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(Prof. Dr. Martin Beer)

A Neuraminidase-negative Variant of Highly Pathogenic Avian Influenza Virus H5N1 – Generation, Characterization and Use as a Model for Early Onset of Immunity

Inaugural-Dissertation zur Erlangung
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2. A model for early onset of protection against lethal challenge with highly pathogenic H5N1 influenza virus Vaccine

Röhrs S., Kalthoff D., Beer M.

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"This is honestly the most exciting period ever for the study of unknown life forms on our planet. The dominant things that exist here we know almost nothing about."

Nathan Wolfe

1. Introduction

Influenza A viruses (IAV) are important pathogens in the field of veterinary medicine, but also in the public health sector as emerging and re-emerging zoonotic disease. They infect a wide range of different species: such as wild birds, poultry and mammalian species including humans. Currently several subtypes are known and differentiated on the basis of their antigenic surface proteins. Influenza A viruses transfer in-between their reservoir host with constantly recurrent spill over infection to livestock animals and humans. Resulting illness in humans extends from mild symptoms to severe acute respiratory infections. Influenza A viruses are also of great importance for the livestock industry particularly for the swine and poultry production sectors. Especially in the poultry industry, IAV can cause enormous economic damages due to the stamping out policy asserted for Influenza A subtypes H5 and H7 in Europe. A characteristic of Influenza A viruses is the ability to adapt quickly and efficient to environmental (host) changes. These changes may result in variants escaping the immune response of the host after the administration of vaccines or become resistant to antiviral agents on an individual host level.

However little is known about molecular mechanisms of Influenza evolution in inadequate vaccinated flocks and resulting escape mutant viruses. This thesis focusses on the characterization of an H5N1 escape mutant generated by passages of a highly pathogenic avian influenza virus of the subtype H5N1 with an antiserum. Using this in vitro approach the multiple replication cycles of an highly pathogenic avian influenza within an insufficiently vaccinated poultry flock was simulated. The characterized attenuated H5 escape mutant was also used as a vaccine model for the induction of an early onset of immunity in birds as well as in mammals. Using this modified live virus vaccine candidate an easy to administer, highly efficacious “single shot” preparation could be generated.

2. Literature Review

2.1. Influenza A viruses

2.1.1. Taxonomy

Influenza viruses are segmented, negative stranded RNA viruses and belong to the virus family *Orthomyxoviridae* (41). Based on the number of segments of linear, single stranded RNA and the antigenic differences of the matrix as well as nucleoprotein the particles are classified by the genera Influenza A, B and C (41). Influenza A viruses are highly heterogeneous and can cause diseases in mammals as well as birds. In contrast influenza B viruses occur in humans and marine mammals, while influenza C viruses infect human as well as pigs (40, 69). Influenza A viruses are further classified by antigenic properties of their surface proteins: the haemagglutinin (HA) and the neuraminidase (NA). To date, a total number of 18 different HA and 11 different NA subtypes are recognized. Two subtypes were quite recently sequenced from samples out of South American bat species (10, 102, 103). Due to a considerable high number of data belonging to different isolates which were collected during the last decades, the introduction of additional terms and classifications was necessary. Therefore, the World Health Organization (WHO) has published a memorandum in 1980 and standardized the nomenclature of influenza A viruses. The nomenclature includes now Influenza genera, host species (omitted if human origin), geographical sites, serial numbers and the year of the isolate as well as the HA and NA subtypes (111). For example, the main Influenza strain included in this script is cited as "A/swan/Germany/R65/2006 (H5N1)". In addition, AIV are classified into highly pathogenic avian influenza viruses (HPAIV), that cause nearly 100% mortality rate in gallinaceous birds, and into low pathogenic avian influenza strains (LPAIV) resulting in milder symptoms or even subclinical infections (97).

2.1.2. The viral genome, virion structure and replication

Orthomyxoviridae are spherical or pleomorphic, meaning that they differ enormously in shape and size. Electron microscopy analyses showed rods and spherical structures with

diameters from 80 nm to 120 nm after *in vitro* cultivation and passaging, while filamentous particles were found for non-passaged virus isolates (41). The genome of Influenza A viruses comprises about 13.600 nucleotides (nt) and is split into eight individual segments, with segment sizes ranging from 890 to 2341 nt. The whole genome encodes for 15 known proteins, some of them with partially unknown functions. Within the virus particle, each RNA segment is associated with one polymerase complex, consisting of the polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA) and multiple copies of the nucleoprotein (NP) (76). The 3' - end and the 5' - end of the RNA strand are complementary to each other and form a coiled panhandle like-structure with the polymerase complex (Figure 1). These so-called "ribonucleoprotein complexes" (RNP) are arranged in a typical rod-like structure (32) - one RNP in the middle surrounded by seven additional RNPs (67).

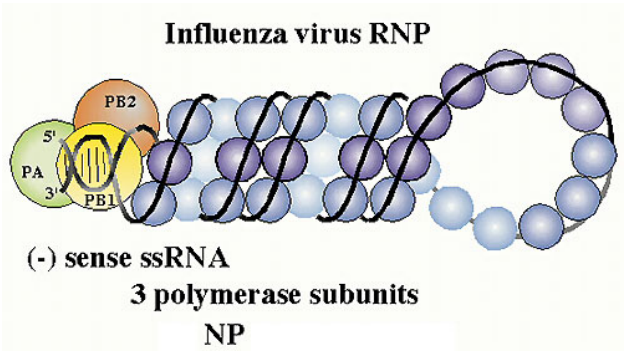

Figure 1: Influenza A ribonucleoprotein complex (© Paul Digard, Dept Pathology, University of Cambridge)

The matrix protein 1 (M1) links RNPs with the viral envelope within the virus particle. The host cell derived lipid membrane encircles all complexes (67) (Figure 2). Within the viral envelope, the matrix protein 2 (M2) is embedded and serves as an ion channel (80). The two glycoproteins HA and NA are also anchored in the viral membrane and create surface projections of 10 to 14 nm out of the lipid bilayer (41).

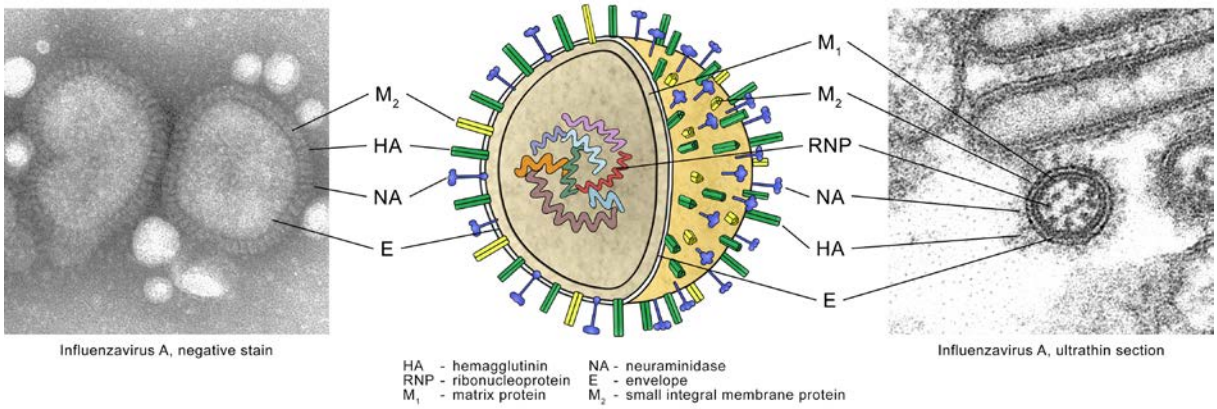

Figure 2: Influenza A virion composition – scheme and electron microscopy (©Friedrich-Loeffler-Institut, Insel Riems Bundesforschungsinstitut für Tiergesundheit; Elektronenmikroskopie: Dr. habil. H. Granzow; Grafikdesign M. Jörn)

The first and essential step for virus replication is the binding of the HA to α 2.6 or α 2.3 sialic acids on the host cell surface (83). The virion gets into the cell via endocytosis, followed by the acidification of the vesicle and conformational change of the HA protein, which induces the membrane fusion and causes the release of the ribonucleoprotein complex into the cytoplasm (83). Furthermore, the M2 channel carries protons to separate the M1 protein from the ribonucleoprotein complex (85) and the RNP complex migrates through nuclear pores into the cell nucleus (54, 68). In the nucleus, the PB2 protein binds to the 3'- end of the cellular messenger RNA (mRNA) and adsorbs the so called 5' - cap group on the 3' - end of the viral RNA (vRNA). This step, called "cap snatching", is essential for viral replication due to the fact, that the viral RNA dependent RNA polymerase is not able to induce the transcription process (24, 62). The so prepared vRNA is transcribed into mRNA by the viral polymerase (67). Viral mRNA is transported from the nucleus to the endoplasmatic reticulum and is used for translation into viral proteins. Those proteins are translocated back into the nucleus. Replication of the viral RNA starts when the concentration of NP is sufficiently high (76). High amounts of M1 together with the polymerase complex trigger NP attachment to M1. The NP-M1 complex is transported by the non-structural protein 2 (NS2) from the nucleus to the cytoplasm and through the Golgi apparatus to the cell membrane (56). All viral components accumulate at the cell membrane and leave the cell via a budding process (67, 81). The NS1 protein inhibits the cellular interferon response and blocks the cellular repair and protection mechanisms throughout the whole replication cycle. Properties and

functions of Influence A segments and their corresponding proteins are shown in table 1 (6, 33, 61, 113).

