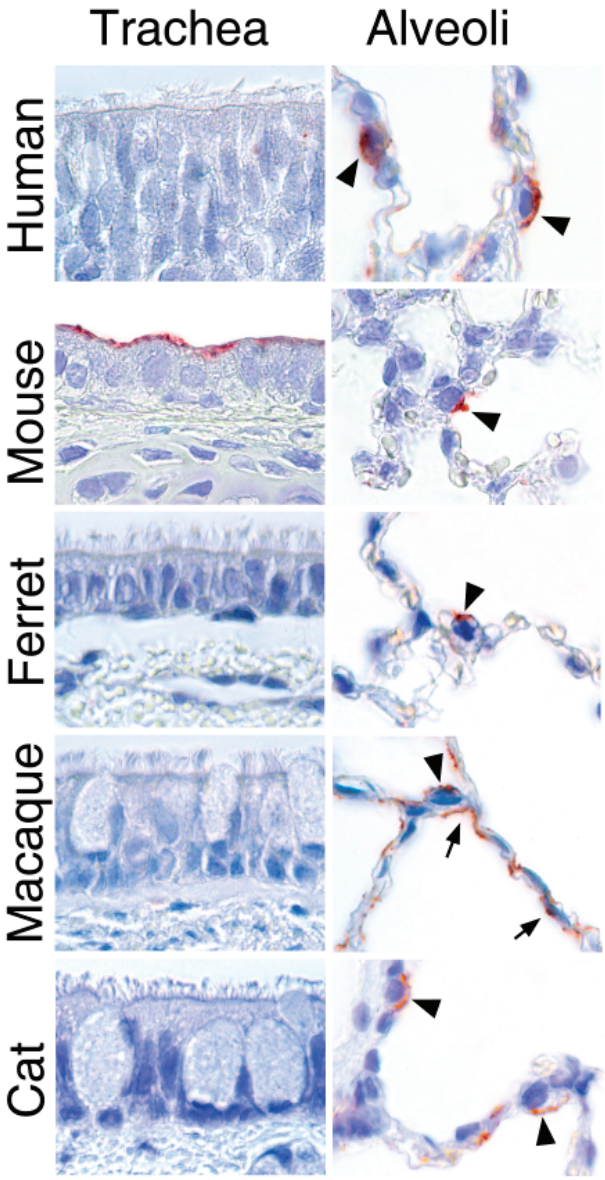


Figure 1. Attachment of H5N1 virus to respiratory tract of humans and four animal species. In the trachea, H5N1 virus -visible as red-brown staining- attached only to epithelial cells of mice. In the alveoli, H5N1 virus attached predominantly to type II pneumocytes (arrowheads) in human and all animal species except the macaque, where attachment was predominantly to type I pneumocytes (arrows).

The pattern of H5N1 virus attachment to cat LRT and, to a lesser extent, ferret LRT most closely resembled that in human tissues (Fig. 1 and table S1[260]). Based on this criterion, we considered these two species as the most suitable models for H5N1 viral pneumonia in humans. However, other factors also need to be considered, such as the availability of reagents and immunologic similarity. In macaque alveoli, H5N1 virus attached predominantly to type I pneumocytes instead of type II pneumocytes, as in human tissues. In mice, H5N1 virus attachment to cells was most abundant in the trachea and became progressively rarer toward the alveoli, whereas the opposite trend was observed in human tissues. The observed pattern of H5N1 virus attachment to the LRT is consistent with the respective pathology and immunohistochemistry results of experimental H5N1 virus infection in mice[262], ferrets[263], macaques[243], and cats[264].

This study demonstrates the attachment of H5N1 virus to the human LRT in a pattern that corresponds with autopsy findings. It also identifies cat and ferret as the most suitable animal models for human H5N1 viral pneumonia, on the basis of the similarity of viral attachment pattern. This technique also could be applied to further determine H5N1 virus attachment to the upper respiratory tract. Failure to attach to this site may be a limiting factor in human-to-human transmissibility of H5N1 virus.

Acknowledgements

We thank W. Lim for providing the H5N1 isolate; M. den Bakker, H. Sharma, M. Vermeij, and J. van den Brand for providing tissues; and F. van der Panne for technical assistance.

13 Summarizing discussion

Summarizing discussion

Wild birds play an essential role in the persistence of avian influenza A viruses. Despite the relatively intense surveillance studies that have been performed for many years in North America and Eurasia, our understanding of the global distribution of LPAI viruses in wild bird populations is still limited. It is reasonable to assume that influenza viruses are distributed globally, wherever competent host species are present. Some subtypes may be rare or not detected annually in current surveillance studies. Simply because of the limitations of our studies, we are currently biased toward species that are easy to sample during migration or wintering. To understand the global patterns of LPAI viruses in wild birds, it will be crucial to integrate virus and host ecology with long-term surveillance studies to provide more insight on the year-round perpetuation of influenza viruses in wild birds. Possible intercontinental contacts among ducks and shorebirds in areas where migrating birds from the northern and southern latitudes mix are of particular interest. Can influenza viruses be perpetuated in ducks alone, or does the interface between ducks and shorebirds as seems to exist in North America, also occur on other continents? With high-throughput sequencing technology, it should also be possible to gain more insight into the genetic variability and evolution of LPAI viruses in wild birds and to integrate this information with epidemiology and virus-host ecology. This thesis focuses on the complex ecology and evolution of avian influenza viruses.

Avian influenza viruses in wild birds

In 1998, a wild bird influenza A surveillance network was initiated at the department of virology of the Erasmus Medical Center. The surveillance network included many expert ornithologists using a wide variety of catching methods to catch and sample different wild bird species. Over the period of 8 years (1998–2005) a total of 36,796 bird samples were analysed for the presence of influenza A virus. Cloacal samples were collected from 321 different bird species and 18 bird orders. Traditionally, influenza A virus surveillance in wild birds was performed by direct virus isolation from fecal samples or cloacal swabs in embryonated hen's eggs[1]. This method is labour intensive due to handling time of each of the individual samples. The implementation of high-throughput molecular screening techniques enabled us to perform more intensive surveillance[71,265]. Despite the increase in intensity (both in species and in samples), compared with other historic and contemporary surveillance studies[15,24,26,62,64] the general pattern of influenza A viruses with respect to seasonal prevalence and host species remained relatively similar. Although historically influenza A viruses have been obtained from more than 105 species of 26 different families[7], no relevant influenza virus prevalence in species other than belonging to the orders of Anseriformes and Charadriiformes was detected (chapter 2), which was in line with other surveillance studies. This suggests that, although multiple bird species can be infected with avian influenza, they do not contribute to the overall influenza A virus ecology and that infection, although potentially with high peak prevalence, is transient within these species (chapter 2 and 3). The large-scale surveillance confirmed the predominant role for dabbling ducks in the perpetuation of most HA subtypes, except H13 – H16. In Canadian studies, cyclic patterns of influenza virus subtypes were reported. Within our surveillance studies, the time

span of our dataset currently limits any decisive analysis on the matter. Although preliminary data show between-year variations in distribution of HA subtypes, further analyses should reveal whether this is cyclic variation in distribution of subtypes, between-(sub)population variations in prevalence of subtypes or a combination of both[26].

Based on the low influenza virus prevalence in ducks, other than during fall migration, some investigators support the hypothesis that frozen lakes are serving as a reservoir from which an epidemic is initiated each year[1]. Recent data on the spring prevalence in ducks in Northern Europe indicate that virus prevalence is sufficiently high to maintain year round influenza virus presence in the population, although year-round surveillance would greatly contribute to our understanding of the perpetuation of influenza A viruses. An additional hypothesis is based on surveillance data from North America (east coast), which suggest a distinct role for wader species in the perpetuation and maintenance of certain influenza A virus subtypes[26]. The seasonal prevalence of influenza viruses in waders seems to be reversed as compared with ducks, with high influenza virus prevalence during spring migration[26]. This led to the hypothesis that, in spring, waders carry the influenza A viruses to the breeding grounds of the ducks. No such pattern was observed in Northern Europe; not a single influenza A virus was detected in 2996 waders sampled. It was suggested that the majority of these samples were collected at a timing at which the prevalence in shorebirds was low (i.e. fall migration)[63], but additional samples obtained during spring migration and breeding season rendered the same negative results. Thus, these data indicate that wader species in Northern Europe do not play any role of significance in the ecology of avian influenza A viruses (chapter 2). This hypothesis is currently further evaluated by use of serological surveillance to determine conclusively whether wader species are involved in influenza A virus ecology in Northern Europe.

Factors contributing to the predominant role of dabbling ducks as influenza A virus host species are population size and mode of transmission. The importance of population size, age structure and herd-immunity on the epidemiology of infectious diseases has been described for human pathogens like measles[17]. Large populations are probably more capable of sustaining a large variety of different influenza A virus subtypes, as observed in dabbling ducks. The dabbling duck populations are estimated at between 5,000,000 and 10,000,000 birds in Northern Europe[21], with Mallards (*Anas platyrhynchos*) being the most abundant species[21]. The estimated yearly turnover rate of Mallards in Northern Europe is roughly 1/3[266]. A large part of the population is therefore rejuvenated and immunologically naive every year, potentially allowing simultaneous cocirculation of multiple genetic lineages and subtypes within one meta-population of potential hosts for influenza A virus. In contrast, the population estimates for the different goose species in Northern Europe are significantly lower compared to the dabbling ducks; with an estimated population size of 1,2 million geese[21].

Influenza A viruses are transmitted via the faecal-oral route, which allows effective transmission of influenza A viruses between susceptible birds. Influenza A viruses can stay infectious for prolonged periods of time in surface water[1], potentially allowing temporal and spatial connectivity of different host sub-populations by their respective virus populations. Differences in behaviour could account for the

differences in prevalence between bird families and species. Whereas dabbling ducks feed on the surface water, geese and certain swan species graze in pastures and agricultural fields. This could potentially lead to a less efficient transmission and subsequently lead to a lower influenza A virus prevalence in these species (chapter 2). The smaller population sizes could limit the perpetuation and maintenance of multiple influenza A virus subtypes and allow only a limited number of influenza A virus subtypes to co-circulate within the population, whereas the continuing sustainability of influenza viruses in geese is further hampered by an inefficient transmission. The overall prevalence in geese (1.8%) and the limited number of HA subtypes identified in influenza A viruses isolated from geese (H1, H2 and H6) is in line with this hypothesis.

Previous surveillance efforts in North America indicated Laridae (gulls) as a distinct host population for the perpetuation of influenza A viruses of the H13 subtype[1,24]. In 1999, surveillance in black-headed gulls in Sweden led to the isolation of influenza A viruses that could not be subtyped using previously existing panels of reference reagents. Using standard classification techniques together with molecular techniques the influenza A viruses were genetically and antigenically characterized into a previously unknown HA subtype; H16 (Chapter 4). The predominant isolation of H13 and H16 influenza A viruses from different gull species confirms the observation that these viruses belong to a group of distinct influenza viruses based on distinct genetic, functional and ecological properties[24]. H16 influenza viruses have so far only been isolated from gulls. Analyses of historical samples suggest that H16 gull viruses have been circulating for at least 30 years (data not shown) and have also been isolated in North America[33], indicating that H16 is not a recently emerging subtype. The identification of a novel HA subtype underlines the value of avian influenza A surveillance. Furthermore, novel subtypes may exist in less intensively sampled host populations or new geographical regions. In addition to the novel influenza A virus subtype, additional influenza A viruses of specific interest were identified. For instance, from Common Guillemots (*Uria aalge*) banded in the auk colony on Bonden Island in the northern Baltic Sea in July 2000 an influenza A virus of subtype H6N2 was isolated. Phylogenetic analyses of the viral genome showed that the PB2, PB1, HA, MA and NS gene segments belonged to the American avian lineage of influenza A viruses, whereas the PA, NP and NA gene segments belonged to the Eurasian lineage. These reassortant influenza A viruses, obtained from Guillemots, showed that, although the virus populations of the Americas and Eurasia are separated, the isolation of the virus populations is not absolute. Influenza A viruses are not only transmitted occasionally between the American and Eurasian lineage of viral populations[27,28], but reassortment between viruses belonging to these different populations can also take place, as shown by the Guillemot isolate (Chapter 5). Reassortment is one of the driving forces behind the variability of influenza viruses and contributes greatly to the phenotypic variability among influenza viruses[34]. Few details are known about the capacity for reassortment of different lineages of influenza A viruses, the rate of reassortment in nature or the effects of reassortment on the virus population. Analyses of reassortment is currently limited by lack of data on the frequency of simultaneous infections, the biological constraints of reassortment and genetic diversity of avian influenza viruses. Although recently results of large scale avian influenza genome sequencing were published, no fine-grained analyses was

performed[33]. Avian influenza viruses used in the genome sequencing study were predominantly American lineage viruses further limiting the analyses to only one geographical region; the inclusion of genomic sequences of Eurasian lineage wild bird isolates would therefore contribute to our knowledge of genetic variability in the wild bird reservoir.

As for most wildlife diseases, little information is available on the relation between virus ecology and host ecology of influenza A viruses. Moreover, very little is known about the impact of disease on the general behavioral ecology of infected birds. In domestic birds, the clinical signs and disease observed following infection with LPAI viruses vary with host species, age, the presence of other micro organisms and environmental factors. In these hosts, LPAI viruses can cause a mild disease. Upon experimental infection of ducks, LPAI viruses replicate in the epithelial cells of the intestine of birds and virus may be shed in high concentrations in the faeces, without inducing apparent signs of disease[12,19,267]. It is hard to extrapolate the effect of infection to wild birds, where mild or subclinical infections may have significant ecological fitness consequences. It is generally believed that LPAI viruses are also non-pathogenic upon natural infection of wild birds, although data are scarce. The observations that infection of swans with LPAI viruses can result in altered migratory and feeding patterns indicates that LPAI virus infections in wild birds have a higher clinical, epidemiological, and ecological impact than previously recognised (Chapter 6). The incorporation of behavioural ecology in influenza virus surveillance enables an interdisciplinary approach to study the complex relation between virus and host ecology. The incorporation of tools such as GPS-collars allows continuous tracking of birds, and subsequently the monitoring of infection status of individual wild birds in real time, allowing detailed questions about the influenza prevalence in individual birds in relation to their social status, migration pattern, condition, habitat choice and aggregation intensity. Furthermore, it will lead to more detailed information on the potential pathological effects of influenza virus infection, on the effect of previous infections on re-infection, on duration of virus shedding, and on analyses of differences between subtypes of influenza A viruses.

Vaccine developments

Vaccination is the primary control strategy for epidemic influenza strains in the human population. While non-adjuvanted human influenza vaccines protect adequately against seasonal influenza, the development of effective pandemic influenza vaccines is still ongoing. The development of pandemic vaccines is targeted at influenza A viruses with known pandemic potential and zoonotic potential; such as H1, H2, H3 strains which caused the three pandemics of the last century, and the H5, H7 and H9 strains which have been transmitted repeatedly to humans and other mammals[35,55,67,161,268,269]. During our wild bird surveillance, only minor genetic and antigenic diversity was observed between the H5 and H7 genes of influenza A viruses isolated from wild birds, and those of HPAI virus strains of recent European outbreaks (chapter 7). This implies that the influenza A virus isolates obtained during wild bird surveillance studies may also serve as prototypic vaccine candidates for human or veterinary use and the avian influenza virus collection could be used as a repository of genomic HA and NA subtypes which will allow the rapid generation of recombinant or reassortant viruses in case of an outbreak. Prototype vaccine strains, representing both the

American and Eurasian genetic lineages of influenza A virus, could be generated to cover a wide range of influenza A virus strains, both directed at LPAI and HPAI viruses. Such vaccine seed strains can be produced well ahead of outbreaks. The disadvantage of the minor antigenic differences between the vaccine strain and the epidemic strains will likely be compensated by the immediate availability of the vaccine. An additional advantage of the use of LPAI strains from wild birds as prototype vaccine strains is that they do not contain a basic cleavage site in the HA gene. Before HPAI strains can be used as vaccine candidates, the basic amino acid residues in the HA gene need to be removed using 'reverse genetics' technology, resulting in an extra modification step, consuming precious time. Moreover, these vaccine strains can only be generated after an outbreak of HPAI has started. The outbreak of HPAI H7N7 in The Netherlands and the subsequent human cases underlined the need for effective human vaccines targeted at HPAI viruses. The limited antigenic differences between the H7 of LPAI viruses circulating in wild birds and the H7 of the viruses causing the HPAI H7N7 outbreak enabled the use of a LPAI H7N7 as donor strain for the generation of a reassortant seed strain by reverse genetics. The efficacy of a classical, non-adjuvanted influenza vaccine preparation containing HA and NA, were compared with an ISCOM-adjuvanted preparation in an experimental mouse model. The comparison of the two vaccine preparations showed that although an antibody response was mounted, even two doses of the classical vaccine administered were not sufficient to protect against a lethal HPAI H7N7 challenge. Two doses of the ISCOM-adjuvanted vaccine preparation were necessary to establish clinical protection against the influenza virus challenge (chapter 8). In the event of a pandemic outbreak, a vaccine offering clinical protection could be sufficient to reduce the clinical impact of infection and to alter the transmission kinetics of the pandemic virus. H5 and H7 HPAI viruses have been shown to be poor inducers of protective levels of antibodies in humans even after two doses with adjuvant[270,271], therefore the development of effective vaccines against HPAI viruses should not only be focused at the selection of seed strains with desirable antigenic properties but also at the development of new adjuvants or improved vaccine-delivery approaches, aimed at better immune responses, improved protection against lethal infection and preferably after administration of a single dose.

Whereas in most HPAI outbreaks (such as the H7N7 HPAI outbreak in The Netherlands) the genetic and antigenic diversity of the viruses is very limited compared to the ancestral strains (chapter 7), the rapid antigenic evolution of HPAI H5N1 renders it virtually impossible to use LPAI prototype vaccine strains for vaccine development against contemporary H5N1 strains. The genetic and antigenic stability of HA of LPAI viruses in their natural reservoir is contrasted by the genetic and antigenic evolution of HPAI H5N1 viruses. The continuous circulation in poultry in Asia of HPAI H5N1 since 1997 has resulted in an antigenic evolution from their original ancestor A/Goose/Guandong/96 to the emergence of at least 5 different antigenic clusters of HPAI H5N1 viruses[272]. Whereas the drift in human H3 influenza A viruses is associated with escape from herd immunity, it is not directly clear whether the evolutionary force behind the rapid genetic and antigenic changes of H5 is due to escape from prior immunity or selected for by continuous replication in poultry. The recently developed antigenic cartography methods allows a detailed quantitative visual interpretation of antigenic variation between circulating clusters of influenza A

viruses[56]. Detailed antigenic analyses are crucial to evaluate the rapid evolution of HPAI H5N1 and to match the antigenic properties of vaccine strains for both human and veterinary use.

The control measures of outbreaks of HPAI in poultry differ markedly from control strategies focused at pandemic outbreaks in humans. The objective is absolute eradication of the causative agent, predominantly by so-called “stamping out” procedures aimed at infected poultry flocks and by pre-emptive culling aimed at the prevention of spread of the causative agent to yet uninfected flocks in order to control the outbreak. These control measures involve mass-culling of poultry, such as seen during the HPAI H5N1 outbreak in Hong Kong in 1997 (estimated 3,000,000 birds culled) and the HPAI H7N7 outbreak in The Netherlands (estimated 30,000,000 birds culled)[9,208,273]. Until recently, prophylactic vaccination in birds against H5 and H7 influenza A viruses was not considered as a control measurement since this would result in export bans on live poultry, birds and poultry products. The recent HPAI H7N7 outbreak in The Netherlands and the current HPAI H5N1 enzootic in Asia and Africa, and subsequent threat of transmission of HPAI to domestic birds in Europe, led to vaccination programs in zoos and small non-commercial backyard flocks. The vaccination program aimed at avoiding pre-emptive culling of the birds kept in zoo collections (often valuable and endangered bird species), and to reduce confinement of birds. Birds in zoos in The Netherlands were vaccinated with a whole inactivated oil-adjuvanted vaccine based on influenza virus A/chicken/Italy/473/99 (H7N1), with high homology to the field strain HPAI H7N7 A/chicken/Netherlands/1/03 (97,4 % nucleotide and 98,7 % amino acid sequence identity), and boosted with the same vaccine 6 weeks later. The vaccination efficacy was determined by analyzing the induction of protective antibodies. After vaccination 81,5 % of the vaccinated animals reached an antibody titer of ≥ 40 (considered a protective level). The high agreement between antibody levels (post vaccination titers against the vaccine strain) and the virus neutralization titers (against the field strain), was used as measure of immunogenicity because testing vaccine efficacy by challenge was not feasible. Generally veterinary vaccines are tested in poultry[274], the broad vaccine efficacy in the 13 different bird orders included in this vaccination program exemplified that vaccination is currently an essential tool for the protection of valuable exotic birds in zoos against HPAI virus infections (chapter 9). Moreover, vaccination of poultry in Asia against HPAI H5N1 is an important additional control option to be applied to the poultry industries in this region, because attempts to control the outbreak by stamping-out and containment alone have not proven to be successful in the control and prevention of HPAI H5N1 outbreaks. However, extensive prophylactic vaccination in Mexico (H5N2) has led to the emergence of influenza strains escaping the immune response generated by the vaccine[275]. Therefore, vaccination programs must be accompanied by appropriate biosecurity measures, sentinel system and continuous evaluation of the antigenic properties of the vaccine strain. Vaccination programs against HPAI H5N1 are currently ongoing in zoos all over Europe.

HPAI H5N1 in wild birds

Although HPAI H5N1 viruses have previously been isolated sporadically from wild birds, the first reported large-scale outbreak in wild migratory birds occurred in April

– June 2005, Lake Qinghai, China[37-39]. The HPAI H5N1 outbreak in wild birds affected large numbers of wild birds such as Bar-headed Geese (*Anser indicus*), Brown-headed Gulls (*Larus brunnicephallus*), Great Black-headed Gulls (*Larus ichthyaetus*), and Great Cormorants (*Phalacrocorax carbo*). After the HPAI H5N1 outbreak in wild birds, the virus rapidly spread westwards across Asia, Europe, Middle East and Africa. Affected wild birds have been reported in several countries, predominantly in Mute Swans (*Cygnus olor*), Whooper Swans (*Cygnus cygnus*), and Tufted Ducks (*Aythya fuligula*)[41], although small numbers of other species have been reported as well (raptors, gulls and herons). It has been much debated whether wild birds play an active role in the geographic spread of the disease. It has been argued that infected birds would be too severely affected to continue migration and thus unlikely to spread the H5N1 virus. Although this may be true for some wild birds, it has been shown that, in experimental infections, several bird species survive infection and shed the H5N1 virus without apparent disease signs[40,255,276]. In addition, many wild birds may be partially immune owing to previous exposures to LPAI influenza viruses, as has been shown for chickens[277]. Finally, recent studies suggest that HPAI viruses may become less pathogenic to ducks infected experimentally, while retaining high pathogenicity for chickens[43,45,278]. The present situation in Europe, where infected wild birds have been found in several countries that have not reported outbreaks among poultry, suggests that wild birds can indeed carry the virus to previously unaffected areas. Although swan deaths have been the first indicator for the presence of the H5N1 virus in several European countries, this does not necessarily imply a role as predominant vectors; they could merely have functioned as sentinel birds infected via other migrating bird species.

The recent H5N1 outbreaks in Eurasia have identified additional gaps in our knowledge of avian influenza viruses in wild birds in general. It should be realized that our knowledge of LPAI viruses in wild birds cannot simply be extrapolated to HPAI viruses; for instance, the most important host species or routes of transmission may be quite different. It is clear that influenza virus surveillance of wild birds could provide “early warning” signals for the introduction of HPAI H5N1 virus in new regions and may provide access to strains for characterization and genetic epidemiology. The value of genetic epidemiology was recently shown in Nigeria, in which phylogenetic analyses of outbreaks of HPAI H5N1 in poultry could be traced back to three independent introductions[279]. Moreover, thorough and fast genetic analyses of outbreaks could give information of the likely source and potential vector, which are both still under dispute in the case of the spread of HPAI H5N1.

The analyses of gene sequence variation has become standard practice in analyzing viral outbreaks, especially with the implementation of high-throughput sequencing technologies. The analyses of outbreaks would greatly be strengthened by the rapid release of genetic data of contemporary HPAI H5N1 strains[280]. For proper risk assessment studies, however, we also need a better understanding of the interface between wild and domestic birds, the possible transmission of influenza viruses between these populations, bird behavior, age-structures of populations, and detailed migration routes. We further need better understanding of the transmission and pathogenesis of H5N1 virus in wild birds, as well as identification of virus-permissive host species and their relative likelihood to develop disease, patterns of virus secretion, and temporal and spatial variations in virus prevalence.

As compared to most existing influenza A virus surveillance networks, several changes need to be implemented in order to achieve the goal of proper risk assessment and to provide a useful early warning system for the introduction of HPAI H5N1 by wild birds. First of all, because of the highly pathogenic nature of the H5N1 viruses, studies should be performed under higher biosecurity levels than usual, if the screening procedure includes attempts to isolate the virus in eggs or cell culture. Secondly, studies should be performed in real-time (Fig. 1), which will complicate the organization of the surveillance network and will likely increase the costs of the surveillance studies significantly. Finally, it is essential to focus on the relevant bird species and include sufficient numbers, suit-to-fit the region in which the surveillance is performed. Whereas it is relatively easy to adjust existing surveillance schemes to comply with the first two points and to optimize the logistics of harmonized sampling and sample handling prior to analysis between different sites, the latter point is a serious challenge that can only be dealt with through proper collaboration between virologists, ornithologists and epidemiologists. This collaboration will allow a better organized international monitoring system, with a strong focus on specific points of interest along the international flyways, where large numbers of target species congregate. This will allow a science-based monitoring without unnecessary duplication along the flyways, but also filling existing gaps in the monitoring system with respect to bird species, geography and timing.

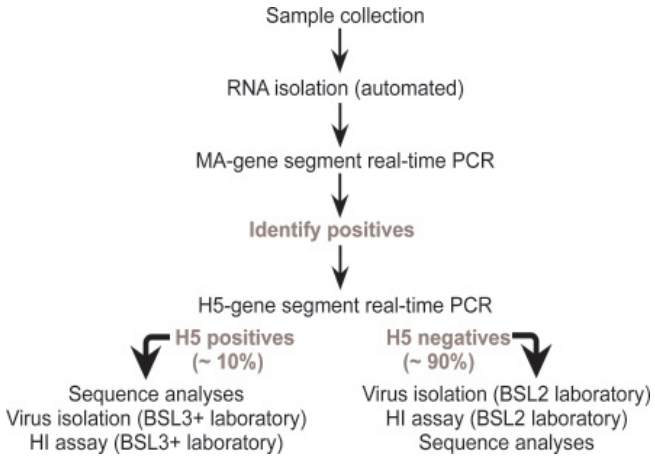


Figure 1. Flowchart depicting real-time influenza A virus screening. Starting with total amount of samples obtained from wild birds, the relative amount of influenza A virus positive samples (based on previous surveillance studies) which are further screened for H5 subtype. The left curved arrow is the relative amount of H5 positive samples expected, further isolation and characterization is performed under $BSL3^{+}$ conditions. The right arrow is the relative amount of influenza A virus positive samples expected, other than H5. Subsequent analyses of these samples can be performed under BSL2 conditions.

HPAI H7N7 pathogenesis

On 28 February 2003, an HPAI outbreak started in The Netherlands caused by an HPAI H7N7 virus. The outbreak started in the “Gelderse Vallei” and spread to adjacent areas. In order to control the outbreak, 30 million chickens were culled. 86 people in close contact with infected poultry (cullers, farmers and veterinarians) became

infected with the HPAI H7N7 virus and there were three additional cases of human-to-human transmission[55,161]. Most of the 89 human cases suffered from conjunctivitis and some from mild influenza-like illness; however, there was also one fatal case of pneumonia leading to acute respiratory distress syndrome. Sequencing analysis revealed that conjunctivitis viruses such as A/Netherlands/33/03 (CC) were almost identical to the first isolated prototype chicken virus, A/chicken/Netherlands/1/03. The virus isolated from the fatal case, A/Netherlands/219/03 (FC), displayed 14 amino acid substitutions in five different gene segments: PB2 (5), PA (1), HA (3), NA (4) and NS1 (1) when compared with A/Netherlands/33/03[55], suggesting rapid evolution within the fatal case of a virus initially originating from poultry. The relatively limited genetic variability coupled to large phenotypical differences (conjunctivitis vs. death) offered an opportunity to study the determinants of pathogenicity of H7 viruses *in vivo* and *in vitro* (chapters 10 and 11).