segment	lenght	encodin protein(s)	protein lenght	properties and functions
1	2341	PB2	759	Polymerase subunit; mRNA cap recognition
2	2341	PB1	757	Polymerase subunit; RNA elongation, endonuclease activity
		PB1-F2	87	Pro-apoptotic activity
		NS40		viral replication but not essential
3	2233	PA	716	Polymerase subunit; protease activity
		PA-X		
		PA-N182		
		PA-N155		
4	1778	HA	550	Surface glycoprotein; major antigen, receptor binding and fusion activities
5	1565	NP	498	RNA binding protein; nudear import regulation
6	1413	NA	454	Surface glycoprotein; sialidase activity, virus release
7	1027	M1	252	Matrix protein; vRNP interaction, RNA nudear export regulation, viral budding
		M2	97	Ion channel; virus uncoating and assembly
8	890	NS1	230	Interferon antagonist protein; regulation of host gene expression
		NEP/NS2	121	Nuclear export of RNA

Table 1: Overview of Influenza A segments and encoding proteins (table adapted from (6))

2.1.3. The Haemagglutinin (HA)

The HA surface protein is a typical type I membrane glycoprotein, which consists of a homotrimeric structure (92). The protein is initially synthesized as precursor protein HA0 (90). The HA serves as receptor binding protein for Influenza A viruses and has to be cleaved to achieve its second biological function - the induction of membrane fusion of virus and endosomal membrane (90). Cellular enzymes are responsible for the cleavage of the precursor HA0 into the subunits HA1 and HA2, which stay connected by disulphide-bonds (90). The cleavage site of the HA is an important pathogenicity factor for avian influenza viruses of subtype H5 and H7 (90). Low pathogenic influenza viruses exhibit a monobasic cleavage site and therefore represent the cleavage motif of trypsin-like proteases, which are present in the avian respiratory and gastrointestinal tract but not in brain (77). In contrast, highly pathogenic avian influenza viruses have a polybasic cleavage site within the HA protein that is recognized by ubiquitous cellular proteases (37). See also figure 3.

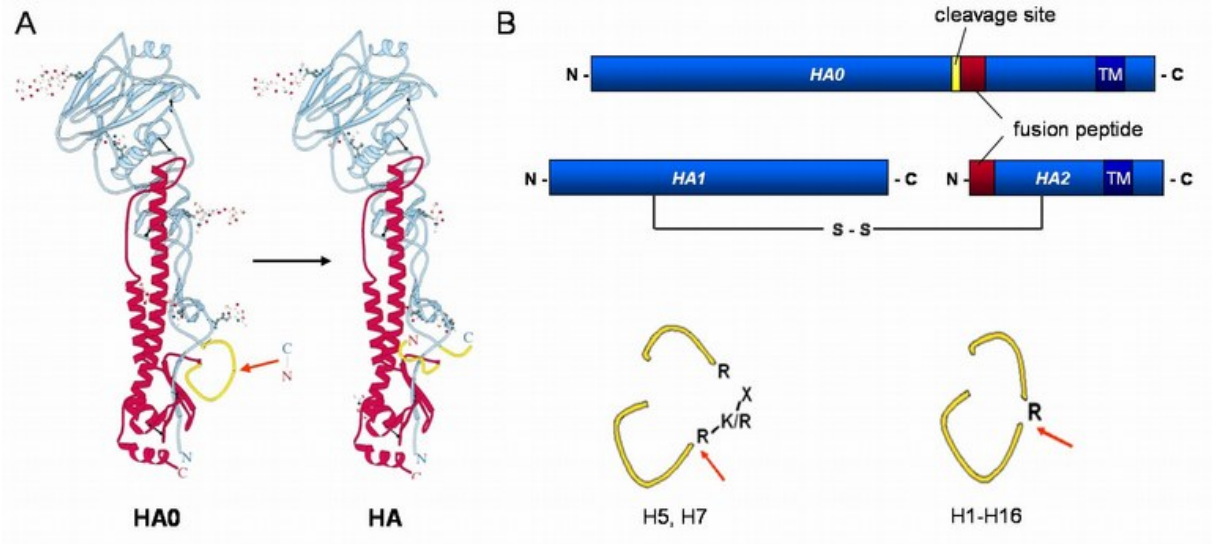

Figure 3: A) Structure of the HA0 monomer and cleaved HA (11). Cleavage site: yellow, HA1: blue, HA2: red. B) Cleavage of HA0 into the disulfide linked subunits HA1 and HA2 at a specific cleavage site. TM: transmembrane domain (figures and text originated from (18)

The globular head of the protein is formed by the HA1 subunit and includes the receptor binding site (RBS) (90). This RBS binds to sialic acids present on glycoproteins and glycolipids implemented within the host cell plasma membrane (115). Avian influenza viruses prefer to

use sialic acids that are linked via α 2.3 sialic acids binding to galactose (115). In contrast, α 2.6 linkage of sialic acids is more accessible for mammalian influenza A viruses (115). Accordingly, avian species primarily exhibit α 2.3 linked sialic acids in their respiratory and intestinal epithelia cells, while respiratory epithelia from mammalian species mainly present α 2.6 linked sialic acids (82). Host species with expression of both types of sialic acid linkage are of special interest since these hosts promote replication of avian influenza viruses as well as mammalian influenza viruses and can act as so-called “mixing vessel” (52). Swine and quail are considered the major host species that enable replication and therefore reassortment of avian influenza and mammalian influenza viruses (further details are presented in chapter “Antigenicity and viral evolution”) (100). After endocytotic uptake of the virus particle and acidification of the endosomal compartment, a conformational change of the HA structure exposes the fusion peptide of the HA2 subunit. Fusion between the viral envelopment and the endosomal membrane occurs after initialisation by insertion of the fusion peptide into the cellular membrane (95).

2.1.4. The Neuraminidase (NA)

The NA surface protein is a tetrameric glycosylated polypeptide of about 50 kilo Dalton (105). The cytoplasmic tail starts with six polar amino acids highly conserved in all Influenza A neuraminidase subtypes (12), followed by a transmembrane domain and a stalk region variable in length (46). The transmembrane region attaches the molecule firstly to the membrane of the endoplasmatic reticulum and later to the viral lipid membrane (1).

On the top of the protein, 4 symmetrical and circularly arranged monomers form the head of the protein (104). This form is stabilized through metal ions in the middle of the symmetrical axes (12). Each monomer is formed by six identical beta sheets, which are collocated to each other similar to a propeller. The catalytic centre of the enzyme is located on the head domain, with functionality only in the tetrameric form (96).

Based on the detailed structure of the active site of the enzyme, the neuraminidase protein subtypes are divided into two groups (96). Group 1 contains N1, N4, N5, and N8 and Group 2 consists of N2, N3, N6, N7, and N9. The NA equivalents of the bat influenza viruses N10 and

N11 differ markedly from N1 to N9, but are not discussed in the context of the present work. (17).

The substrates of the neuraminidase are α 2.3 - and α 2.6 - linked sialic acids and the enzyme releases the free sialic acid (1). As these substrates are the receptor of Influenza A viruses, the NA serves as receptor-destroying enzyme cleaving the receptors elements from the host cell plasma membrane and the viral envelopment (1). This cleavage step facilitates the budding process of progeny virions, release from the host cell and prohibition of agglutination of particles among themselves (70). In general, the NA protein seems to be essential for efficient initiation (55) and spread of the infection (60), but not for viral entry, replication, assembly, or budding (70). NA-negative viruses have been described (87) and those cases supplementation of bacterial sialidases is sufficient to allow effective replication of NA negative mutants (11, 30).

2.1.5. Antigenicity and viral evolution

Both viral glycoproteins HA and NA determine the antigenic subtype of influenza A viruses. The characterization of the different subtypes is achieved by the use of mono-specific antisera, which are reactive only against one a single subtype (43). Accordingly, HA and NA are the only proteins inducing neutralizing antibodies, and are therefore highly important for a protective immunity within the host (63). However, this implicates that humoral immunity is primarily subtype specific and does not prevent from infection with a heterologous subtype. In addition, antibodies against the HA protein are the main determinant for protection against the disease, while NA specific antibodies are less important (63). In addition antibody-mediated immune responses from the host can results in adaptive reactions (“immune escape”) of Influenza viruses and the consequence is further evolution of the respective strains. Influenza A viruses evolve by two different mechanisms: antigenic drift and antigenic shift (107). These mechanisms are responsible for an enormous plasticity of the genome of influenza A viruses. Antigenic drift refers to the adaption of viruses by point mutation due to the intrinsic error rate of the viral polymerase (91). This high error rate leads to the development of a viral quasispecies including many different genotypes (16). The initiation of a sorting process within the host depends on the level of fitness of the

different virus genotypes and promotes replication of adapted viral genotypes. Therefore, a certain immunogenic pressure from the host will always lead to an adapting quasispecies drift. These changes over time occur more likely within the antigenic relevant genes of HA and NA. Changes within the binding sites of neutralizing antibodies may result in viral mutants that escape from the immunogenic pressure. These so called “escape mutants” are able to replicate in the presence of the formerly protective antibodies (34). Antigenic shift may result in any of three scenarios: (a) the transmission of an antigenically distinct virus from one animal species to another that is immunologically naïve to the virus (e.g. the recent avian H7N9 virus spill-over infections in humans), (b) the emergence of an antigenically distinct virus from an intermediate host to a naïve population, which is particularly associated with a reassortment event (e.g. the pandemic H1N1 virus 2009 resulted from reassortment events in swine and was then introduced into the human population), or (c) a reassortment event within the same host species (89). Reassortment allows the exchange of viral gene segments of two co-circulating viruses. Genetically new assembled viral particles from a dually infected cell are a result of exchange of viral segments by chance and called reassortants. In theory, this results in a possible number of 2^8 different segment combinations from one dually infected cell. In practice, only a subset of combinations is viable due to dependencies between the viral segments (primarily within the polymerase encoding segments)(45). Some of the substitutions of the antigenically important proteins HA and NA, that occur due to reassortment events, can lead to the development of potentially pandemic influenza A viruses. The second scenario of antigenic shift refers to the mediation of reassortment in a special intermediate host. These hosts are able to promote the replication of mammalian and avian influenza A viruses, because of their receptor environment (see Chapter “Haemagglutinin”). Therefore, those “mixing vessel” species bring up most favourable reassortants from a combined avian and mammalian origin (52).