HPAI H7N7 isolated from a case of conjunctivitis (CC) and from the fatal case (FC), LPAI H7N7 and HPAI H5N1 viruses were shown to attach to epithelium of the cornea and conjunctiva, in contrast to human H3N2 viruses. The attachment of avian influenza viruses to the ocular epithelium and the human lower respiratory tract (LRT) corresponds with the receptor distribution in these tissues and the receptor affinity of avian viruses (α2,3-linked sialic acids). This indicates that both the eye and the LRT are a potential porte d'entrée for avian influenza viruses. Although differences were observed in attachment pattern in the LRT between the HPAI H7N7 viruses isolated from a case of conjunctivitis and from the fatal case, both viruses attached to tissues of the human respiratory tract, suggesting that the observed differences in phenotype can not be explained solely by differences in attachment pattern.

In mice, it was shown that the difference in pathogenicity between the CC and FC virus was primarily determined by PB2 of the FC virus. Virus titers in mice infected with the FC virus or a reassortant virus containing the FC PB2 gene segment were significantly higher, suggesting a role in enhanced replication of the FC PB2 gene segment. The role of FC PB2 in enhanced replication was confirmed by *in vitro* studies. Thus, the increase in both pathogenicity and replication was caused by the glutamic acid to lysine substitution at position 627 (E627K) in PB2 of the FC virus. The E627K substitution was previously described as an adaptation towards efficient replication at lower temperatures[250], where the glutamic acid in avian viruses would explain the inability of these viruses to replicate in the human upper respiratory tract (URT). The increased replication of the FC was shown not to be related to temperature nor to host restriction, but merely to represent an adaptation to efficient replication in mammals. Recent studies on the pathogenesis of HPAI H5N1 showed a prominent role for the lysine at position 627 of PB2[190,234]. Moreover, the lysine at position 627 is also present in the 1918 'Spanish' influenza virus[53], all human lineages of influenza A viruses and a variety of recent mammalian H5 HPAI isolates, whereas, in virtually all LPAI viruses the amino acid residue at position 627 is a glutamic acid (911 out of 912). Together, these data indicate the necessity of this residue for efficient viral replication of avian influenza viruses in mammals. The fact that polymerase complexes with the 'human-like' lysine at position still replicate efficiently in cells of avian origin may explain why there appears to be no negative selection on the lysine at position 627 in contemporary HPAI H5N1 viruses which were recently isolated from wild birds[38]. No effect on pathogenicity in mice was observed in any of the other gene segments

of the FC virus. However, the HA of the FC virus caused increased virus titers in the lungs and was not confined to the lungs but was also detected in other tissues (liver, kidney and brain). This was caused by the amino acid substitution from alanine to threonine at position 143 that introduces a potential glycosylation site at position 141 of HA, and that caused increased replication *in vitro* and affected the attachment pattern of the FC virus. However, no difference was observed between the CC and FC virus in hemagglutination of turkey erythrocytes, expressing α2,3- and α2,6-linked sialic acids, or horse erythrocytes, expressing predominantly α2,3-linked sialic acids[281], indicating that no large differences in receptor binding specificity, but more subtle differences in receptor affinity may exist. Although no contribution of the PA, NA and NS in the pathogenicity was observed in mice, all 3 gene segments resulted in increased growth kinetics *in vitro* as compared with the wild type CC virus. Only recently, a chicken H7N7 virus isolated from chickens at the farm that was visited by the deceased veterinarian was sequenced. Sequence analysis revealed that 12 out of 14 amino acid substitutions in the FC virus were already present in this virus, except for the E627K substitution in PB2 and the K416R substitution in HA. The circulation of the H7N7 HPAI virus during the 2003 outbreak potentially facilitated adaptation of the viruses from replication in their original host (wild birds) towards replication in poultry. These replication-enhancing mutations potentially allowed the virus to enter the lower respiratory tract of the veterinairian, replicate and acquire the E627K substitution. This therefore suggests that adaptations facilitating transmission across the species barrier can already occur in poultry.

Taken together, the data presented here lead us to suggest that the virus isolated from the fatal case either entered via the ocular epithelium and gained access to the lower respiratory tract, e.g. via the lacrimal duct, or that the virus accessed the lower respiratory tract directly. During the initial infection the virus acquired the PB2 E627K substitution, which allowed efficient replication of this virus in the lower respiratory tract, leading to pneumonia, acute respiratory distress syndrome and eventually death. Moreover, it was shown that avian influenza A viruses can bind to the human ocular tissues and respiratory tract without prior adaptation, indicating that although attachment is a prerequisite for virus replication, it does not determine outcome of infection.

Great similarities were observed in the attachment pattern to the human respiratory tract of the H7N7 fatal case virus and of A/Vietnam/1194/04, a recent H5N1 HPAI isolate (chapter 12). The attachment pattern observed is in line with the distribution of the receptor in the human respiratory tract, used by avian influenza A viruses (α2,3-linked SA)[232]. Like A/Vietnam/1194/04, the FC virus attaches to type II pneumocytes, alveolar macrophages and non-ciliated cuboidal epithelial cells in the bronchioles. The attachment pattern of these viruses corresponds with the pathological findings in fatal cases of H5N1 and H7N7 infection[52,55,282], which show diffuse alveolar damage in the lower pulmonary lobes. However, they contrast with the idea that avian influenza viruses generally have little affinity for human respiratory tissues. Attachment of HPAI viruses to the lower respiratory tract may be a limiting factor in human-to-human transmissibility of H5N1 and H7N7 viruses and effective transmission may only take place once avian influenza viruses are capable of replication in the upper respiratory tract. While the mechanism behind the ability of avian influenza viruses to transmit efficiently to humans has recently become more clear (chapters 10, 11

and 12), the mechanism behind acquiring determinants of efficient human-to-human transmission remains elusive. Effective human-to-human transmission has not yet been established by HPAI H5N1 viruses. Whereas mutations in H1, H2 and H3 have been identified that cause a shift from avian to human receptor specificity, these same mutations do not cause a equivalent shift in receptor specificity in HPAI H5 viruses[283], indicating that there are viral constraints which inhibit the viral adaptation to efficient shift from avian to human receptor affinity. Recent experiments with reassortment of HPAI H5N1 viruses and contemporary human H3N2 influenza viruses showed that these reassortant viruses did not exhibit efficient transmission abilities, showing that although reassortment might render viable strains in terms of replication, it is not an indication of the pandemic potential of the strain. Interestingly, a reassortant bearing the internal genes of the avian virus and the HA and NA of a recent human H3N2 virus was able to be excreted from the upper respiratory tract but failed to transmit sufficiently[283]. This indicates, that although efficient replication and excretion from the upper respiratory tract enhance the likelihood of transmission, other molecular determinants in influenza viruses (and hosts) determine the efficiency of transmissibility.

General conclusion

Despite the fact that H5 and H7 subtype influenza A viruses are currently responsible for the outbreaks and epizootics in poultry, it is important to remember that all known pandemics in humans and several problems in poultry had their origin in other subtypes of LPAI viruses. From this historical point of view, the current HPAI H5N1 threat is not an isolated problem for public health, animal health and agriculture. A complete inventory of the influenza A viruses detected in wild birds is therefore important. There is a possibility that virtually all influenza A viruses presenting as low or even non-pathogenic in their natural reservoir may have the capacity to become more pathogenic upon transmission to ‘non-natural’ hosts. The long term and harmonized surveillance in wild birds, poultry and humans in a worldwide multidisciplinary network with a non prestigious exchange of information between the actors, may help assessing the capacity of viruses of subtypes H5 and H7 as well as other subtypes to cross the species barrier and reduce the threats posed to human and animal health.

References

R

1. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992) Evolution and ecology of influenza A viruses. Microbiol Rev 56: 152-179.
2. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, editors. Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses, 2005.
3. Lamb RA, Krug RM (2001) Orthomyxoviridae: the viruses and their replication. In: Knipe DM, Howley PM, editors. Fields Virology. 4th ed. Philadelphia, PA: Lippincott Williams & Wilkins. pp. 1487-1531.
4. Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, et al. (2001) A novel influenza A virus mitochondrial protein that induces cell death. Nat Med 7: 1306-1312.
5. Wright PF, Webster RG (2001) Orthomyxoviruses. In: Knipe DM, Howley PM, editors. Fields Virology. 4th ed. Philadelphia, PA: Lippincott Williams & Wilkins. pp. 1533-1579.
6. Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, et al. (2005) Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 79: 2814-2822.
7. Alexander DJ (2000) A review of avian influenza in different bird species. Vet Microbiol 74: 3-13.
8. Webster RG, Rott R (1987) Influenza virus A pathogenicity: the pivotal role of hemagglutinin. Cell 50: 665-666.
9. Capua I, Alexander DJ (2006) The challenge of avian influenza to the veterinary community. Avian Pathol 35: 189-205.
10. Science (2006) Supporting Online Material for Global Patterns of Influenza A Virus in Wild Birds. http://www.sciencemag.org/cgi/data/312/5772/384/DC1/1.
11. Del Hoyo J, Elliot A, Sargatal J, Eds. (1996) Handbook of the Birds of the world. Barcelona: Lynx Edicions.
12. Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KG (1978) Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology 84: 268-278.
13. van de Kam J, Ens B, Piersma T, Zwarts L (2004) Shorebirds: An Illustrated Behavioural Ecology. Utrecht: KNNV Publishers.
14. Alerstam T, Lindstrom A (1990) In bird Migration: Physiology and Ecophysiology; Gwinner E, editor. Berlin: Springer-Verlag. 331-351.
15. Okazaki K, Takada A, Ito T, Imai M, Takakuwa H, et al. (2000) Precursor genes of future pandemic influenza viruses are perpetuated in ducks nesting in Siberia. Arch Virol 145: 885-893.
16. Hanson BA, Stallknecht DE, Swayne DE, Lewis LA, Senne DA (2003) Avian influenza viruses in Minnesota ducks during 1998-2000. Avian Dis 47: 867-871.
17. Rohani P, Earn DJ, Grenfell BT (1999) Opposite patterns of synchrony in sympatric disease metapopulations. Science 286: 968-971.
18. Nguyen DC, Uyeki TM, Jadhao S, Maines T, Shaw M, et al. (2005) Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in Hanoi, Vietnam, in 2001. J Virol 79: 4201-4212.
19. Kida H, Yanagawa R, Matsuoka Y (1980) Duck influenza lacking evidence of disease signs and immune response. Infect Immun 30: 547-553.
20. Sharp GB, Kawaoka Y, Jones DJ, Bean WJ, Pryor SP, et al. (1997) Coinfection of wild ducks by influenza A viruses: distribution patterns and biological significance. J Virol 71: 6128-6135.
21. Scott DA, Rose PM (1996) Atlas of Anatidae populations in Africa and Western Eurasia. Wageningen, Netherlands: Wetlands International.
22. Brown LH, Urban EK, Newman K (1982) The Birds of Africa. London: Academic Press.
23. Becker WB (1961) The isolation and classification of Tern virus: influenza A-Tern South Africa-1961. Journal of Hygiene (London) 64: 309-320.
24. Kawaoka Y, Chambers TM, Sladen WL, Webster RG (1988) Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? Virology 163: 247-250.
25. Piersma T (1997) Do global patterns of habitat use and migration strategics co-evolve with relative investments in immunocompetence due to spatial variation in parasite pressure? Oikos 80: 623-631.
26. Krauss S, Walker D, Pryor SP, Niles L, Chenghong L, et al. (2004) Influenza A viruses of migrating wild aquatic birds in North America. Vector Borne Zoonotic Dis 4: 177-189.
27. Widjaja L, Krauss SL, Webby RJ, Xie T, Webster RG (2004) Matrix gene of influenza a viruses isolated from wild aquatic birds: ecology and emergence of influenza a viruses. J Virol 78: 8771-8779.
28. Spackman E, Stallknecht DE, Slemons RD, Winker K, Suarez DL, et al. (2005) Phylogenetic analyses of type A influenza genes in natural reservoir species in North America reveals genetic variation. Virus Res 114: 89-100.
29. Gill RE, Butler RW, Tomkovich PS, Mundkur T, Handel CM. (1994) Trans. North Am. Wildlife Nat. Recouces conf. 59-63.
30. Wallensten A, Munster VJ, Elmberg J, Osterhaus AD, Fouchier RA, et al. (2005) Multiple gene segment reassortment between Eurasian and American lineages of influenza A virus (H6N2) in Guillemot (*Uria aalge*). Arch Virol 150: 1685-1692.
31. Makarova NV, Kaverin NV, Krauss S, Senne D, Webster RG (1999) Transmission of Eurasian avian H2 influenza virus to shorebirds in North America. J Gen Virol 80 (Pt 12): 3167-3171.
32. Liu JH, Okazaki K, Bai GR, Shi WM, Mweene A, et al. (2004) Interregional transmission of the internal protein genes of H2 influenza virus in migratory ducks from North America to Eurasia. Virus Genes 29: 81-86.
33. Obenauer JC, Denson J, Mehta PK, Su X, Mukatira S, et al. (2006) Large-scale sequence analysis of avian influenza isolates. Science 311: 1576-1580.

34. Hatchette TF, Walker D, Johnson C, Baker A, Pryor SP, et al. (2004) Influenza A viruses in feral Canadian ducks: extensive reassortment in nature. J Gen Virol 85: 2327-2337.
35. de Jong JC, Claas EC, Osterhaus AD, Webster RG, Lim WL (1997) A pandemic warning? Nature 389: 554.
36. Ellis TM, Bousfield RB, Bissett LA, Dyrting KC, Luk GS, et al. (2004) Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. Avian Pathol 33: 492-505.
37. Liu J, Xiao H, Lei F, Zhu Q, Qin K, et al. (2005) Highly pathogenic H5N1 influenza virus infection in migratory birds. Science 309: 1206.
38. Chen H, Smith GJ, Zhang SY, Qin K, Wang J, et al. (2005) Avian flu: H5N1 virus outbreak in migratory waterfowl. Nature 436: 191-192.
39. Chen HX, Shen HG, Li XL, Zhou JY, Hou YQ, et al. (2006) Seroprevalance and identification of influenza A virus infection from migratory wild waterfowl in China (2004-2005). J Vet Med B Infect Dis Vet Public Health 53: 166-170.
40. Sturm-Ramirez KM, Ellis T, Bousfield B, Bissett L, Dyrting K, et al. (2004) Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. J Virol 78: 4892-4901.
41. OIE (2006) Update on avian influenza in animals (type H5). http://www.oie.int/downld/AVIAN%20INFLUENZA/A_AI-Asia.htm.
42. WHO (2006) Cumulative Number of Confirmed Human Cases of Avian Influenza A (H5N1), since October 3 2006. http://wwwwhoint/csr/disease/avian_influenza/country/cases_table_2006_10_03/en/indexhtml.
43. Li KS, Guan Y, Wang J, Smith GJ, Xu KM, et al. (2004) Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature 430: 209-213.
44. Martin V, Sims L, Lubroth J, Pfeiffer D, Slingenbergh J, et al. (2006) Epidemiology and ecology of highly pathogenic avian influenza with particular emphasis on South East Asia. Dev Biol (Basel) 124: 23-36.
45. Gilbert M, Chaitaweesub P, Parakamawongsa T, Premashthira S, Tiensin T, et al. (2006) Free-grazing ducks and highly pathogenic avian influenza, Thailand. Emerg Infect Dis 12: 227-234.
46. Scholtissek C, Rohde W, Von Hoyningen V, Rott R (1978) On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology 87: 13-20.
47. Kawaoka Y, Krauss S, Webster RG (1989) Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol 63: 4603-4608.
48. Webster RG, Geraci J, Petursson G, Skirnisson K (1981) Conjunctivitis in human beings caused by influenza A virus of seals [letter]. N Engl J Med 304: 911.
49. Bridges CB, Lim W, Hu-Primmer J, Sims L, Fukuda K, et al. (2002) Risk of influenza A (H5N1) infection among poultry workers, Hong Kong, 1997-1998. J Infect Dis 185: 1005-1010.
50. Puzelli S, Di Trani L, Fabiani C, Campitelli L, De Marco MA, et al. (2005) Serological analysis of serum samples from humans exposed to avian H7 influenza viruses in Italy between 1999 and 2003. J Infect Dis 192: 1318-1322.
51. Meijer A, Bosman A, van de Kamp EE, Wilbrink B, van Beest Holle Mdu R, et al. (2006) Measurement of antibodies to avian influenza virus A(H7N7) in humans by hemagglutination inhibition test. J Virol Methods 132: 113-120.
52. Beigel JH, Farrar J, Han AM, Hayden FG, Hyer R, et al. (2005) Avian influenza A (H5N1) infection in humans. N Engl J Med 353: 1374-1385.
53. Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, et al. (2005) Characterization of the 1918 influenza virus polymerase genes. Nature 437: 889-893.
54. Reid AH, Taubenberger JK, Fanning TG (2004) Evidence of an absence: the genetic origins of the 1918 pandemic influenza virus. Nat Rev Microbiol 2: 909-914.
55. Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, et al. (2004) Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci U S A 101: 1356-1361.
56. Smith DJ, Lapedes AS, de Jong JC, Bestebroer TM, Rimmelzwaan GF, et al. (2004) Mapping the antigenic and genetic evolution of influenza virus. Science 305: 371-376.
57. Swayne DE, Garcia M, Beck JR, Kinney N, Suarez DL (2000) Protection against diverse highly pathogenic H5 avian influenza viruses in chickens immunized with a recombinant fowlpox vaccine containing an H5 avian influenza hemagglutinin gene insert. Vaccine 18: 1088-1095.
58. Lüschow D, Werner O, Mettenleiter TC, Fuchs W (2001) Protection of chickens from lethal avian influenza A virus infection by live-virus vaccination with infectious laryngotracheitis virus recombinants expressing the hemagglutinin (H5) gene. Vaccine 19: 4249-4259.
59. Veits J, Wiesner D, Fuchs W, Hoffmann B, Granzow H, et al. (2006) Newcastle disease virus expressing H5 hemagglutinin gene protects chickens against Newcastle disease and avian influenza. Proc Natl Acad Sci U S A 103: 8197-8202.
60. van der Goot JA, Koch G, de Jong MC, van Boven M (2005) Quantification of the effect of vaccination on transmission of avian influenza (H7N7) in chickens. Proc Natl Acad Sci U S A 102: 18141-18146.
61. Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, et al. (2006) Global patterns of influenza A virus in wild birds. Science 312: 384-388.
62. De Marco MA, Foni E, Campitelli L, Raffini E, Delogu M, et al. (2003) Long-term monitoring for avian influenza viruses in wild bird species in Italy. Vet Res Commun 27 Suppl 1: 107-114.
63. Fouchier RA, Olsen B, Bestebroer TM, Herfst S, van der Kemp L, et al. (2003) Influenza A virus surveillance in wild birds in Northern Europe in 1999 and 2000. Avian Dis 47: 857-860.

64. Süss J, Schafer J, Sinnecker H, Webster RG (1994) Influenza virus subtypes in aquatic birds of eastern Germany. Arch Virol 135: 101-114.
65. Hinshaw VS, Air GM, Gibbs AJ, Graves L, Prescott B, et al. (1982) Antigenic and genetic characterization of a novel hemagglutinin subtype of influenza A viruses from gulls. J Virol 42: 865-872.
66. Chambers TM, Yamnikova S, Kawaoka Y, Lvov DK, Webster RG (1989) Antigenic and molecular characterization of subtype H13 hemagglutinin of influenza virus. Virology 172: 180-188.
67. Butt KM, Smith GJ, Chen H, Zhang LJ, Leung YH, et al. (2005) Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. J Clin Microbiol 43: 5760-5767.
68. Keawcharoen J, Oraveerakul K, Kuiken T, Fouchier RA, Amonsin A, et al. (2004) Avian influenza H5N1 in tigers and leopards. Emerg Infect Dis 10: 2189-2191.
69. WHO (2004) WHO manual on animal influenza diagnosis and surveillance. http://www.who.int/csr/resources/publications/influenza/WHO_CDS_CSR_NCS_2002_5/en/index.html.
70. Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, et al. (2002) Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol 40: 3256-3260.
71. Ward CL, Dempsey MH, Ring CJ, Kempson RE, Zhang L, et al. (2004) Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. J Clin Virol 29: 179-188.
72. Noordwijk AJv, Speek G, Clark JA, Rohde Z, Wassenaar RD (2003) The EURING exchange code 2000. Journal of Ornithology: 479-483.
73. Munster VJ, Wallensten A, Baas C, Rimmelzwaan GF, Schutten M, et al. (2005) Mallards and highly pathogenic avian influenza ancestral viruses, Northern Europe. Emerg Infect Dis 11: 1545-1551.
74. Kawaoka Y, Yamnikova S, Chambers TM, Lvov DK, Webster RG (1990) Molecular characterization of a new hemagglutinin, subtype H14, of influenza A virus. Virology 179: 759-767.
75. Rohm C, Zhou N, Suss J, Mackenzie J, Webster RG (1996) Characterization of a novel influenza hemagglutinin, H15: criteria for determination of influenza A subtypes. Virology 217: 508-516.
76. Woolcock PR, Suarez DL, Kuney D (2003) Low-pathogenicity avian influenza virus (H6N2) in chickens in California, 2000-02. Avian Dis 47: 872-881.
77. Kinde H, Read DH, Daft BM, Hammarlund M, Moore J, et al. (2003) The occurrence of avian influenza A subtype H6N2 in commercial layer flocks in Southern California (2000-02): clinicopathologic findings. Avian Dis 47: 1214-1218.
78. Chen H, Smith GJ, Li KS, Wang J, Fan XH, et al. (2006) Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. Proc Natl Acad Sci U S A 103: 2845-2850.
79. Munster VJ, Veen J, Olsen B, Vogel R, Osterhaus AD, et al. (2006) Towards improved influenza A virus surveillance in migrating birds. Vaccine.
80. Fouchier RA, Bestebroer TM, Herfst S, Van Der Kemp L, Rimmelzwaan GF, et al. (2000) Detection of influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene. J Clin Microbiol 38: 4096-4101.
81. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001) Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 146: 2275-2289.
82. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL (2005) GenBank. Nucleic Acids Res 33: D34-38.
83. Hinshaw VS, Webster RG, Bean WJ, Sriram G (1980) The ecology of influenza viruses in ducks and analysis of influenza viruses with monoclonal antibodies. Comp Immunol Microbiol Infect Dis 3: 155-164.
84. Austin FJ, Webster RG (1993) Evidence of ortho- and paramyxoviruses in fauna from Antarctica. J Wildl Dis 29: 568-571.
85. Morgan IR, Westbury HA (1981) Virological studies of Adélie Penguins (Pygoscelis adeliae) in Antarctica. Avian Dis 25: 1019-1026.
86. Baumeister E, Leotta G, Pontoriero A, Campos A, Monalti D, et al. (2004) Serological evidence of influenza A virus infection in Antarctica migratory birds. International Congress Series 1263: 737-740.
87. Ebel GD, Dupuis AP, 2nd, Nicholas D, Young D, Maffei J, et al. (2002) Detection by enzyme-linked immunosorbent assay of antibodies to West Nile virus in birds. Emerg Infect Dis 8: 979-982.
88. Wallensten A, Munster VJ, Fouchier RAM, Olsen B (2004) Avian influenza A virus in ducks migrating through Sweden. International Congress Series 1263: 771-772.
89. Briggs LC, Ashton RM, Metcalf P (2003) Development of a highly sensitive screen for influenza A in guano and its application in the search for ancient RNA preserved under Antarctic Adelie penguin colonies. Avian Dis 47: 1200-1202.
90. Cannon RM, Roe RT (1982) Livestock disease surveys. A field manual for veterinairians. Canberra: Australian Government Publishing Service.
91. Sinnecker R, Sinnecker H, Zilske E, Kohler D (1983) Surveillance of pelagic birds for influenza A viruses. Acta Virol 27: 75-79.
92. Lvov DK (1978) Viruses and environment. London: Academic Press. 351-380 p.
93. Stallknecht DE, Kearney MT, Shane SM, Zwank PJ (1990) Effects of pH, temperature, and salinity on persistence of avian influenza viruses in water. Avian Dis 34: 412-418.
94. Stallknecht DE, Shane SM, Kearney MT, Zwank PJ (1990) Persistence of avian influenza viruses in water. Avian Dis 34: 406-411.
95. Pearce DA, Wilson WH (2003) Viruses in antarctic ecosystems. Antarctic Science 15: 319-331.

96. Cox NJ, Fuller F, Kaverin N, Klenk HD, Lamb RA, et al. (2000) Orthomyxoviridae. In: van Regenmortel MHV, Fauquet CM, Bishop DH, Carstens EB, Estes MK et al., editors. Virus Taxonomy; Seventh Report of the International Committee on Taxonomy of Viruses. San Diego: Academic Press. 585-597.
97. Osterhaus AD, Rimmelzwaan GF, Martina BE, Bestebroer TM, Fouchier RA (2000) Influenza B virus in seals. Science 288: 1051-1053.
98. Guo YJ, Jin FG, Wang P, Wang M, Zhu JM (1983) Isolation of influenza C virus from pigs and experimental infection of pigs with influenza C virus. J Gen Virol 64: 177-182.
99. Murphy BR, Webster RG (1996) Orthomyxoviruses. In: Fields BN, Knipe DM, Howley PM, editors. Virology. third ed. Philadelphia: Lippincott-Raven publishers. 1397-1445.
100. Memorandum W (1980) A revision of the system of nomenclature for influenza viruses: a WHO memorandum. Bull World Health Organ 58: 585-591.
101. Memorandum W (1971) A revised system of nomenclature for influenza viruses. Bull World Health Organ 45: 119-124.
102. Skehel JJ, Wiley DC (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem 69: 531-569.
103. Wallensten A, Munster VJ, Fouchier RAM, Olsen B (2004) Avian influenza A virus in ducks migrating through Sweden. In: Kawaoka Y, editor. Options for the control of influenza V. Amsterdam: Elsevier Science B.V. 771-772.
104. Rimmelzwaan GF, Baars M, Claas EC, Osterhaus AD (1998) Comparison of RNA hybridization, hemagglutination assay, titration of infectious virus and immunofluorescence as methods for monitoring influenza virus replication in vitro. J Virol Methods 74: 57-66.
105. Salk JE (1944) Simplified procedure for titrating hemagglutinating capacity of influenza virus and the corresponding antibody. J Immunol 49: 87-98.
106. Aymard-Henry M, Coleman MT, Dowdle WR, Laver WG, Schild GC, et al. (1973) Influenza neuraminidase and neuraminidase-inhibition test procedures. Bull World Health Organ 48: 199-202.
107. Hall A (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis prgram for Windows 95/98/NT. Nucl Acids Symp Ser 41: 95-98.
108. Felsenstein J (1989) PHYLIP - Phylogeny Inference Package (Version 3.2). Cladistics 5: 164-166.
109. Suarez DL, Schultz-Cherry S (2000) The effect of eukaryotic expression vectors and adjuvants on DNA vaccines in chickens using an avian influenza model. Avian Dis 44: 861-868.
110. Bokhout BA, van Gaalen C, van der Heijden PJ (1981) A selected water-in-oil emulsion: composition and usefulness as an immunological adjuvant. Vet Immunol Immunopathol 2: 491-500.
111. Hornbeck P (2001) Double-immunodiffusion assay for detecting specific antibodies. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, editors. Current protocols in immunology. New York: John Wiley & Sons. Chapter 2.3. 1-4.
112. Hamming RW (1950) Error detecting and error correcting codes. Bell System Tech Journal 9: 147-160.
113. Hinshaw VS, Bean WJ, Geraci J, Fiorelli P, Early G, et al. (1986) Characterization of two influenza A viruses from a pilot whale. J Virol 58: 655-656.
114. Yamnikova SS, Gambaryan AS, Tuzikov AB, Bovin NV, Matrosovich MN, et al. (2003) Differences between HA receptor-binding sites of avian influenza viruses isolated from Laridae and Anatidae. Avian Dis 47: 1164-1168.
115. Kawaoka Y, Gorman OT, Ito T, Wells K, Donis RO, et al. (1998) Influence of host species on the evolution of the nonstructural (NS) gene of influenza A viruses. Virus Res 55: 143-156.
116. Suarez DL, Perdue ML (1998) Multiple alignment comparison of the non-structural genes of influenza A viruses. Virus Res 54: 59-69.
117. Ito T, Gorman OT, Kawaoka Y, Bean WJ, Webster RG (1991) Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. J Virol 65: 5491-5498.
118. Kawaoka Y, Krauss S, Webster RG (1989) Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol 63: 4603-4608.
119. Gorman OT, Donis RO, Kawaoka Y, Webster RG (1990) Evolution of influenza A virus PB2 genes: implications for evolution of the ribonucleoprotein complex and origin of human influenza A virus. J Virol 64: 4893-4902.
120. Gorman OT, Bean WJ, Kawaoka Y, Donatelli I, Guo YJ, et al. (1991) Evolution of influenza A virus nucleoprotein genes: implications for the origins of H1N1 human and classical swine viruses. J Virol 65: 3704-3714.
121. Sneath PHA, Sokal RR (1973) Numerical taxonomy: the priniciples and practice of numerical classification. San Fransisco: Freeman. 573.
122. Clements JF (2000) Birds of the world, a checklist. Sussex: Pica Press.
123. Nettleship DN, Birkhead TR (1985) The atlantic alcidae: The evolution, distribution and biology of the auks inhabiting the Atlantic and adjacent water areas. London: Academic Press.
124. Newton I (1998) Population limits in birds. San Diego: Academic Press.
125. Hatch SA, Meyers PM, Mulcahy DM, Douglas DC (2000) Seasonal movements and pelagic habitat use of murres and puffins determined by satellite telemetry. Condor 102: 145-154.
126. Väisänen RA, Lammi E, Koskimies P (1998) Muuttuva pesimälinnusto [Finnish bird atlas, in Finnish]. Helsinki: Otava.
127. Halley DJ, Harris MP (1993) Intercolony Movement and Behavior of Immature Guillemots Uria-Aalge. Ibis 135: 264-270.