2.1.6. Ecology and Pathogenesis of avian influenza A viruses

A large variety of low pathogenic avian influenza viruses (LPAIV) circulate in wild birds. Most of them are apathogenic or induce only mild clinical signs (89). The role of ducks and other aquatic birds as the main reservoir hosts is discussed (112). They seem to be responsible for the spread and transmission of avian influenza to poultry farms.

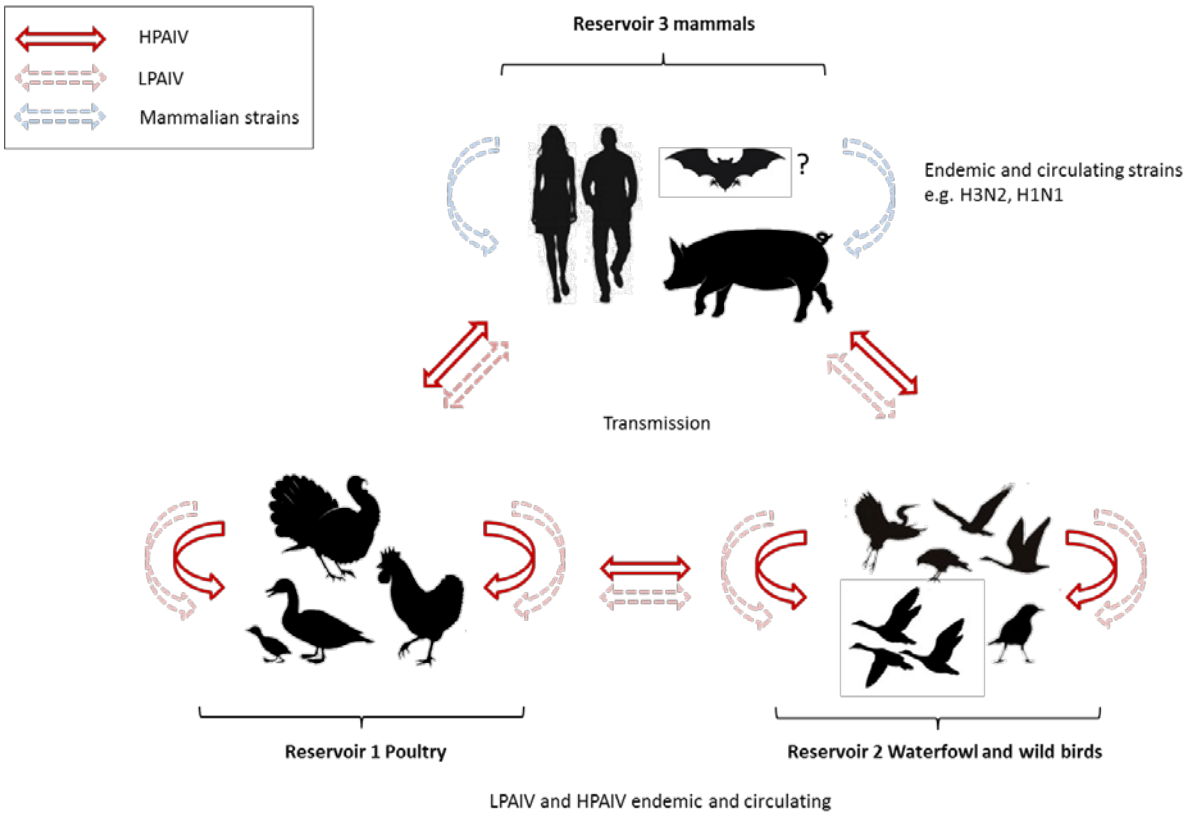

Figure 4: Reservoir and transmission of Influenza A viruses. Figure adapted from (89).

Ducks get infected with LPAI viruses via the oral route. The viruses mainly replicate in the intestinal tract and can be shed in high doses within the faeces (13). Therefore, transmission occurs primarily by the faecal–oral route by direct or indirect contact with contaminated surface water (89). In general, chickens infected with LPAIV only show mild clinical signs, if any, but secondary infections or the infection of juvenile birds may also cause clinical cases (97). Typical clinical signs in LPAIV-infected chickens are associated with the respiratory tract and include coughing, sneezing, rales, rattles and excessive lacrimation. Furthermore, animals can show unspecific signs like huddling, ruffled feathers, listlessness, decreased activity, decreased water and feed consumption and diarrhoea (97).

Up to today, only AIV of subtype H5 and H7 have caused outbreaks of highly pathogenic avian influenza viruses (HPAIV) previously known as 'fowl plague'. These outbreaks emerge after transmission of a former LPAIV to poultry and subsequent mutation to and selection of HPAI by multiple bird passages (59). A well characterized example is the development of HPAIV H7N1 from a LPAIV precursor in Italy in 1999 (4).

HPAIV infections in gallinaceous birds result in mortalities of up to 100% within 48 h (97). Chicken with peracute HPAIV infection show sever symptoms such as a comatose state, dehydration, diarrhea (see figure 3) and can die peracutely without any clinical sign. In case of an acute course of disease, individuals often also exhibit neurological dysfunction. Furthermore, especially gallinaceous birds show edematous (see figure 3), hemorrhagic and necrotic lesions in multiple organs as described by Swayne et al. (97). In contrast to LPAIV, the HPAIVs cause viremia and can be found in several organs. In addition, HPAIV replicates in the upper and lower respiratory tract and is also shed by the oronasal route (58).

Before 2002, HPAIV have very rarely been isolated from wild birds and if so, only in close vicinity of ongoing outbreaks in poultry. However, this has recently dramatically changed with the HPAIV H5N1 outbreaks in wild birds like swans and geese in Europe and Asia (89). Experimental infection of ducks with HPAIV demonstrated that several species are able transmit the virus to chickens without showing any detectable clinical sign (31, 36). Hence, wild birds are now also considered as a reservoir host for HPAIV.

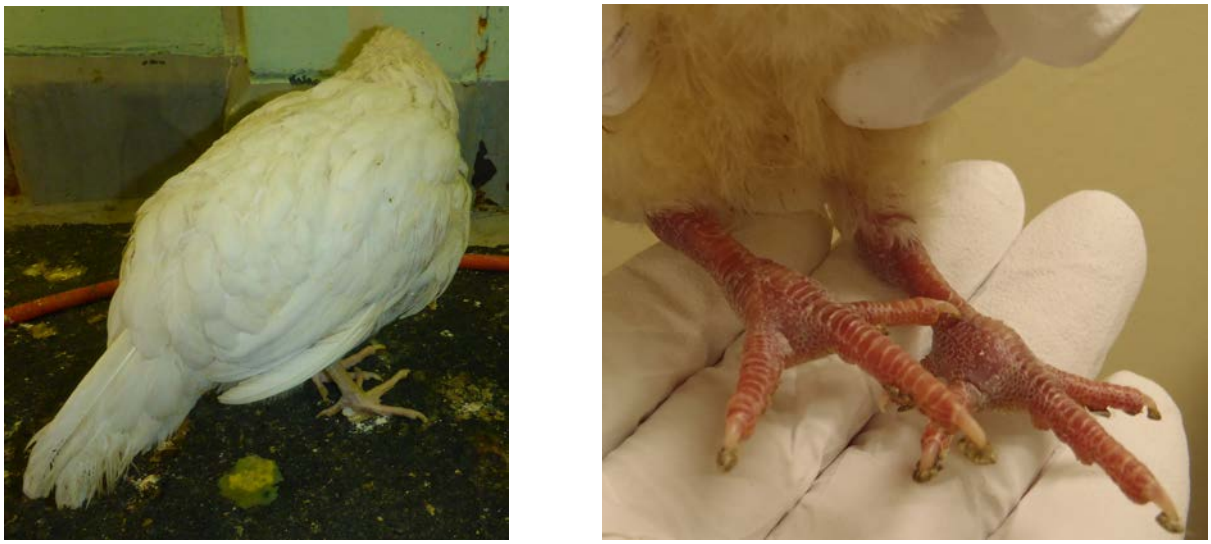

Figure 5: Diarrhoea, apathy and oedema of chickens infected with HPAIV H5N1.

2.1.7. HPAIV H5N1

HPAI viruses of subtype H5N1 originated from south China 1996 (106). During this time different LPAIVs circulated in wild waterfowls and reassortment events lead to the first HPAIV H5N1 strain – Goose/Guangdong/1/1996 - with a high mortality in domestic geese (116). Probably, infected commercial poultry were transported to Hong Kong and there the virus strain reassorted with LPAIV strains of subtype H9N2 and H6N1 (A/quail/HK/G1/97-like; A/teal/HKW312/97-like). The new virus infected domestic birds as well as humans (88). In 2005 and 2006 this strain was detected in wild birds for the first time (89). The H5N1 strain from 1996 was further circulating and caused several outbreaks in China. Additionally mutations in the stalk deletion changed the receptor specificity from the intestinal to the respiratory tract and domestic geese started to be the reservoirs host (15). In 2002 a novel H5N1 outbreak appeared with a new genotype of the Chinese strain, called “Z” (22). The “Z” strain was also introduced from China into poultry holdings in Hong Kong and could be isolated from wild birds (22). Since 2002, HPAIV H5N1 started to spread through South East Asia showing a variation into different clades (Thailand/Vietnam with clade 1 and Indonesia clade 2.1) (108). The main reservoir for these viruses was duck farms, and characteristically, only mild clinical signs were seen in ducks. However, these HPAIV H5N1 were shed and transmitted to other bird species, with very high mortality rates, especially in chickens and related bird species (108).