128. Sazonov AA, Lvov DK, Webster RG, Sokolova TV, Braude NA, et al. (1977) Isolation of an influenza virus, similar to A/Port Chalmers/1/73 (H3N2) from a common murre at Sakhalin Island in U.S.S.R. (Strain A/common murre/Sakhalin/1/74). Arch Virol 53: 1-7.
129. Donis RO, Bean WJ, Kawaoka Y, Webster RG (1989) Distinct lineages of influenza virus H4 hemagglutinin genes in different regions of the world. Virology 169: 408-417.
130. Schafer JR, Kawaoka Y, Bean WJ, Suss J, Senne D, et al. (1993) Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir. Virology 194: 781-788.
131. Guan Y, Peiris JS, Poon LL, Dyrting KC, Ellis TM, et al. (2003) Reassortants of H5N1 influenza viruses recently isolated from aquatic poultry in Hong Kong SAR. Avian Dis 47: 911-913.
132. Olsson O, Nilsson T, Fransson T (2000) Long-term mortality in the common guillemot in the Baltic Sea. Swedish Environmental Protection Agency. 5057 5057.
133. Bakken V, Runde O, Tjörve E (2004) Norweigian bird ringing atlas, vol 1. Stavanger: Stavanger Museum.
134. Wernham CV, Toms MP, Marchant JH, Clark JA, Siriwardena GM, et al. (2002) The migration atlas, movement of the birds of Britain and Ireland. London: T. & A.D. Poyser.
135. Viney ME, Riley EM, Buchanan KL (2005) Optimal immune responses: immunocompetence revisited. Trends in Ecology & Evolution 20: 665-669.
136. Hedenström A, Alerstam T (1997) Optimum fuel loads in migratory birds: Distinguishing between time and energy minimization. Journal of Theoretical Biology 189: 227-234.
137. Martin P, Bateson P (1993) Measuring behaviour: An introductory guide. Cambridge: Camebridge University Press.
138. Bowler JM (1994) The condition of Bewick's swans *Cygnus columbianus bewickii* in winter as assessed by their abdominal profile. Ardea 82: 241-248.
139. Madsen J, Klaassen M (2006) Assessing body condition and energy budget components by scoring abdominal profiles in free-ranging pink-footed geese Anser brachyrhynchus. Journal of Avian Biology 37: 283-287.
140. Klaassen M, Bauer S, Madsen J, Tombre I (2005) Modelling behavioural and fitness consequences of disturbance for geese along their spring flyway. J Appl Ecol 43: 92-100.
141. Software S (2000) Systat Version 1.0. Point Richmont, CA: Systat Software.
142. Wallensten A, Munster VJ, Osterhaus ADME, Waldenström J, Bonnedahl J, et al. (2006) Mounting evidence for the presence of influenza A virus in the avifauna of the Antarctic region. Antarctic Science 18: 353-356.
143. Rising JD, Somers KM (1989) The Measurement of Overall Body Size in Birds. Auk 106: 666-674.
144. Choudhury S, Black JM, Owen M (1996) Body size, fitness and compatibility in barnacle geese Branta leucopsis. Ibis 138: 700-709.
145. Rees EC, Lievesly P, Pettifor RA, Perrins C (1996) Partnership in Birds: the study of monogamy; Black JM, editor. Oxford: Oxford University Press. 118-137.
146. Coleman JT, Spray CJ, Percival SM, Rickeard AT, Yeoman P (2002) The dynamics of a flock of mute swans at Berwick-upon-Tweed with particular reference to the effects of age, sex, social status and body condition on molt. Waterbirds 25: 346-351.
147. Rees E (2006) Bewick's swan. London: T. & A.D. Poyser.
148. Sedinger JS (1997) Adaptations to and consequences of an herbivorous diet in grouse and waterfowl. Condor 99: 314-326.
149. Piersma T, Drent J (2003) Phenotypic flexibility and the evolution of organismal design. Trends in Ecology & Evolution 18: 228-233.
150. Van Gils JA, Klaassen M, Beekman JH (Unpublished Data) Based on dissections of 11 carcasses we found an increase in gut length throughout the winter of 31.0 cm/month (SE = 7.1 cm/month; $r = 0.82; F_{1,9}=19.15; p<0.005$.
151. Evans MJ, Kear J (1978) Weights and measurments of Bewick's swans during winter. Wildfowl 29: 118-122.
152. Nolet BA, Drent RH (1998) Bewick's swans refuelling on pondweed tubers in the Dvina Bay (White Sea) during their spring migration: firts come, first served. Journal of Avian Biology 29: 574-581.
153. Kokko H (1999) Competition for early arrival in migratory birds. Journal of Animal Ecology 68: 940-950.
154. Both C, Visser ME (2005) The effect of climate change on the correlation between avian life-history traits. Global Change Biology 11: 1606-1613.
155. Cramps S, Simmons KEL, Eds (1977) The birds of the western Palearctic. Oxford: Oxford University Press.
156. Horimoto T, Rivera E, Pearson J, Senne D, Krauss S, et al. (1995) Origin and molecular changes associated with emergence of a highly pathogenic H5N2 influenza virus in Mexico. Virology 213: 223-230.
157. Capua I, Marangon S, dalla Pozza M, Terregino C, Cattoli G (2003) Avian influenza in Italy 1997-2001. Avian Dis 47: 839-843.
158. Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, et al. (1998) Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 351: 472-477.
159. Everett C (2004) USDA Confirms Highly Pathogenic Avian Influenza in Texas. ProMed.
160. Jones YL, Swayne DE (2004) Comparative pathobiology of low and high pathogenicity H7N3 Chilean avian influenza viruses in chickens. Avian Dis 48: 119-128.
161. Koopmans M, Wilbrink B, Conyn M, Natrop G, van der Nat H, et al. (2004) Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. Lancet 363: 587-593.

162. Hirst M, Astell CR, Griffith M, Coughlin SM, Moksa M, et al. (2004) Novel avian influenza H7N3 strain outbreak, British Columbia. Emerg Infect Dis 10: 2192-2195.
163. Alexander DJ, Brown IH (2000) Recent zoonoses caused by influenza A viruses. Rev Sci Tech 19: 197-225.
164. Webster RG (1993) Are equine 1 influenza viruses still present in horses? Equine Vet J 25: 537-538.
165. Lang G, Gagnon A, Geraci JR (1981) Isolation of an influenza A virus from seals. Arch Virol 68: 189-195.
166. Banks J, Speidel E, Alexander DJ (1998) Characterisation of an avian influenza A virus isolated from a human--is an intermediate host necessary for the emergence of pandemic influenza viruses? Arch Virol 143: 781-787.
167. Update: influenza activity--United States and worldwide, 2003-04 season, and composition of the 2004-05 influenza vaccine. MMWR Morb Mortal Wkly Rep 53: 547-552.
168. Promed (2004) Avian influenza A (H7N2) virus, human - USA (New York). Available from http://www.promedmail.org [archive no. 20040420.1104].
169. Promed (2004) Avian influenza A (H7N3) virus, human - Canada (BC) (02) Available from http://www.promedmail.org [archive no. 20040402.0908].
170. WHO (2005) Cumulative number of confirmed human cases of avian influenza A (H5N1) since 28 January 2004. http://who.int/csr/disease/avian_influenza/country/cases_table_2005_06_28/en/index.html
171. Promed (2004) Avian influenza, porcine, H5N1 - China (06): OIE. Available from http://www.promedmail.org [archive no. 20040905.2483].
172. Promed (2004) Avian influenza H5N1, mammals - East Asia. Available from http://www.promedmail.org [archive no. 20040221.0560].
173. Kuiken T, Rimmelzwaan G, van Riel D, van Amerongen G, Baars M, et al. (2004) Avian H5N1 influenza in cats. Science 306: 241.
174. Keawcharoen J, Oraveerakul K, Kuiken T, Fouchier RAM, Amonsin A, et al. (2004) Avian influenza H5N1 in tigers and leopards. Emerg Infect Dis In Press.
175. Nicolson C, Major D, Wood JM, Robertson JS (2005) Generation of influenza vaccine viruses on Vero cells by reverse genetics: an H5N1 candidate vaccine strain produced under a quality system. Vaccine 23: 2943-2952.
176. Li S, Liu C, Klimov A, Subbarao K, Perdue ML, et al. (1999) Recombinant influenza A virus vaccines for the pathogenic human A/Hong Kong/97 (H5N1) viruses. J Infect Dis 179: 1132-1138.
177. Webby RJ, Perez DR, Coleman JS, Guan Y, Knight JH, et al. (2004) Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. Lancet 363: 1099-1103.
178. Liu M, Wood JM, Ellis T, Krauss S, Seiler P, et al. (2003) Preparation of a standardized, efficacious agricultural H5N3 vaccine by reverse genetics. Virology 314: 580-590.
179. Wood JM, Major D, Newman RW, Dunleavy U, Nicolson C, et al. (2002) Preparation of vaccines against H5N1 influenza. Vaccine 20 Suppl 2: S84-87.
180. Rimmelzwaan GF, Baars M, van Beek R, van Amerongen G, Lovgren-Bengtsson K, et al. (1997) Induction of protective immunity against influenza virus in a macaque model: comparison of conventional and iscom vaccines. J Gen Virol 78 (Pt 4): 757-765.
181. Rimmelzwaan GF, Claas EC, van Amerongen G, de Jong JC, Osterhaus AD (1999) ISCOM vaccine induced protection against a lethal challenge with a human H5N1 influenza virus. Vaccine 17: 1355-1358.
182. Morein B, Sundquist B, Hoglund S, Dalsgaard K, Osterhaus A (1984) Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. Nature 308: 457-460.
183. Mumford JA, Jessett D, Dunleavy U, Wood J, Hannant D, et al. (1994) Antigenicity and immunogenicity of experimental equine influenza ISCOM vaccines. Vaccine 12: 857-863.
184. Rimmelzwaan GF, Nieuwkoop N, Brandenburg A, Sutter G, Beyer WE, et al. (2000) A randomized, double blind study in young healthy adults comparing cell mediated and humoral immune responses induced by influenza ISCOM vaccines and conventional vaccines. Vaccine 19: 1180-1187.
185. de Wit E, Spronken MI, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, et al. (2004) Efficient generation and growth of influenza virus A/PR/8/34 from eight cDNA fragments. Virus Res 103: 155-161.
186. Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, et al. (1999) Rescue of influenza A virus from recombinant DNA. J Virol 73: 9679-9682.
187. Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, et al. (1999) Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci U S A 96: 9345-9350.
188. Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG (2000) A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci U S A 97: 6108-6113.
189. Stittelaar KJ, Boes J, Kersten GF, Spiekstra A, Mulder PG, et al. (2000) In vivo antibody response and in vitro CTL activation induced by selected measles vaccine candidates, prepared with purified Quil A components. Vaccine 18: 2482-2493.
190. Lu X, Tumpey TM, Morken T, Zaki SR, Cox NJ, et al. (1999) A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. J Virol 73: 5903-5911.
191. Takada A, Kuboki N, Okazaki K, Ninomiya A, Tanaka H, et al. (1999) Avirulent Avian influenza virus as a vaccine strain against a potential human pandemic. J Virol 73: 8303-8307.
192. Nicholson KG, Colegate AE, Podda A, Stephenson I, Wood J, et al. (2001) Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. Lancet 357: 1937-1943.