In 2005 a huge outbreak of a new HPAIV H5N1 reassortant of clades 2.2 and 1 emerged in the Chinese Qinghai region. The novel virus spread efficiently via the flyways of migratory wild birds to Europe and Africa, and the first European outbreaks of HPAIV H5N1 in 2006 were caused by migratory birds from the East (20). However, multiple sub lineages of the H5N1 strain of 1996 are still circulating till date. Since 2008 Egypt has an endemic status for HPAIV H5N1 of clade 2.2. Furthermore, different strains are also co-circulating in China until today (mainly clades 2.3.2, 2.3.4, 2.5, 4, 7 and the Qinghai lineage clade 2.2), and also Indonesia is endemic for H5N1 since 2006 (clade 2.1.3, 2.1.2 and 2.3.2.1) (89).

Different reasons for the ongoing circulation and the strain variability can be designated: 1. Use of vaccines including antigens of clades which are genetically far away from the

circulating strains. 2. Backyard poultry farming and live bird markets with high risk of reassortment and transmission to animals as well as humans. 3. Inadequate surveillance. 4. on-going circulating of virus strains in waterfowl and wild birds (8, 98). Nevertheless, numerous effected regions like Europe, Hong Kong, Japan or Nigeria successfully eradicated H5N1 (89) by stamping out strategies and emerging vaccination.

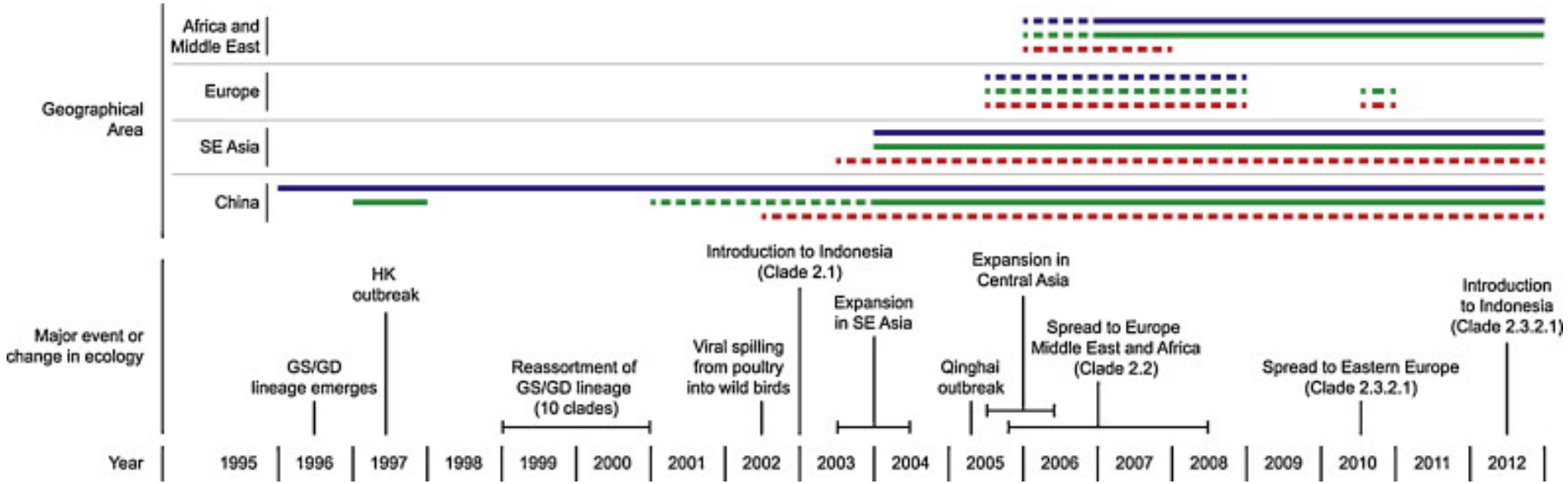

Figure 6: Timeline of major events of Goose/Guangdong/1/1996 HPAIV H5N1 evolution (89).

Dates of major changes in the evolution of highly pathogenic H5N1, Goose/Guangdong/1/1996 – lineage, are shown. The expansion into different geographical areas is depicted, as is status in various hosts in different locations: solid lines show stable interactions between virus and hosts and dashed lines depict transient interactions. Blue lines represent aquatic poultry hosts, green lines terrestrial poultry hosts, and red lines wild birds hosts. SE Asia – South East Asia, GS/GD – goose/Guangdong, HK – Hong Kong (89)

2.2. Control and vaccination strategies

2.2.1. Non-pharmaceutical intervention strategies

Apart from vaccination and therapeutic treatment, non-pharmaceutical interventions are actions that help to reduce the risk of infection for individuals as well as for communities or animal holdings. While in human medicine the recommended methods target the informational communication to individuals and communities, the veterinary sector is also dealing with legally fixed sanctions (e.g. in Germany “Verordnung zum Schutz gegen die Geflügelpest (Geflügelpest-Verordnung)”)(7).

In the poultry sector, the main control strategies for the prevention of Influenza A virus infection are based on (a) continuous surveillance of livestock, (b) training of veterinarians and stockmen, (c) rapid diagnosis of H5 and H7 subtypes, (d) quarantine and movement bans, € disinfection of stabling and equipment and most importantly (f) stamping out strategies (8). These radical measures especially serve as a tool of transmission control. Therefore, the introduction of IAVs particularly of the subtypes H5 or H7, might cause enormous economic impacts as well.

2.2.2. Vaccination

Vaccines against influenza A virus infection that are licensed include inactivated whole virus vaccine preparations consisting of either one viral subtype (monovalent) or three viral subtypes (trivalent) (114). So-called split vaccines are also inactivated preparations where detergent mediated subvirion preparations are produced. These formulations show a reduction of reactivity and therefore are the most commonly used inactivated influenza vaccines for humans (44). Inactivated vaccines are safe as the agent is not able to replicate and therefore even in immunocompromised individuals an infection is not established (71). The immune response initiated by inactivated preparation is mostly humoral and needs to be boosted regularly (8). In contrast live attenuated virus formulations consist of replication competent particles. Although

the vaccine virus replicates, it does not induce disease as it was attenuated e.g. through cold adaptation(3).

Live attenuated vaccines induce a broader immune response including cell mediated and mucosal immunity and are in some cases also able to induce cross protection against heterologous challenge infection (25, 35). However, drawbacks are the possibilities of transmission and reassortment of the vaccine virus strain with circulating strains and therefore live attenuated virus preparations are not an option for pre-pandemic control strategies (71). Recombinant vector vaccines are genetically engineered live non-influenza viruses (e.g. Fowlpox virus) that express influenza specific antigens (109). Therefore, these preparations are able to induce a broader immune response, but do not cause the risk of reassortment. The major disadvantage of some vectors is, that vector-specific antibodies including maternally derived antibodies can neutralize the vector and limit its efficacy with repeated use (8).

2.2.3. Human vaccine

Human influenza vaccines can be divided into two groups – pre-pandemic and seasonal vaccine preparations.

The most widely used seasonal influenza vaccines include influenza A virus from subtype H3N2 and H1N1 and influenza B strains. Twice a year the World Health Organization (WHO) determines the circulating influenza strains and decides which strains are included in the seasonal vaccine formulation (110). The decision is based on the global Influenza surveillance data of 137 WHO centres in 107 member states. Recently different researchers were working on mathematical models to forecast evolutional changes of circulating strains (51).

Most of the formulations for humans are trivalent inactivated whole virus vaccines, “detergent”-split or subunit vaccines and in some cases adjuvanted for a stronger immune response. Mainly, the vaccines are produced in embryonated chicken eggs, concentrated, inactivated with formalin or beta-propiolactone and purified (114). Some newer vaccines are also produced in cell culture systems (72). Furthermore one trivalent live attenuated vaccine for

intranasal application is available in Europe (9). Seasonal influenza vaccines are mostly used for older or immunosuppressed people, except the live attenuated formulation, this is especially dedicated to children (114).

In addition, in many countries, pre-pandemic vaccines, e.g. for H5 viruses, are stockpiled, these vaccines are in most cases monovalent, inactivated preparations using a single subtype, which was predicted to be responsible for future pandemic events (23). Actually such formulations are licensed for the subtype H5N1. The main challenge of these vaccines is to keep the preparations antigenically actual as e.g. H5N1 is ongoing to evolve (23).

2.2.4. Poultry vaccines

Since 1959, HPAIVs have caused 32 epizootics world wide (98). At the moment 59 vaccines against avian influenza are commercialized (27). In contrast to human vaccination strategies, there is no frequent update of the used vaccines and regulations are defined more locally. In Germany the generalized application of vaccines against avian influenza of subtype H5 and H7 is forbidden (7). There is the possibility to vaccinate only as an emerging strategy using preparations that allow the differentiation of vaccinated from infected animals (7). This was used for H5 e.g. in chickens in the Netherlands and for zoo birds in Germany (73). For the differentiation of vaccinated from infected animals certain serologic assays have to be on the market with corresponding vaccine preparations (94). Some of the inactivated vaccine preparations as well as vector vaccines full fill these requirements as the vaccines induce a serological response against a selected NA type (inactivated vaccines) or the vector-presented antigens (mostly the HA protein) (94). Serological tests for antibodies against internal proteins such as M1 or NP in combination with serological test against the selected NA type allows the differentiation of vaccinated animals from infected animals (Figure 5).