193. Crouch CF, Daly J, Hannant D, Wilkins J, Francis MJ (2004) Immune responses and protective efficacy in ponies immunised with an equine influenza ISCOM vaccine containing an 'American lineage' H3N8 virus. Vaccine 23: 418-425.
194. Lipatov AS, Webby RJ, Govorkova EA, Krauss S, Webster RG (2005) Efficacy of H5 influenza vaccines produced by reverse genetics in a lethal mouse model. J Infect Dis 191: 1216-1220.
195. Capua I, Alexander DJ (2004) Avian influenza: recent developments. Avian Pathol 33: 393-404.
196. Alexander DJ, Parsons G, Manvell RJ (1986) Experimental assesment of pathogenicity of eight avian influenza viruses of H5 subtype for chickens, turkeys, ducks and quail. Avian Pathol 15: 647-662.
197. Uys CJ, Becker WB (1967) Experimental infection of chickens with influenza A-Tern/South Africa/1961 and Chicken/Scotland/1959 viruses. II. Pathology. J Comp Pathol 77: 167-173.
198. Gylstorff I (1987) Orthomyxoviren; Gylstorff I, Grimm F, editors. Stuttgart: Verlag Eugen Ulmer. 249-253.
199. Slemons RD, Cooper RS, Orsborn JS (1973) Isolation of type-A influenza viruses from imported exotic birds. Avian Dis 17: 746-751.
200. Alexander DJ (1988) Influenza A isolations from exotic caged birds. Vet Rec 123: 442.
201. Easterday BC, Hinshaw VS (1991) Influenza; Calnek BW, Barnes HJ, Beard LR, D. M, Saifl YM, editors. Ames: Iowas state University Press. 532-551.
202. Lowenstine LJ (1986) Emerging viral diseases of psittacine birds; Kirk RW, editor. Philadelphia: Saunders Co. 705-710.
203. Senne DA, Pearson JE, Miller LD, Gustafson GA (1983) Virus isolations from pet birds submitted for importation into the United States. Avian Dis 27: 731-744.
204. Shivaprasad HL, Woolcock PR, Sakas PS (1994) Avian Inluenza Psittacines. Passerine: 259-260.
205. Ottis K, Bachmann PA (1983) Isolation and characterization of ortho- and paramyxoviruses from feral birds in Europe. Zentralblad fur Veterinairmedizin 30: 22-35.
206. Capua I, Mutinelli F, Terregino C, Cattoli G, Manvell RJ, et al. (2000) Highly pathogenic avian influenza (H7N1) in ostriches farmed in Italy. Veterinary Record 146: 356-356.
207. Promed (2004) Avian influenza, ostriches - South Africa. Available from http://www.promedmail.org [archive no. 20040807.2176].
208. Elbers AR, Fabri TH, de Vries TS, de Wit JJ, Pijpers A, et al. (2004) The highly pathogenic avian influenza A (H7N7) virus epidemic in The Netherlands in 2003--lessons learned from the first five outbreaks. Avian Dis 48: 691-705.
209. Stegeman A, Bouma A, Elbers AR, de Jong MC, Nodelijk G, et al. (2004) Avian influenza A virus (H7N7) epidemic in The Netherlands in 2003: course of the epidemic and effectiveness of control measures. J Infect Dis 190: 2088-2095.
210. Karunakaran D, Newman JA, Halvorson DA, Abraham A (1987) Evaluation of inactivated influenza vaccines in market turkeys. Avian Dis 31: 498-503.
211. Brugh M, Beard CW, Stone HD (1979) Immunization of chickens and turkeys against avian influenza with monovalent and polyvalent oil emulsion vaccines. Am J Vet Res 40: 165-169.
212. Capua I, Terregino C, Cattoli G, Mutinelli F, Rodriguez JF (2003) Development of a DIVA (Differentiating Infected from Vaccinated Animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. Avian Pathol 32: 47-55.
213. Johnsgard PA (1980) Ducks, geese and swans of the world. Lincoln: University press.
214. Masurel N, Ophof P, de Jong P (1981) Antibody response to immunization with influenza A/USSR/77 (H1N1) virus in young individuals primed or unprimed for A/New Jersey/76 (H1N1) virus. J Hyg (Lond) 87: 201-209.
215. Palmer DF, Dowle WR, Coleman MT, Schild GC (1975) Haemagglutination inhibition test. Atlanta: US Department of health, Education and Welfare. 25-62.
216. Couch RB, Kasel JA (1983) Immunity to influenza in man. Annu Rev Microbiol 37: 529-549.
217. Swayne DE, Senne DA, Beard CW (1992) Influenza; Swayne DE, Glisson JR, Jackwood JR, Pearson JE, Read WM, editors. Pennsylvania: American Association of Avian Pathologists. 150-155.
218. Stone HD (1987) Efficacy of avian influenza oil-emulsion vaccines in chickens of various ages. Avian Dis 31: 483-490.
219. Lee CW, Senne DA, Suarez DL (2004) Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. J Virol 78: 8372-8381.
220. Ellis TM, Leung CY, Chow MK, Bissett LA, Wong W, et al. (2004) Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. Avian Pathol 33: 405-412.
221. Capua I, Terregino C, Cattoli G, Toffan A (2004) Increased resistance of vaccinated turkeys to experimental infection with an H7N3 low-pathogenicity avian influenza virus. Avian Pathol 33: 158-163.
222. Garcia A, Johnson H, Srivastava DK, Jayawardene DA, Wehr DR, et al. (1998) Efficacy of inactivated H5N2 influenza vaccines against lethal A/Chicken/Queretaro/19/95 infection. Avian Dis 42: 248-256.
223. Swayne DE, Beck JR, Perdue ML, Beard CW (2001) Efficacy of vaccines in chickens against highly pathogenic Hong Kong H5N1 avian influenza. Avian Dis 45: 355-365.
224. Note for guidance on harmonization of requirements for influenza vaccines (1997), CPMP/BWP/214/96. Committee for proprietary medicinal products.
225. Tumpey TM, Kapczynski DR, Swayne DE (2004) Comparative susceptibility of chickens and turkeys to avian influenza A H7N2 virus infection and protective efficacy of a commercial avian influenza H7N2 virus vaccine. Avian Dis 48: 167-176.
226. Tizard IR (2004) Veterinairy immunology - an introduction. Philadelphia, PA: Saunders.

227. Higgins DA (1996) Comparative immunology of avian species; Davison TF, Morris LN, editors. Abingdon: Carfax Publishing Co. 149-205.
228. Lu H, Castro AE (2004) Evaluation of the infectivity, length of infection, and immune response of a low-pathogenicity H7N2 avian influenza virus in specific-pathogen-free chickens. Avian Dis 48: 263-270.
229. Choi YK, Nguyen TD, Ozaki H, Webby RJ, Puthavathana P, et al. (2005) Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004. J Virol 79: 10821-10825.
230. Butler D (2006) Thai dogs carry bird-flu virus, but will they spread it? Nature 439: 773.
231. Banks J, Speidel ES, Moore E, Plowright L, Piccirillo A, et al. (2001) Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. Arch Virol 146: 963-973.
232. Shinya K, Ebina M, Yamada S, Ono M, Kasai N, et al. (2006) Avian flu: influenza virus receptors in the human airway. Nature 440: 435-436.
233. van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, et al. (2006) H5N1 Virus Attachment to Lower Respiratory Tract. Science 312: 399.
234. Hatta M, Gao P, Halfmann P, Kawaoka Y (2001) Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 293: 1840-1842.
235. Salomon R, Franks J, Govorkova EA, Ilyushina NA, Yen HL, et al. (2006) The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. J Exp Med 203: 689-697.
236. Gabriel G, Dauber B, Wolff T, Planz O, Klenk HD, et al. (2005) The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc Natl Acad Sci U S A 102: 18590-18595.
237. Seo SH, Hoffmann E, Webster RG (2002) Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat Med 8: 950-954.
238. Krug RM, Yuan W, Noah DL, Latham AG (2003) Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein. Virology 309: 181-189.
239. Olofsson S, Kumlin U, Dimock K, Arnberg N (2005) Avian influenza and sialic acid receptors: more than meets the eye? Lancet Infect Dis 5: 184-188.
240. Couceiro JN, Paulson JC, Baum LG (1993) Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. Virus Res 29: 155-165.
241. de Wit E, Munster VJ, Spronken MI, Bestebroer TM, Baas C, et al. (2005) Protection of mice against lethal infection with highly pathogenic H7N7 influenza A virus by using a recombinant low-pathogenicity vaccine strain. J Virol 79: 12401-12407.
242. Karber G (1931) Beitrag zur kollektiven behandlung pharmakologischer reihenversuche. Exp Pathol Pharmakol 162: 480-483.
243. Rimmelzwaan GF, Kuiken T, van Amerongen G, Bestebroer TM, Fouchier RA, et al. (2001) Pathogenesis of influenza A (H5N1) virus infection in a primate model. J Virol 75: 6687-6691.
244. Cheung CY, Poon LL, Lau AS, Luk W, Lau YL, et al. (2002) Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet 360: 1831-1837.
245. Perdue ML, Latimer JW, Crawford JM (1995) A novel carbohydrate addition site on the hemagglutinin protein of a highly pathogenic H7 subtype avian influenza virus. Virology 213: 276-281.
246. Iwatsuki-Horimoto K, Kanazawa R, Sugii S, Kawaoka Y, Horimoto T (2004) The index influenza A virus subtype H5N1 isolated from a human in 1997 differs in its receptor-binding properties from a virulent avian influenza virus. J Gen Virol 85: 1001-1005.
247. Chen G-W, Chang, S.-C., Mok, C.-K., Lo, Y.-L., Kung, Y.-N., Huang, J.-H., Shih, Y.-H.,Wang, J.-Y., Chiang, C., Chen, C-J., Shih, S.-R. (2006) Genomic signatures of human versus avian influenza A viruses. Emerg Infect Dis 12: 1353-1360.
248. WHO (2006) Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO. http://wwwwhoint/csr/disease/avian_influenza/country/cases_table_2006_10_03/en/indexhtml.
249. Subbarao EK, London W, Murphy BR (1993) A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol 67: 1761-1764.
250. Massin P, van der Werf S, Naffakh N (2001) Residue 627 of PB2 is a determinant of cold sensitivity in RNA replication of avian influenza viruses. J Virol 75: 5398-5404.
251. Connor RJ, Kawaoka Y, Webster RG, Paulson JC (1994) Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology 205: 17-23.
252. Mitnaul LJ, Matrosovich MN, Castrucci MR, Tuzikov AB, Bovin NV, et al. (2000) Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus. J Virol 74: 6015-6020.
253. Dunn JJ, Krippl B, Bernstein KE, Westphal H, Studier FW (1988) Targeting bacteriophage T7 RNA polymerase to the mammalian cell nucleus. Gene 68: 259-266.
254. Isenberg SJ, Green BF (1985) Changes in conjunctival oxygen tension and temperature with advancing age. Crit Care Med 13: 683-685.
255. Chen H, Li Y, Li Z, Shi J, Shinya K, et al. (2006) Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in western China. J Virol 80: 5976-5983.
256. Ito T, Suzuki Y, Mitnaul L, Vines A, Kida H, et al. (1997) Receptor specificity of influenza A viruses correlates with the agglutination of erythrocytes from different animal species. Virology 227: 493-499.

257. Wagner R, Matrosovich M, Klenk HD (2002) Functional balance between haemagglutinin and neuraminidase in influenza virus infections. Rev Med Virol 12: 159-166.
258. Perez DR, Lim W, Seiler JP, Yi G, Peiris M, et al. (2003) Role of Quail in the Interspecies Transmission of H9 Influenza A Viruses: Molecular Changes on HA That Correspond to Adaptation from Ducks to Chickens. J Virol 77: 3148-3156.
259. Baigent SJ, McCauley JW (2003) Influenza type A in humans, mammals and birds: determinants of virus virulence, host-range and interspecies transmission. Bioessays 25: 657-671.
260. Science (2006) Supporting Online Material for H5N1 Virus Attachment to Lower Respiratory Tract. http://wwwsciencemagorg/cgi/data/1125548/DC1/1.
261. Uiprasertkul M, Puthavathana P, Sangsiriwut K, Pooruk P, Srisook K, et al. (2005) Influenza A H5N1 replication sites in humans. Emerg Infect Dis 11: 1036-1041.
262. Lee CW, Suarez DL, Tumpey TM, Sung HW, Kwon YK, et al. (2005) Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. J Virol 79: 3692-3702.
263. Maines TR, Lu XH, Erb SM, Edwards L, Guarner J, et al. (2005) Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. J Virol 79: 11788-11800.
264. Rimmelzwaan GF, van Riel D, Baars M, Bestebroer TM, van Amerongen G, et al. (2006) Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts. Am J Pathol 168: 176-183; quiz 364.
265. Spackman E, Senne DA, Bulaga LL, Myers TJ, Perdue ML, et al. (2003) Development of real-time RT-PCR for the detection of avian influenza virus. Avian Dis 47: 1079-1082.
266. Bentz P-G (1985) Studies on some urban Mallard Anas platyrhynchos populations in Scandinavia. Part I: Causes of death, mortality and longevity among Malmö Mallards as shown by ringing recoveries. Fauna norv Ser C C, Cinclus: 44-56.
267. Slemons RD, Easterday BC (1978) Virus replication in the digestive tract of ducks exposed by aerosol to type-A influenza. Avian Dis 22: 367-377.
268. Zhu QY, Qin ED, Wang W, Yu J, Liu BH, et al. (2006) Fatal infection with influenza A (H5N1) virus in China. N Engl J Med 354: 2731-2732.
269. Joseph T, Subbarao K (2005) Human infections with avian influenza viruses. Md Med 6: 30-32.
270. Stephenson I, Nicholson KG, Colegate A, Podda A, Wood J, et al. (2003) Boosting immunity to influenza H5N1 with MF59-adjuvanted H5N3 A/Duck/Singapore/97 vaccine in a primed human population. Vaccine 21: 1687-1693.
271. Stephenson I, Bugarini R, Nicholson KG, Podda A, Wood JM, et al. (2005) Cross-Reactivity to Highly Pathogenic Avian Influenza H5N1 Viruses after Vaccination with Nonadjuvanted and MF59-Adjuvanted Influenza A/Duck/Singapore/97 (H5N3) Vaccine: A Potential Priming Strategy. J Infect Dis 191: 1210-1215.
272. Fouchier R *et al*. antigenic cartography of influenza A H5N1 virus. submitted for publication.
273. Sims LD, Ellis TM, Liu KK, Dyrting K, Wong H, et al. (2003) Avian influenza in Hong Kong 1997-2002. Avian Dis 47: 832-838.
274. Swayne DE, Lee CW, Spackman E (2006) Inactivated North American and European H5N2 avian influenza virus vaccines protect chickens from Asian H5N1 high pathogenicity avian influenza virus. Avian Pathol 35: 141-146.
275. Villarreal-Chavez C, Rivera-Cruz E (2003) An update on avian influenza in Mexico. Avian Dis 47: 1002-1005.
276. Hulse-Post DJ, Sturm-Ramirez KM, Humberd J, Seiler P, Govorkova EA, et al. (2005) Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proc Natl Acad Sci U S A 102: 10682-10687.
277. Seo SH, Peiris M, Webster RG (2002) Protective cross-reactive cellular immunity to lethal A/Goose/Guangdong/1/96-like H5N1 influenza virus is correlated with the proportion of pulmonary CD8(+) T cells expressing gamma interferon. J Virol 76: 4886-4890.
278. Sturm-Ramirez KM, Hulse-Post DJ, Govorkova EA, Humberd J, Seiler P, et al. (2005) Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? J Virol 79: 11269-11279.
279. Ducatez MF, Olinger CM, Owoade AA, De Landtsheer S, Ammerlaan W, et al. (2006) Avian flu: multiple introductions of H5N1 in Nigeria. Nature 442: 37.
280. Capua I, Brown I, Johnson M, Senne D, Swayne D (2006) Veterinary virologists share avian flu data. Science 312: 1597.
281. Stephenson I, Wood JM, Nicholson KG, Zambon MC (2003) Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. J Med Virol 70: 391-398.
282. de Jong MD, Bach VC, Phan TQ, Vo MH, Tran TT, et al. (2005) Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. N Engl J Med 352: 686-691.
283. Maines TR, Chen LM, Matsuoka Y, Chen H, Rowe T, et al. (2006) Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. Proc Natl Acad Sci U S A 103: 12121-12126.