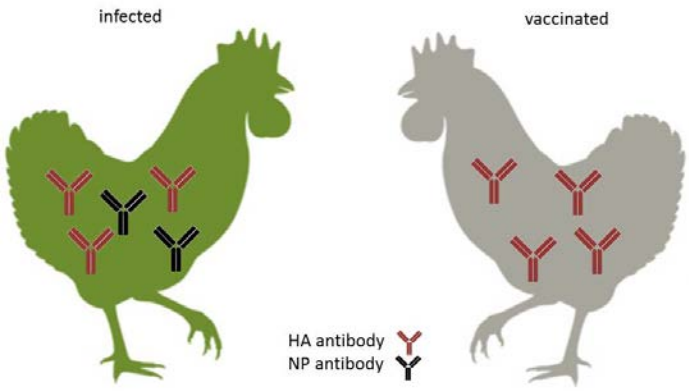

Figure 7: Differentiation of vaccinated animals from infected animals (DIVA strategy). Infected animals show NP and HA antibodies, whereas e.g. vector-vaccinated ones only show HA-specific antibodies.

Vaccines as a control strategy against HPAIV are used since 1994/95 (98). At this time public authorities of Mexico and Pakistan were forced to develop new strategies to control HPAIV H5N2 and H7N3 (42). One major reason was that the usual culling strategies led to food shortage in the affected countries. Since this time poultry stocks in thirteen countries on the European, Asian and African continents were immunized against HPAIV strains of subtype H7 and H5 (8). The majority of the used vaccines – about 99% - were used in areas enzootic for HPAIV H5N1 (98), this includes for example China, Egypt, Indonesia and Vietnam (5, 8, 66, 89).

Most of the vaccines used at the moment are inactivated and adjuvanted full-virus vaccines of the circulating HPAIV as well as LPAIV H5/H7/H9 strains (95,5%) (98). However, these vaccine types do not induce a complete protection to the poultry, e.g. from virus replication and shedding ("sterile immunity"). In fact, this leads to the development of escape mutants by antigenic drift and therefore these vaccines should be adapted frequently (98). Furthermore, the induced immune response to these vaccines is reduced (14) and therefore also non-permanent.

Further used vaccines are live vector formulations licensed e.g. for chickens (Mexico/China Avian Paramyxovirus type 1; USA/ Mexico Fowlpox virus; USA/Egypt Herpesvirus Turkey)(98). Liu et. al developed a duck enteritis virus vector vaccine for ducks and submitted it for license in 2012 (49).

Although vaccines were able to minimize the total number of infected countries, there are still regions endemic for HPAIV (8). In addition these vaccination campaigns lead to substantial antigenic drift of the circulating influenza viruses (21, 38).

Further vector vaccines are under development (Avian Leukosis virus; replication-defective Adenovirus; Infectious Laryngotracheitis virus; Salmonella typhimurium; Equine-Encephalitis virus (98)) as well as live vaccines, which based on LPIAV H5 or H7 strains or – similar to human strains – temperature sensitive, attenuated H5 and H7 strains (8).

2.2.5. Antivirals

Antiviral drugs are in addition to vaccine strategies used as control agents in humans.

Especially for new strains there are no other options for infection control. During the last decades some influenza A strains developed resistance to antiviral drugs like Oseltamivir, Amantadine and Flumadine the usage of these drugs has to be well considered, controlled and as far as practicable minimized (75).

Oseltamivir phosphate (Tamiflu®) and Zanamivir (Relanza®) inhibit the NA of influenza A and B viruses and prohibit the viral spreading in the early infection stage (60). It must be administered as early as possible, while the peak of replication of Influenza A in the respiratory tract is between 24 and 72 hours after the onset of illness (60). Zanamivir is available as a dry powder to inhale the substance orally, whereas Oseltamivir can only be absorbed orally, when absorbed in the gastro intestinal tract, hepatic esterases convert it to the active form. It was shown that application of neuraminidase inhibitors leads to a lower frequency of secondary complications, shorter periods of influenza typical symptoms like depression and fever (60). However, escape from antivirals like NA-inhibitors are well known and several responsible mutations have been demonstrated (28, 99, 101). Detected example strains and substitutions are listed in table 2.

Influenza type	Amino acid substitution	Strain
N1	H274Y	H1N1 pandemic 2009
N2	E119V	endemic H3N2
N2	R292K	Endemic H3N2
Influenza B	D198N/E	
N9	R292K	H7N9

Table 2: Strains and amino acid substitutions (50, 65) detected 2003/04; 2007 to 09 and 2014.

M2 channel inhibitors like Amantadine (Symmetrel®) or Rimantandine (Flumadine®) can also be used against influenza, but they are only effective against Influenza A viruses and several toxic side effects are described. Additionally they induce rapid emergence of drug resistant (74).

The manuscripts are presented in the form accepted for publication.

Each manuscript has its own reference section; references and abbreviations from the manuscripts are not included in the relevant sections at the end of this document.

Figures and tables are numbered individually within each manuscript.

3. Truncation and sequence shuffling of segment 6 generate replication-competent neuraminidase-negative influenza H5N1 viruses

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3.1. Abstract

Influenza viruses are highly genetically variable and escape from immunogenic pressure by antigenic changes in their surface proteins referred to as antigenic drift and antigenic shift. To assess the potential genetic plasticity under strong selection pressure, highly pathogenic avian influenza virus (HPAIV) of subtype H5N1 was passaged in embryonated chicken eggs in the presence of a neutralizing, polyclonal chicken serum for 50 times. The resulting mutant had acquired major alterations in the neuraminidase (NA)-encoding segment. Extensive deletions and rearrangements were detected in contrast to only 12 amino acid substitutions within all other segments. Interestingly, this new neuraminidase segment resulted from complex sequence shuffling and insertion of a short fragment originating from the PA segment. Characterization of that novel variant revealed a loss of the neuraminidase protein and enzymatic activity, but its replication efficiency remained comparable to the wild-type. Using reverse genetics, a recombinant virus consisting of the wild-type backbone and the shortened NA-segment could be generated, however, required the polybasic hemagglutinin cleavage site. Two independent repetitions starting with egg passage 30 in the presence of alternative chicken-derived immune sera selected mutants with similar but different large deletions within the NA segment without any neuraminidase activity, indicating a general mechanism. In chicken, these virus variants were avirulent despite the still present HPAIV polybasic hemagglutinin cleavage site. Overall, the variants reported here are the first HPAIV H5N1-viruses without functional neuraminidase which grow efficiently without any helper factor. These novel HPAIV variants may facilitate future studies shedding light on the neuraminidase in virus replication and pathogenicity.

3.2. Introduction

Highly pathogenic avian influenza viruses (HPAIV) of the subtype H5N1 have been circulating in many regions in Asia and Africa for up to 10 years (1) raising concerns of an influenza pandemic.

While wild waterfowl serves as a virus reservoir, poultry - primarily chicken - infected with HPAIV H5N1 succumb to death due to a devastating disease. In addition, the currently used control measures (2) like culling of infected birds, restriction of movement, enforcement of biosecurity, and surveillance lead to severe economic losses in the poultry industry worldwide. Vaccination against HPAIV H5N1 using inactivated virus preparations was implemented particularly in developing countries to combat the disease. However, as influenza A viruses continue to change their antigenicity by antigenic drift, due to base exchanges introduced during the error prone genome replication by the viral polymerase complex, and by antigenic shift, which results from reassortment of genome segments from two viruses (3), vaccines have to be adapted regularly. For application in humans, the world health organization (WHO) predetermines the vaccine composition each season. In the veterinary field, non-homologous vaccines are used, often resulting in non-sterile immunity in the vaccinated poultry flocks and thus without disruption of infection chains. In consequence, infection of those partially protected birds by circulating recent HPAIV H5N1 leads to the continuous emergence of escape variants (4-6) with an altered antigenic repertoire (6). These viruses are not neutralized by the antibodies present in the vaccinated flocks; hence the animals are not fully protected as demonstrated by the re-occurrence of morbidity and mortality (4).

The phenomenon of antigenic escape was classically investigated by the characterization of escape variants generated in vitro by virus passaging in the presence of monoclonal antibodies (7, 8). While antigenic sites were hereby identified successfully, such a rather artificial selection is limited to epitope-specific variation only. However, in silico analysis of the evolution of both viral surface proteins, i. e. the hemagglutinin (HA) and neuraminidase (NA), revealed several epistatic mutations, highlighting that immunoescape is a polygenic trait (9). In addition, we could show recently that cell-culture passaging of HPAIV H5N1 under the selection pressure of a

polyclonal chicken-derived serum resulted in attenuated viruses with numerous point mutations in several segments (10). To assess the immunoescape enabled by the considerable genetic plasticity of influenza A viruses under strong, more authentic selection pressure closer to conditions in vivo, we passaged an HPAIV H5N1 strain 50 times in the presence of a polyclonal antiserum in embryonated chicken eggs. In contrast to our previous in vitro study (10), this experimental approach resulted in replication-competent and stable neuraminidase-negative attenuated H5N1 viruses with large intra-segmental deletions in segment 6 causing a complete loss of neuraminidase activity. Their generation along with the in vitro and in vivo features are subject of this study.