Nederlandse samenvatting

NL

Influenza A virus behoort tot de familie van *Orthomyxoviridae*. Influenza A virussen zijn onregelmatig gevormde virussen van ongeveer 120 nm groot. Het genoom van influenza A virussen is gesegmenteerd en bestaat uit negatief-strengs RNA. De acht gensegmenten coderen voor 11 verschillende eiwitten. Influenza A virussen worden onderverdeeld op basis van de oppervlakte eiwitten; hemagglutinine (HA, een eiwit dat zorg draagt voor de binding van het virus aan en binnendringen van de gastheercel) en neuraminidase (NA, een eiwit dat zorgt voor de efficiënte verspreiding na vermenigvuldiging van het virus). Er waren tot voor kort 15 verschillende subtypen van HA geïdentificeerd en 9 verschillende subtypen van NA. Dit leidt tot 135 potentiële subtype combinaties zoals H1N1, H3N2, H5N1 en H7N7. Aviaire influenza virussen zijn verder in te delen op grond van biologische eigenschappen: laag pathogene aviaire influenza virussen (LPAI), die geen tot milde ziekteverschijnselen veroorzaken en hoog pathogene aviare influenza virussen (HPAI), die massale sterfte onder pluimvee kunnen veroorzaken (ook bekend als vogelpest).
Influenza A virus is voornamelijk bekend als veroorzaker van de drie pandemieën (wereldwijde uitbraken) van de afgelopen eeuw: de H1N1 Spaanse griep in 1918, de H2N2 Aziatische griep in 1957 en de H3N2 Hong Kong griep in 1968. Daarnaast is het ook bekend van de jaarlijks terugkerende griepepidemieën (lokale uitbraken) en van uitbraken van vogelpest. Hoewel het bekend is dat alle influenza A virussen hun oorsprong hebben in wilde vogels, is er weinig bekend over het voorkomen van aviaire influenza virussen in wilde vogels in Europa. Het onderzoek beschreven in dit proefschrift richtte zich in eerste instantie op het in kaart brengen van de complexe ecologie van aviaire influenza virussen in hun natuurlijke gastheer, de wilde vogels, en vervolgens op de veranderingen die ten grondslag liggen aan het vermogen van deze virussen om overgedragen te kunnen worden naar de mens.
Hoofdstuk 1 geeft een algemene inleiding en een overzicht van de huidige kennis over het voorkomen van influenza A virussen in wilde vogels. Hierbij wordt ingegaan op de rol die verschillende vogelfamilies spelen in de ecologie van influenza A virussen zoals de Anseriformes (eenden, ganzen en zwanen) en Charadriformes (meeuwen, alken en steltlopers).
Om inzicht te krijgen in het voorkomen van influenza virussen in wilde vogels in Europa, werd in 1998 in samenwerking met talrijke ornithologen een influenza A virus surveillance netwerk opgezet. Door de toepassing van moleculair biologisch diagnostische technieken werd het mogelijk om grote aantallen cloacale vogelmonsters te testen op de aanwezigheid van influenza A virus. De resultaten van deze wilde vogel surveillance studies worden beschreven in hoofdstuk 2. Watervogels uit de familie van Anseriformes bleken over het algemeen het vaakst influenza A virussen bij zich te dragen, waarbij het percentage geïnfecteerde dieren afhankelijk was van soort, leeftijd, seizoen, locatie en tijdpunt in de najaarsmigratie. Binnen de familie van Anseriformes bleken de grondeleenden (wilde eend (*Anas platyrhynchos*), smient (*Anas penelope*), wintertaling (*Anas crecca*), pijlstaart (*Anas acuta*), slobeend (*Anas clypeata*) en krakeend (*Anas strepera*)) de voornaamste eenden te zijn die een rol spelen bij de circulatie van influenza virussen in de wilde vogelpopulatie. Influenza virussen werden ook aangetroffen bij diverse andere vogelsoorten zoals ganzen, zwanen, meeuwen, alken, steltlopers en koeten. Alle tot op heden bekende HA subtypen, behalve H14 en H15, werden in onze influenza A

surveillance van wilde vogels aangetroffen. Behalve monsters van wilde vogels in Europa werden er ook monsters genomen van vogels op minder gangbare plekken, zoals Afrika, Azië, Noord– en Zuid-Amerika en de poolgebieden..
In hoofdstuk 3 werd het voorkomen van influenza A virussen in pinguïns op Antarctica bestudeerd. In 2002 werden er op Antarctica cloacale monsters genomen in de broedkolonies van drie pinguïn soorten: de keelbandpinguïn (*Pygoscelis antarctica*), de ezelspinguïn (*Pygoscelis papua*) en de Adélie pinguïn (*Pygoscelis adeliae*). Hoewel geen influenza A virus kon worden aangetoond in de monsters genomen van deze pinguïns, bleek uit vervolgonderzoek dat er in een aantal van deze pinguïns wel antistoffen tegen influenza A virussen aanwezig waren. Hieruit bleek dat er nu en dan influenza A virussen circuleren in deze pinguïnkolonies. Hoewel Antarctica op het eerste gezicht zeer afgelegen lijkt, worden deze vogelpopulaties met andere continenten verbonden door middel van vogels die over lange afstanden migreren zoals sternen. Deze lange afstand migranten zouden een rol kunnen spelen bij de introductie van influenza virussen in vogelpopulaties op afgelegen plekken.
Hoofdstuk 4 beschrijft de karakterisering van een nieuw subtype influenza A virus verkregen uit kokmeeuwen (*Larus ridibundus*). Bij het vogelringstation Ottenby, in Öland, Zweden werden in Augustus 1999 tien jonge kokmeeuwen gevangen. Zes van de tien kokmeeuwen bleken geïnfecteerd te zijn met een influenza A virus. Uit deze zes monsters werden 5 influenza A virussen geïsoleerd, waarvan er vier niet konden worden getypeerd met conventionele identificatiemethoden. Na uitvoerige antigene en genetische analyse bleken deze influenza A virussen een nieuw HA subtype te vormen, dat H16 werd genoemd. Deze H16 virussen bleken genetisch nauw verwant aan H13 virussen. Beide virus subtypen worden bijna uitsluitend in meeuwachtigen gevonden, en hebben zich waarschijnlijk volledig aangepast aan deze vogelsoorten.
De uitgebreide surveillance van vogelsoorten op influenza virussen leverde ook de identificatie van een nieuwe gastheersoort voor influenza A virussen op die beschreven wordt in hoofdstuk 5. Van zeekoeten (*Uria aalge*), zwart-witte zeevogels die alleen aan land komen om te broeden, werden tijdens het ringen ook monsters voor influenza A virus surveillance afgenomen. De virussen geïsoleerd uit de monsters van deze zeekoeten bleken zogenaamde reassortanten (mengvarianten) van virussen uit de Amerikaanse genetische lijn en de Euro-Aziatische genetische lijn van influenza virussen te zijn.
Door samenwerking met ecologen kon er ook gekeken worden naar het effect van een influenza A virus infectie op het gedrag van wilde vogels. Uit onderzoek aan kleine zwanen (*Cygnus colombianus bewickii*) bleek dat een infectie met een LPAI H6 virus niet een direct aantoonbaar pathogeen effect had, maar wel degelijk een invloed had op het gedrag van het dier. De kleine zwanen die geïnfecteerd waren, stelden hun migratie naar het zuiden met meer dan een maand uit ten opzichte van niet-geïnfecteerde kleine zwanen. Dit onderzoek, beschreven in hoofdstuk 6, toonde aan dat, in tegenstelling tot de algemeen heersende opvatting, wilde vogels wel degelijk ziek kunnen worden door een infectie met influenza virus en dat deze infecties een groot effect kunnen hebben op gedrags-ecologisch niveau.
In Nederland brak in 2003 vogelpest uit, veroorzaakt door een HPAI virus van het H7N7 subtype. Tijdens deze uitbraak werd het virus overgedragen op 89 mensen, waarvan de meeste in contact waren geweest met besmet pluimvee. Uitbraken

van HPAI virussen, zoals in 2003 in de Gelderse vallei, worden veroorzaakt door de introductie van H5 en H7 influenza virussen uit wilde vogels. Na introductie in pluimvee, kunnen deze H5 en H7 van LPAI virussen veranderen in HPAI virussen, de zogenaamde vogelpest virussen. Het hoog pathogene karakter van HPAI virussen wordt veroorzaakt door de aanwezigheid van meerdere basische aminozuren die de splitsing van het hemagluttinine eiwit vergemakkelijken. In hoofdstuk 7 wordt beschreven dat er tijdens de influenza A surveillance studies in wilde vogels verschillende H5 en H7 LPAI virussen werden geïsoleerd uit wilde eenden in Nederland en Zweden. De analyse van de antigene en genetische eigenschappen van deze virussen liet zien dat voor elk van de vogelpest uitbraken van de afgelopen 10 jaar in Europa (H5N2 en H7N1 Italië, H7N7 in Nederland) een LPAI voorlopervirus circuleerde in de wilde vogel populatie. Doordat de antigene eigenschappen van de H7 LPAI voorloper virussen weinig verschilden ten opzichte van het influenza virus dat de vogelpestuitbraak in Nederland veroorzaakte was het mogelijk het HA gen van een uit een wilde eend geïsoleerd LPAI H7N3 virus te gebruiken voor een vaccin tegen het HPAI vogelpest virus. Dit onderzoek wordt beschreven in hoofdstuk 8. Met moleculair biologische technieken werd een vaccinstam gemaakt, die gebruikt werd voor de productie van de vaccin componenten HA en NA. Het experimentele vaccin werd getest in een door ons opgezet muismodel. Hieruit bleek dat het vaccin bescherming bood tegen een letale infectie met het vogelpest virus indien er werd gevaccineerd met twee doses van een geadjuveerd vaccin. Het is dus mogelijk voorafgaand aan een uitbraak een voorraad vaccinstammen te genereren, afgeleid van stammen geïsoleerd uit wilde vogels, die gebruikt kunnen worden voor vaccinproductie wanneer er een uitbraak van vogelpest is of een pandemie dreigt. Hiermee kan kostbare tijd worden bespaard omdat tijdens een pandemische dreiging of vogelpest uitbraak meteen begonnen kan worden met de vaccinproductie.

Tijdens de vogelpestuitbraak werden ook niet-geïnfecteerde pluimveehouderijen en hobbydieren geruimd om de verspreiding van het vogelpest virus naar nieuwe gebieden te voorkomen. Om de waardevolle collecties van dierentuinen, onder meer bestaande uit bedreigde en zeldzame vogelsoorten, te kunnen beschermen tegen het H7N7 vogelpest virus én tegen mogelijke preventieve ruiming werd besloten tot een grootschalige vaccinatiecampagne in de Nederlandse dierentuinen. De resultaten hiervan worden beschreven in hoofdstuk 9.

Zoals hierboven beschreven, werden tijdens de Nederlandse vogelpest uitbraak in 2003 89 mensen geïnfecteerd met het HPAI H7N7 virus. De meeste van deze mensen kregen conjunctivitis, ofwel oogvliesontsteking, en een klein aantal ook griep-achtige symptomen. Een dierenarts die een besmette pluimveehouderij bezocht ontwikkelde een zware longontsteking met daaropvolgende complicaties, uiteindelijk met de dood als gevolg. De influenza A virus isolaten verkregen uit patienten met conjunctivitis en de overleden dierenarts verschilden op 14 aminozuur posities, verdeeld over 5 gensegmenten. Vervolgonderzoek richtte zich op de vraag welke van deze 14 aminozuursubstituties, of combinatie van aminozuursubstituties verantwoordelijk waren voor het verschil in pathogeniteit (conjunctivitis versus een letale longontsteking) tussen deze twee virussen.

In hoofdstuk 10 werd gekeken of verschillen in het bindingspatroon aan weefsels van het humane oog en de humane luchtwegen een verklaring zou kunnen zijn voor het verschil in pathogeniteit. Zowel het conjunctivitis virus als het letale virus

konden binden aan conjunctiva en cornea, in tegenstelling tot humane influenza virussen. Dit onderzoek toonde aan dat het oog een mogelijke *porte d'entrée* vormt voor HPAI H7N7 virussen, in overeenstemming met de klinische symptomen van het meerendeel van de humane infecties tijdens de HPAI H7N7 uitbraak. Naast binding aan het oog werd er ook gekeken naar binding van beide virussen aan cellen van de humane luchtwegen om na te gaan of verschil in binding de verklaring was voor het verschil in pathogeniteit. Beide virussen bleken te kunnen binden aan de cellen van onderste luchtwegen van de mens. Hoewel er wel verschillen waren in binding tussen de twee virussen, was dit waarschijnlijk niet de enige verklaring voor de verschillen in pathogeniteit tussen de twee virussen. Om de bijdrage van de verschillende aminozuursubstituties in het letale virus aan de pathogeniteit te onderzoeken werd een muismodel opgezet. Muizen werden geïnfecteerd met lage doses van het conjunctivitis virus en het letale virus. Muizen die geïnfecteerd werden met het conjunctivitis virus werden niet ziek, terwijl muizen geïnfecteerd met het letale virus ernstige ziekteverschijnselen ontwikkelden. Door het uitwisselen van de verschillende gensegmenten tussen het conjunctivitis virus en het letale virus bleek dat PB2 het verschil in pathogeniteit in belangrijke mate veroorzaakte. Vervolgens werden mutaties gemaakt in het PB2 gen. Hieruit bleek dat één mutatie, van glutamaat naar lysine op positie 627 in het PB2 gen een cruciaal verschil in pathogeniteit bewerkstelligde. Dezelfde mutatie is ten dele ook verantwoordelijk voor de pathogeniteit van HPAI H5N1 virussen en was aanwezig in het "Spaanse" griep virus en in alle latere humane influenza virussen. Deze mutatie lijkt een efficiënte replicatie in zoogdieren, en dus ook de mens mogelijk te maken. Naast het *in vivo* onderzoek naar de moleculaire basis van de pathogeniteit, werden de effecten van de verschillende aminozuur substituties ook *in vitro* geanalyseerd. Hoofdstuk 11 beschrijft de analyse van de invloed van de 14 aminozuur substituties die verschillen tussen het conjunctivitis virus en het letale virus op de virus replicatie in cellen in weefselkweek. Door de replicatiekinetiek van het conjunctivitis virus en het letale virus te vergelijken in verschillende cellijnen, bleek dat het letale virus efficiënter repliceert in cellijnen van humane en zoogdier herkomst, maar ook in cellen van aviare herkomst. Elk van de vijf gensegmenten waarin het letale virus verschilde van het conjunctivitis virus droegen bij aan de efficiëntere replicatie van het letale virus. De aminozuur substitutie van glutamaat naar lysine op positie 627 in het PB2 gen, die in het muis model verantwoordelijk was voor de verhoogde pathogeniteit van het letale virus, zorgde *in vitro* voor een verhoogde polymerase activiteit. Virussen met het HA gen van het letale virus bleken vooral efficiënter te repliceren door een aminozuur substitutie van alanine naar threonine op positie 143. Deze aminozuur substitutie bleek bovendien verantwoordelijk te zijn voor de bindingsverschillen aan weefsels van de humane lagere luchtwegen tussen het conjunctivitis virus en het letale virus. Uit sequentie analyse van een influenza A virus isolaat, afkomstig uit de door de overleden dierenarts bezochte pluimveehouderij, bleek dat het merendeel van de 14 aminozuur verschillen tussen het conjunctivitis virus en het letale virus al aanwezig waren in pluimvee. HPAI virussen kunnen dus al tijdens de circulatie in pluimvee eigenschappen verwerven die niet alleen voordelig zijn voor virus replicatie in pluimvee, maar ook de overdracht naar de mens kunnen vergemakkelijken.