3.3. Material and Methods

All experiments using HPAIV H5N1 were conducted in biosafety level 3+ containment facilities at the Friedrich-Loeffler-Institut (FLI), Greifswald – Insel Riems, Germany.

Viruses and sera

Ancestor virus for passaging was the 3rd (egg culture) passage of the reference strain A/cygnus cygnus/Germany/R65/2006 (H5N1) (11). The initial serum (serum A) used to implement neutralizing pressure originated from an individual chicken vaccinated twice with a commercial inactivated vaccine of the H5N2 subtype (Nobilis Influenza H5N2, Intervet, Unterschleißheim, Germany) and afterwards boostered with a challenge infection using HPAIV H5N1 R65/p17 (a passaged, but highly related variant of the original R65 strain). This immunization procedure was selected to allow the development of a maximum of serum antibodies against immunogenic influenza virus proteins, which would enable an efficient immunogenic pressure to HPAIV H5N1 (immunization schedule data available upon request). The immune-serum from another chicken (serum B) also vaccinated twice with the inactivated H5N2 vaccine and challenged with HPAIV R65/p17 was used as a second test serum for both the neutralization test and for passaging the 30th passage of H5N1 R65 in the repetition experiment generating the second escape variant virus EscEgg50B. A third serum (serum C) originating from a chicken vaccinated once with the commercial inactivated H5N2 vaccine and afterwards challenged with

the original HPAIV H5N1 R65/06 (serum C) was used for the generation of the third escape variant virus EscEgg50C. All three sera were further characterized using the hemagglutination inhibition test (HI; see below) against the ancestor virus H5N1 R65 scoring with the same HI-titer of 1:128. The HI-value of the three sera is comparable to titers reported for similar experiments (12), and also for chicken-sera from an evaluation study under field conditions using the H5N2 vaccine and the HPAIV H5N1 R65 challenge infection (13). Furthermore, sera B and C (serum A was completely used for passaging, the neutralization testing, and the HI-assay, and was therefore not longer available) were tested using a commercial ELISA assay for the detection of antibodies against the N1-protein (ID Screen® Influenza N1 Antibody Competition ELISA kit, ID-vet, Montpellier, France). Both sera scored negative in the N1-ELISA.

In addition, sera B and C as well as a negative commercially available chicken serum (Sigma Aldrich) were tested in dilutions from 1:2 to 1:512 against a defined amount of ancestor virus H5N1 strain R65 using a neuraminidase activity test (see below). Interestingly, there was an inhibitory effect to the neuraminidase activity detectable in the antibody positive sera that was dilutable, and values recorded for the non-immunized control chicken serum were statistically different from the data collected for the immune sera B and C (data not shown Students T-test).

Passaging in egg-culture

The principle steps for passaging virus under positive serum pressure were as follows: Virus was incubated with eight different antiserum dilutions at room temperature in 0.2 ml Dulbecco modified Eagle medium supplemented with 5% fetal calf serum for one hour with gentle agitation. Subsequently, eight embryonated specific pathogen free (SPF) chicken eggs (10 days old) were inoculated with one of the antiserum-incubated virus preparations each via the allantoic cavity and checked daily for embryonic death. Five days after inoculation, allantois fluid was harvested and MDCK cells (collection of cell lines in veterinary medicine, FLI Insel Riems, RIE1061) were inoculated with 50 µl of the allantois fluid. After incubation of the cells for three days, the cytopathic effect was assessed via light microscopy. The virus from that allantois fluid with the maximum serum amount still allowing viral growth was chosen for the next egg passage.

In total, 50 egg passages using a single polyclonal antiserum (serum A) were done resulting in the virus EscEgg50A (A/hen's egg/Germany/[A/cygnus cygnus/Germany/R65/2006]-EscEgg50-escape/2009 (H5N1)). A control virus was mock-passaged without serum in egg culture 50 times in parallel (CoEgg50). Starting with the 30th passage of the experiment two additional distinct escape variants (EscEgg50B, EscEgg50C) were generated by passaging in egg culture (until 50 passages were achieved) in the presence of two different polyclonal sera from chickens (serum B and C).

Whole genome sequencing

EscEgg50A and CoEgg50 were sequenced with a Genome Sequencer FLX (GS FLX; Roche, Mannheim, Germany) according to the protocol of Höper and co-workers (14) with the modifications of Leifer and colleagues (15). EscEgg50A was in addition sequenced after preparation of a randomly primed cDNA sequencing library for Titanium sequencing with the GS FLX according to the manufacturer's protocol. Moreover, after RT-PCR amplification of segment 6 of the ancestor virus, EscEgg50A, and viruses from intermediate passages (primer sequences available upon request), DNAs were sequenced with the Genome Sequencer FLX according to the manufacturer's recommendations. Raw data was analyzed using software provided with the Genome Sequencer FLX. In addition, the NA-segments of EscEgg50A, EscEgg50B, and EscEgg50C and the HA-segment of EscEgg50Arec were sequenced using classical Sanger sequencing (16). Moreover, the 3'- and 5'-termini of the EscEgg50A segment 6 were determined by classical Sanger sequencing after rapid amplification of cDNA ends (3' and 5' RACE System for Rapid Amplification of cDNA Ends; Invitrogen, Darmstadt, Germany).

The rearrangement of the EscEgg50A, B, C segments 6 was analyzed using Mauve (v 2.3.1.; (17)) with the MauveAligner algorithm. The coordinates of the rearrangements were then used for plotting the rearrangement graph in R (18).

Generation of recombinant viruses

The ancestor virus R65/06 and the mutants were generated by reverse genetics techniques according to (19, 20). To obtain a plasmid encoding the EscEgg50A NA, we performed target-

primed plasmid amplification using the pHW2000-R65NP plasmid (26) and the full-length PCR-amplicon.

We constructed a pHW2000 EscEgg50A NAdel plasmid by mutating the start codon ATG (nt 21-23) to ACG (primer sequence available upon request). Furthermore, we generated a pHW2000 segment 6 EGFP-expressing plasmid carrying the first 118 nucleotides from segment 6 (like EscEgg50C) upstream to the EGFP encoding sequence followed by the 77 terminal nucleotides from segment 6 (like EscEgg50A). The four ATG-triplets within the first 118 nucleotides were silently mutated resulting in a first start codon at position 116 for expression of the EGFP protein.

Neutralization assay

The virus neutralization test (VNT) was performed according to a previously described procedure (21) with a few modifications. In brief, serum samples were heat inactivated for 30 min at 56°C, and 3-fold serial dilutions were prepared in a 50 µl volume of cell culture medium in 96-well plates. The diluted serum samples were mixed with an equal volume of medium containing R65/06, the escape mutant EscEgg50A, or the passaged control virus CoEgg50 at a concentration of 102 TCID50/well. After 1 h incubation at 37°C in a 5% CO2 humidified atmosphere, 100 µl of MDCK cells at 1.5 × 105/ml were added to each well. The plates were incubated for 3 days at 37°C and 5% CO2. Viral replication was assessed by visually scoring the cytopathic effect without staining. Each assay was validated by comparison with positive and negative control sera from chicken and by titration of the used virus dilutions. Results were statistically evaluated by using a one way ANOVA analysis.

Viral growth kinetics and Plaque size measurement

Growth kinetics were assed on MDCK cells by infecting the cells with a multiplicity of infection (MOI) of 1 or 0.01 respectively. At the indicated times after infection, intra- and extracellular virus titres were determined.

To examine virus induced plaque sizes, MDCK cells were seeded in six-well plates (Nunc, Thermo Fisher Scientific, Langenselbold, Germany), and infected with R65/06, EscEgg50A and

EscEgg50Arec using a MOI of 0.01. Twenty-four hours after infection under an agarose overlay, plaque diameters of 50 randomly selected plaques of each virus were determined (after fixation and staining against NP protein, see below) and mean diameters and standard errors were calculated. Values for the parental strain R65/06 were set to 100 % and the plaque diameters observed for the mutant viruses were expressed relative to this value.

Viral growth analysis after supplementation of bacterial sialidase was performed on MDCK cells incubated for 2h in the presence of 1U Clostridium perfringens neuraminidase (Roche Diagnostics, Mannheim, Germany) per ml. Afterwards, the cell culture was infected with an MOI of 0.1, still in the presence of the bacterial sialidase. The viral titers of the supernatants were determined after 24h and 48h incubation time. Analysis of variance by Kruskal-Wallis was used to determine the statistical relevance of the collected data.

Hemagglutination assay

Hemagglutination activity was determined in microtiter plates by using 0.5% chicken erythrocytes. The reactions were performed in phosphate-buffered saline (PBS) at room temperature (approximately 20°C).

Hemagglutination inhibition assay

The hemagglutination inhibition assay was conducted as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (22).

Indirect immunofluorescence assay

MDCK cells grown on coverslips in 4-well culture plates (Lab-Tek™ Chamber Slide™ System, Nunc, Thermo Fisher Scientific, Langenselbold, Germany) were infected with EscEgg50A virus or the ancestor virus R65/06, respectively, and incubated for 72 h. Subsequently, the cells were fixed with methanol acetone (1:1) for 30 min. For the detection of NA and NP protein, the fixed cells on the coverslips were incubated in the given order with the following monoclonal antibodies each for 60 min at room temperature: (i) Mouse anti-N1 monoclonal antibody (N1 18.2.5 Malte Dauber, Friedrich-Loeffler-Institut, Greifswald, Germany) diluted 1:5 in PBS. (ii)

Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen, Life technologies, Darmstadt, Germany) diluted 1:1,000 in PBS as secondary antibody. (iii) Anti-NP monoclonal antibody (ATCC, HB-65) diluted 1:20 in PBS. (iv) Alexa Fluor® 546 donkey anti-mouse IgG secondary antibody (Invitrogen) diluted 1:1,000 in PBS. Fluorescence was detected using an Axioskop (Zeiss, Jena, Germany).

Western blot analysis

Forty-eight hours after infection, MDCK cells infected with EscEgg50A or the ancestor virus R65/06 were lysed by a freeze/thaw procedure in extraction buffer (1% Triton X-100, 2 mM EDTA, 0.15 M NaCl, 20 mM Na2HPO4, pH 7.6) containing proteinase inhibitor "Complete Mini" (Roche Diagnostics, Mannheim, Germany). The resulting protein extracts and PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific) were separated on a 10% SDS-polyacrylamide gel under reducing conditions and transferred to nitrocellulose membranes (Whatman, Dassel, Germany) by wet western methodology. After blocking overnight in Tris-buffered saline supplemented with 0.1% Tween (TBS-T) and 5% skim milk, the blot was incubated with the anti-HA antibody (23) diluted 1:5,000 in TBS-T or the anti-N1 antibody (23) diluted 1: 5,000 in TBS-T. As secondary antibody, a horseradish peroxidase conjugated anti-mouse antibody (Dianova, Hamburg, Germany) diluted 1:20,000 in TBS-T was used. Antibody binding was visualized by chemiluminescence (Supersignal West Pico Chemiluminescence Kit; Pierce, Bonn, Germany) using ChemoCam System (Intas, Göttingen, Germany).

Reverse transcription real-time PCR assays

RNA extraction from allantois fluid was performed using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The NA-truncation specific reverse transcription real-time PCR (RT-qPCR) assay was optimized to work with a probe complementary to an ancestor sequence maintained within the EscEgg50A segment 6 sequence and EscEgg50A virus segment 6 specific primers (sequences available upon request). In addition, H5 and N1 specific sequences were detected in a duplex RT-qPCR (24). A heterologous internal control system (25) was detected as extraction control. The EscEgg50A-specific RT-qPCR assay

was carried out using the AgPath-ID™ One-Step RT-PCR Kit (Ambion, Applied Biosystems, Life Technologies, Darmstadt, Germany). A total reaction volume of 25 µl containing 4 µl RNase-free water, 12.5 µl 2X AgPath-ID™ One-Step RT-PCR master mix, 1 µl AgPath-ID™ One-Step RT-PCR enzyme mix, 1.25 µl NA-specific FAM-labeled primer-probe-mix, 1.25 µl extraction control-specific HEX-labeled primer-probe-mix and finally 5 µl RNA template were merged. The assay was run on the ABI 7500 real-time PCR system (Applied Biosystems, Life Technologies, Darmstadt, Germany). The following thermal profile was used: reverse transcription at 45°C for 10 min, PCR initial activation step at 95°C for 10 min, 42 cycles of three-step cycling consisting of denaturation at 95°C for 15 sec, annealing at 55°C for 20 s, and extension at 72°C for 30 s.

Neuraminidase activity assay

The ancestor virus R65, the control virus CoEgg50, the escape variant EscEgg50A, and the recombinant escape variant EscEgg50Arec (see below) were inactivated using 1.5 mM binary ethylene-imine (26) before downstream processing. All virus preparations were adjusted to the same genome load quantified by RT-qPCR analysis of viral segment 7 (27). Subsequently, the neuraminidase activity was measured using the NA-XTD™ Influenza Neuraminidase Assay Kit (Applied Biosystems) according to the manufacturer's instructions. The luminescence was determined on a Tecan infinite 200 instrument (Tecan, Crailsheim, Germany). The neuraminidase activity was examined with or without applying the neuraminidase inhibitor oseltamivir carboxylate (10.56 nM, 256 nM, and 6600 nM; Hoffmann – La Roche Inc., Nutley, USA). Except for the recombinant virus EscEgg50Arec neuraminidase activity was determined from two independent virus preparations in each four replicates. For EscEgg50Arec, only a single virus preparation was measured in 4 replicates for each inhibitor concentration. A "no virus control" was included for every inhibitor concentration. After normalization of the raw data, the signal intensities were divided by the respective "no virus control" average signal to get adjusted values for every inhibitor/virus combination. For comparison, the reference viruses A/Mississippi/3/2001 (H1N1) wild type (274H) and the neuraminidase inhibitor resistant A/Mississippi/3/2001 (H1N1) Mutant (274Y) kindly provided by the Neuraminidase Inhibitor Susceptibility Network (NISN) were included.

Animal experiments

The animal trials gained governmental approval under the registration number LVL MV/TSD/7221.3-1.1-003/07.

Intravenous pathogenicity index (IVPI)

The determination of the intravenous pathogenicity index (IVPI) according to the OIE standard protocol (22) is the appropriate test to assess the pathogenicity of a certain influenza virus strain in avian species. The IVPI indicates the mean clinical score of ten 6-weeks-old chickens after intravenous inoculation. Thereto, groups of ten SPF chickens (Lohmann Tierzucht, Cuxhaven, Germany) were infected intravenously with the indicated viruses at 104.5 TCID50/animal. Birds are scored on a scale ranging from 0 to 3, with 0 (healthy), 1 (sick), 2 (severely sick), 3 (dead). After ten days of evaluation, the IVPI is calculated as the average scoring of 10 birds on 10 days. Viruses are classified as highly pathogenic if their IVPI is 1.2 or higher (22).

Oronasal infection

Ten SPF-chicken were housed together and infected with the indicated viruses by oronasal application of 104.5 TCID50/animal. Another group of ten SPF chickens was infected oronasally with 104.5 TCID50/animal of the control virus CoEgg50. The chickens were checked daily for clinical symptoms and fourteen days after inoculation serum samples of individual chicken were taken. Thereafter, surviving chickens were challenged by oronasal application of 106 TCID50/animal of the ancestor virus R65/06. Serum samples from surviving animals were taken nine days post challenge.

Serological analysis

Pre-experimental serum samples of individual chickens and samples from surviving animals were heat-inactivated at 56°C for 30 min and examined for the presence of antibodies against the nucleoprotein of avian influenza A virus (ID Screen® Influenza A Antibody Competition ELISA kit, ID-vet, Montpellier, France), antibodies against the H5-protein (ID Screen® Influenza H5 Antibody Competition ELISA kit, ID-vet), and finally antibodies against the N1-protein (ID

Screen® Influenza N1 Antibody Competition ELISA kit, ID-vet). Sera were also tested by an in vitro neutralization assay (described above) using the ancestor virus R65/06 to be neutralized.

3.4. *Results*

To simulate the selection and emergence of an escape H5 virus in a vaccinated flock of chicken, an H5N1 virus was forced to replicate under the multifarious immunogenic pressure applied by a polyclonal chicken serum. The ancestor virus HPAIV A/Swan/Germany/R65/2006 (H5N1) (R65/06) (11) was passaged fifty times in egg-culture in the presence of a polyclonal chicken serum (serum A) resulting in the escape variant virus "EscEgg50A". A control virus that was mock-passaged fifty times in egg culture without serum was designated "CoEgg50".

EscEgg50A is a novel H5N1 variant with a unique truncation and sequence shuffling within segment 6 including inter-segmental recombination

Expecting several individual amino acid (AA) substitutions, all genome segments of EscEgg50A were sequenced and deposited in GISAID (www.gisaid.org), (accession EPI338332 – EPI338339). Twelve individual amino acid (AA) substitutions within the whole genome (Table 1) were detected for EscEgg50A, while 8 AA substitutions were found for the control virus CoEgg50 (Table 1). For both viruses - EscEgg50A and CoEgg50 - the AA substitutions were scattered across the genome. Interestingly, the EscEgg50A HA-protein acquired only a single amino acid exchange (E385K) during 50 passages, while the identical number of passages without immune serum pressure led to 3 AA substitutions within the HA of the control virus CoEgg50 (Table 1). Besides the aforementioned AA substitutions, EscEgg50A carries a prominent sequence variation within segment 6 in which major parts of the coding region were deleted and the remaining parts were shuffled (Fig. 1 A, Table 2). In addition, 29 bases from the PA-coding segment 3 were incorporated into segment 6. In total, the segment 6 of EscEgg50A had a length of 680 nt with one single open reading frame from nt 21 to nt 209 (62 AA), encoding the 51 N-terminal amino acids of the original NA protein followed by 11 additional unrelated AA. To rule

out that sequences shuffled to regions not covered by the amplification product generated by the segment 6-specific primers, the sequence was confirmed using the standard random RNA sequencing protocol for the GS FLX. In addition, RACE-PCRs were performed to sequence the termini of segment 6. The determined terminal sequences did not differ from the ancestor segment termini.

		AA residue		
protein	AA	R65/06	EscEgg50A	CoEgg50
PB2	368	Q	Q	K
	700	E	K	E
PB1	738	E	E	G
PB1F2	56	A	V	A
	77	L	W	L
PA	208	T	I	T
	595	M	I	M
HA	143	A	A	V
	210	P	P	S
	313	L	L	H
	385	E	K	E
NP	351	R	K	R
NA			deletions and re-arrangement	none
M1	26	Q	Q	R
	50	P	P	T
	88	G	G	R
	125	A	T	A
	175	H	Q	H
M2	14	E	A	E
	68	V	A	V
NS1			none	none
NS2	22	A	E	A

Table 1: AA substitutions acquired by the viruses after 50 passages in egg culture with (EscEgg50A) or without (CoEgg50) use of a polyclonal chicken antiserum. For comparison the ancestor AA are given (R65/06).