Infecties met vogelpest virus in de mens worden vaak gekarakteriseerd door een zware longontsteking in de lagere luchtwegen. De in hoofdstuk 12 beschreven

bindingsstudie met het HPAI H5N1 virus liet zien in welk deel van de luchtwegen, en aan welke cellen, dit virus bindt. Dit HPAI H5N1 virus bleek vooral te binden aan cellen in de lagere luchtwegen. Dit bindingspatroon zou de veel voorkomende longontsteking die deze virussen veroorzaken kunnen verklaren. Bovendien zou het op een beperking van dit virus kunnen duiden om via hoesten en niezen van mens op mens te worden overgebracht.

Het onderzoek beschreven in dit proefschrift heeft geleid tot een verbreding en verdieping van onze kennis over aviaire influenza virussen. Het onderzoek was niet alleen gericht op de natuurlijke gastheer van het influenza A virus (hoofdstuk 2-7), maar richtte zich ook op het ontstaan van vogelpestvirussen en de pathogeniteit van deze virussen in mensen (hoofdstuk 8-12). Dit onderzoek heeft daarmee een belangrijke bijdrage geleverd aan een groter inzicht in de ecologie, evolutie en pathogenese van influenza A virussen en kan bovendien bijdragen aan de bestrijding van vogelpestuitbraken en vaccinontwikkeling.

Dankwoord

Het is duidelijk dat het tot standkomen van dit proefschrift te danken is aan vele bijdragen, binnen en buiten de afdeling virologie. Iedereen die hieraan heeft bijgedragen wil ik hartelijk bedanken, en een aantal mensen in het bijzonder.

Ab, bedankt voor de mogelijkheid tot promoveren op de afdeling virologie en voor het scheppen van de secundaire arbeidsvoorwaarden (Curaçao, kerstfeest, biertap) die mijn verblijf op de afdeling zo aangenaam maken. Ron, zowel het traject van AIO als dat van PI bevat veel leermomenten, graag wil ik je bedanken voor het genoten vertrouwen, het stimuleren van de 'fun factor' in ons onderzoek en de nachtelijke discussies over influenza (enzo).

Mijn paranimfen Chantal en Roel. Chantal, hoewel in het begin misschien niet van harte, heb je je de afgelopen jaren keihard ingezet voor het vogelproject. Naar een ruwe schatting ben ik je de 22ste 10.000 bier verschuldigd. Roel, jouw anale fixatie had veel raakvlakken met mijn onderzoek, toch zijn het je vriendschap en gezelligheid die het meest worden gewaardeerd. Ik wil graag de FLU/hMPV groep bedanken. Alle bijdragen, zowel op werkvlak (het uitvullen van duizenden buisjes medium) als op het sociale vlak (de etentjes, BBQ's en borrels) worden zeer gewaardeerd. Bernadette, Eefje, Femke, Gerrie, Guus, Jacco, Jan, Joost, Juthatip, Leo, Miranda, Monique, Nella, Sander, Theo en Tiny: bedankt! En natuurlijk Pascal, jouw onverstoorbare natuur maakt je een geweldige 'team player', ZSSNA! De ondersteunende hulp van Angela, Geert, Georges, Ger, Hans, Robert, Simone en Wim wordt ook zeer gewaardeerd, bedankt daarvoor. Ook de wildlife groep heeft een belangrijke rol gespeelt in mijn promotietraject, van de monumentale "mass seal necropsy" tot het outbreak team. Debby, Fiona, Jolianne, Joost en Thijs: bedankt voor de samenwerking op vele fronten.
Grootschalige exercities zoals de vogelsurveillance kunnen niet plaatsvinden zonder de medewerking van vele handen. Ik wil de afdeling diagnostiek, in het bijzonder Judith Guldemeester en Martin Schutten, zonder wie de hele vogelsurveillance knarsend tot stilstand zou komen, bedanken voor onze samenwerking.

I would like to thank my Swedish colleagues Anders, Björn, Jonas and Neus for our great, fruitful and continuing collaboration.

Het zal duidelijk zijn dat het een AIO niet alleen aan tijd, maar ook aan expertise ontbreekt om meer dan 36,000 vogels te vangen en te bemonsteren. Veel dank gaat dus uit naar alle ornithologen die de afgelopen jaren een bijdrage hebben geleverd. In het bijzonder de ornithologen van het eerste uur: Bert Pellegrom, Hans Zantinge, Hans Vader en Dick Jonkers. Daarbij komen de ornithologen die van grote invloed zijn geweest op mijn groeiende kennis over onze gevederde vrienden: Marcel Klaassen en Maarten Loonen.

Lieve ouders, Ronald, Cindy, Shanna, Jari & Mika, de de Witjes en alle vrienden, bedankt voor jullie interesse, steun en gezelligheid.

Emmie, jij bent de beste!

Curriculum vitae

De auteur van dit proefschrift werd op 9 januari 1973 in Hoorn geboren. In 1985 werd begonnen aan de HAVO aan het Werenfridus te Hoorn. In 1991 werd begonnen met de studie Veterinaire Microbiologie aan het Friesland College te Leeuwarden. In 1994 werd deze opleiding vervolgd met de studie Hoger Laboratorium Onderwijs aan de Hogeschool Utrecht te Utrecht, afstudeerrichtingen moleculaire biologie en zoölogie. In 1999 werd gestart met de studie Biologie aan de Universiteit te Utrecht. De stage ten behoeve van deze studie betrof onderzoek naar het moleculaire mechanisme dat ten grondslag ligt aan fasevariatie van het oppervlakte eiwit Agn43 van *Escherichia coli*, onder begeleiding van prof. Marjan van der Woude aan de Department of Microbiology van de University of Pennsylvania te Philadelphia. Na het behalen van het doctoraal diploma werd in 2002 begonnen als AIO aan de afdeling virologie van de Erasmus Universiteit Rotterdam, resulterend in dit proefschrift.

Publicaties

J. Correnti, **V.J. Munster**, T. Chan and M. van der Woude. 2002. Dam-dependent phase variation of Ag43 in Escherichia coli is altered in a seqA mutant. Mol Microbiol 44: 521-32

A. Wallecha, **V.J. Munster**, J. Correnti, T. Chan and M. van der Woude. 2002. Dam- and OxyR-dependent phase variation of Ag43: essential elements and evidence for a new role of DNA methylation. J Bacteriol 84: 3338-47

A. Wallecha, J. Correnti, **V.J. Munster** and M. van der Woude. 2003. Phase variation of Ag43 is independent of the oxidation state of OxyR. J Bacteriol 185: 2203-9

R.A.M. Fouchier, P.M. Schneeberger, F.W. Rozendaal, J.M. Broekman, S.A. Kemink, **V.J. Munster**, T. Kuiken, G.F. Rimmelzwaan, M. Schutten, G.J. van Doornum, G. Koch, A. Bosman, M. Koopmans and A.D.M.E. Osterhaus. 2004. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci U S A101: 1356-61

R.A.M. Fouchier, **V.J. Munster**, A. Wallensten, T.M. Bestebroer, S. Herfst, D.J. Smith, G.F. Rimmelzwaan, B. Olsen and A.D.M.E. Osterhaus. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 79: 2814-22

A. Wallensten, **V.J. Munster**, J. Elmberg, A.D.M.E. Osterhaus, R.A.M. Fouchier and B. Olsen. 2005. Multiple gene segment reassortment between Eurasian and American lineages of influenza A virus (H6N2) in Guillemot (Uria aalge).Arch Virol 150: 1685-92

E. de Wit, **V.J. Munster**, M.I.J. Spronken, T.M. Bestebroer, C. Baas, W.E.P. Beyer, G.F. Rimmelzwaan, A.D.M.E. Osterhaus and R.A.M. Fouchier. 2005. Protection of mice against lethal infection with highly pathogenic H7N7 influenza A virus by using a recombinant low-pathogenicity vaccine strain. J Virol 79: 12401-7

J.D. Philippa, **V.J. Munster**, H. Bolhuis, T.M. Bestebroer, W. Schaftenaar, W.E.P. Beyer, R.A.M. Fouchier, T. Kuiken and A.D.M.E. Osterhaus. 2005. Highly pathogenic avian influenza (H7N7): vaccination of zoo birds and transmission to non-poultry species. Vaccine 23: 5743-50

V.J. Munster, A. Wallensten, C. Baas, G.F. Rimmelzwaan, M. Schutten, B. Olsen, A.D.M.E. Osterhaus and R.A.M. Fouchier. 2005. Mallards and highly pathogenic avian influenza ancestral viruses, northern Europe. Emerg Infect Dis 11: 1545-51

V.J. Munster, A. Wallensten, B. Olsen, G.F. Rimmelzwaan, A.D.M.E. Osterhaus and R.A.M. Fouchier. 2005. Influenza A virus surveillance in wild birds. In: Avian influenza, prevention and control. R.S. Schrijver and G. Koch, editors. Springer.

D. van Riel, **V.J. Munster**, E. de Wit, G.F. Rimmelzwaan, R.A.M. Fouchier, A.D.M.E. Osterhaus and T. Kuiken. 2006. H5N1 Virus Attachment to Lower Respiratory Tract. Science 312: 399

B. Olsen, **V.J. Munster**, A. Wallensten, J. Waldenstrom, A.D.M.E. Osterhaus and R.A.M. Fouchier. 2006. Global patterns of influenza A virus in wild birds. Science 312: 384-8

V.J. Munster, J. Veen, B. Olsen, R. Vogel, A.D.M.E. Osterhaus and R.A.M. Fouchier. Towards improved influenza A virus surveillance in migrating birds. Vaccine, Epub June 2006

A. Wallensten, **V.J. Munster**, M. Karlsson, A. Lundkvist, M. Brytting, M. Stervander, A.D.M.E. Osterhaus, R.A.M. Fouchier and B. Olsen. High prevalence of influenza A virus in ducks caught during spring migration through Sweden. Vaccine, Epub June 2006

A. Wallensten, **V.J. Munster**, A.D.M.E. Osterhaus, J. Waldenström, J. Bonnedahl, T. Broman, R.A.M. Fouchier and B. Olsen. 2006. Mounting evidence for the presence of influenza A virus inthe avifauna of the Antarctic regio. J Antarctic Science, 18: 353-6

J. van Gils, **V.J. Munster**, R. Radersma, D. Liefhebber, R.A.M. Fouchier and M. Klaassen. Hampered foraging and migratory performance in swans infected with low-pathogenic avian influenza A virus. Submitted

V.J. Munster, E. de Wit, D. van Riel, W.E.P. Beyer, G.F. Rimmelzwaan, A.D.M.E. Osterhaus, T. Kuiken and R.A.M. Fouchier. The molecular basis of pathogenicity of the Dutch highly pathogenic H7N7 human influenza A viruses. Submitted

V.J. Munster, C. Baas, P. Lexmond, J. Waldenström, A. Wallensten, T. Fransson, G.F. Rimmelzwaan, W.E.P. Beyer, M. Schutten, B. Olsen, A.D.M.E. Osterhaus and R.A.M. Fouchier. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. Submitted

E. de Wit, **V.J. Munster**, D. van Riel, B.N. van Uden, W.E.P. Beyer, G.F. Rimmelzwaan, T. Kuiken, A.D.M.E. Osterhaus and R.A.M. Fouchier. Determinants of enhanced replication of human H7N7 influenza A virus isolates. In preparation

Photographs

Cover	Barnacle Geese (*Branta leucopsis*) during fall migration at Öland, Sweden
Page 6	Arctic Tern (*Sterna paradisaea*) defending its' breeding site at Spitsbergen, Norway
Page 22	Eurasian Wigeon (*Anas penelope*) at wintering site at Ouderkerk a/d IJssel, The Netherlands
Page 42	Chinstrap Penguin (*Pygoscelis antarctica*) at breeding site at the Antarctic Peninsula. Courtesy of Jonas Waldenström
Page 50	Black-headed Gull (*Larus ridibundus*) in Rotterdam, The Netherlands
Page 68	Brünnich's Guillemot (*Uria lomvia*) at breeding site at Spitsbergen, Norway
Page 76	Bewick's Swan (*Cygnus columbianus bewickii*) at Wieringerwerf, The Netherlands. Courtesy of W. Tijssen
Page 88	Mallard (*Anas platyrhynchos*) at wintering site at Ouderkerk a/d IJssel, The Netherlands
Page 100	Mallard (*Anas platyrhynchos*) at wintering site at Ouderkerk a/d IJssel, The Netherlands
Page 116	Vaccination of zoo birds at Blijdorp Zoo, Rotterdam, The Netherlands
Page 126	Presence of HPAI H7N7 virus antigen in lung of infected mouse. Courtesy of Debby van Riel
Page 140	Attachment of HPAI H7N7 virus to alveoli in human lung.Courtesy of Debby van Riel
Page 156	Attachment of HPAI virus to human trachea. Courtesy of Debby van Riel
Page 162	Kittiwake (*Rissa tridactyla*) breeding at Spitsbergen, Norway