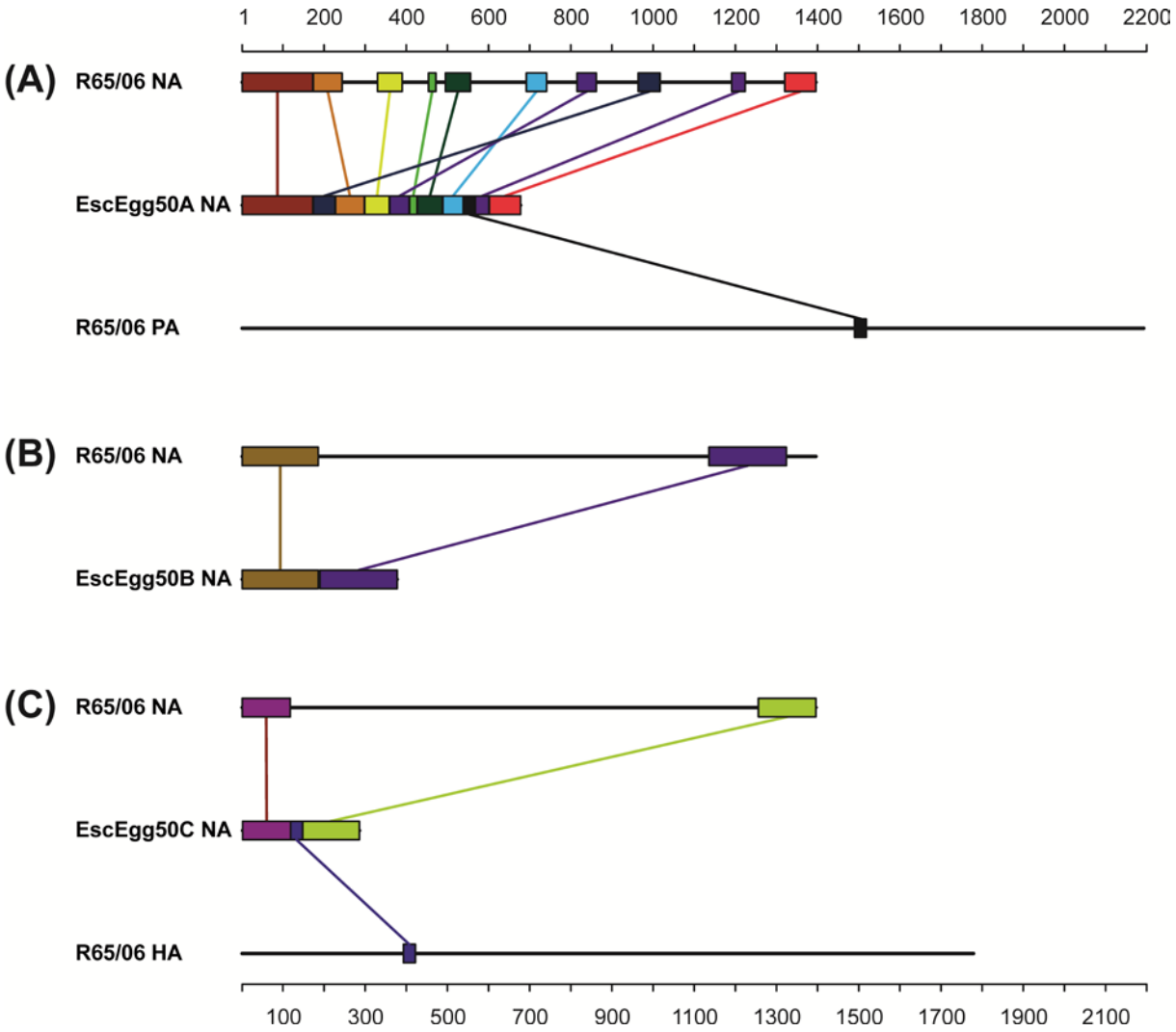

Figure 1: Nucleotide sequence rearrangements in segment 6 of virus variants EscEgg50A, EscEgg50B, and EscEgg50C. (A) Rearranged EscEgg50A segment 6 together with the R65/06 donor segments 6 and 3. The differently colored rectangles depict the different portions of the donor segments that were rearranged in the EscEgg50A segment 6. Those parts of the donor segments that were not incorporated into the EscEgg50A segment 6 are depicted as a black line only. (B) Rearranged EscEgg50B NA with donor segment 6 (C) Rearranged EscEgg50C NA with donor segments 6 and 4.

Position within mutant virus strain			Position within ancestor virus R65/06			Sequence identity
	First nt	Last nt	Segment	First nt	Last nt	
EscEgg50A	1	173	6	1	173	172/173
	174	227	6	964	1017	54/54
	228	298	6	174	244	70/71
	299	359	6	330	390	61/61
	360	407	6	815	862	48/48
	408	426	6	454	472	17/19
	427	488	6	495	556	62/62
	489	538	6	692	741	51/51
	539	567	3	1490	1518	29/29
	568	601	6	1191	1224	34/34
	602	678	6	1320	1396	75/77
EscEgg50B	1	186	6	1	186	186/186
	190	378	6	1136	1324	188/189
EscEgg50C	2	119	6	1	118	118/118
	119	148	4	393	422	30/30
	146	286	6	1256	1396	140/141

Table 2: Compilation of the source sequences of the different EscEgg50 segment 6 variants.

EscEgg50A is negative for the neuraminidase protein and for neuraminidase activity

Indirect immunofluorescence staining and western blotting using monoclonal as well as polyclonal NA-specific antibodies were used to confirm the loss of the NA protein. In cell cultures infected with EscEgg50A, no fluorescence was detected after staining with a monoclonal antibody against NA (Fig. 2). The cell cultures were co-stained with anti-NP antibodies resulting in fluorescence showing the presence of nucleoprotein in the EscEgg50A-

infected cell cultures (Fig. 2). Western blot analysis using a polyclonal anti-NA serum demonstrated a distinct band at 56 kDa in the protein preparation of the ancestor virus R65/06. This band was absent from the EscEgg50A virus preparation (Fig. 3A). Protein detection using a polyclonal anti-HA serum confirmed the presence of viral protein in the used protein preparations (Fig. 3B).

To verify the loss of the enzymatic function, a neuraminidase activity assay was performed. The ancestor virus R65/06, the control virus CoEgg50, the EscEgg50A virus, the recombinant EscEgg50Arec virus, and two reference viruses of the subtype H1N1 were tested in parallel. Neuraminidase activity was determined with or without addition of the neuraminidase inhibitor oseltamivir. Independently of the inhibitor concentration, the NA-deleted preparations EscEgg50A and EscEgg50Arec showed no neuraminidase activity at all (Fig. 4), while the reference viruses as well as R65/06 and CoEgg50 exhibited neuraminidase activities as anticipated (Fig 4). Taken together, these results demonstrate the loss of neuraminidase enzyme activity of EscEgg50A and the recombinant EscEgg50Arec.

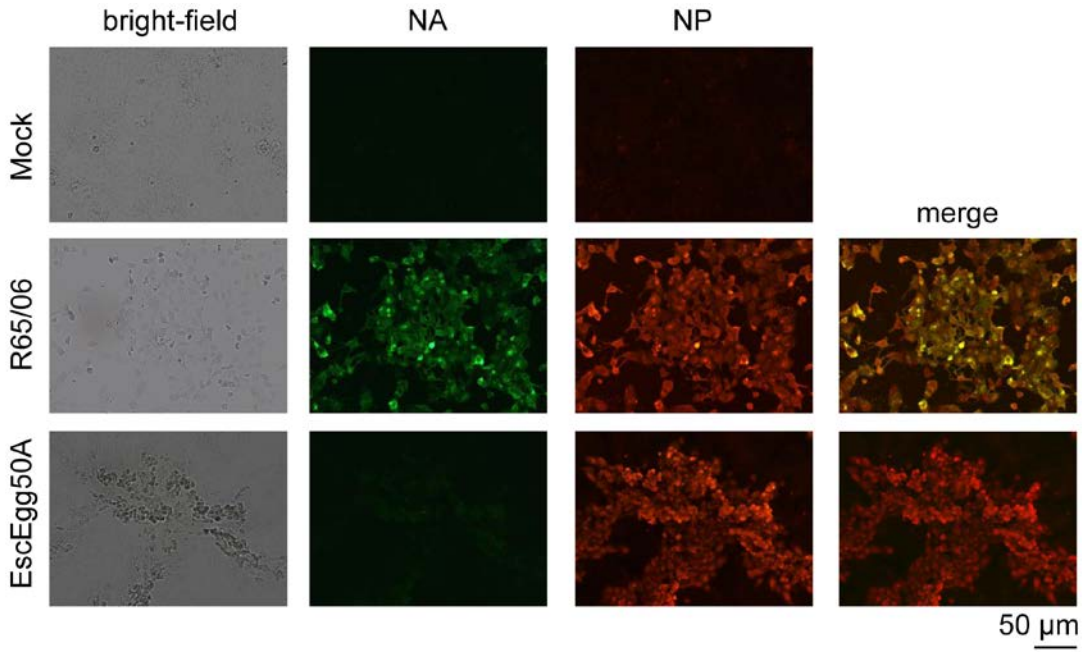

Figure 2: Microscopic analysis of MDCK cells infected with the ancestor virus R65/06 or EscEgg50A. For immunofluorescence, cells were stained 48 hours post infection with monoclonal antibodies specific for NA or NP.

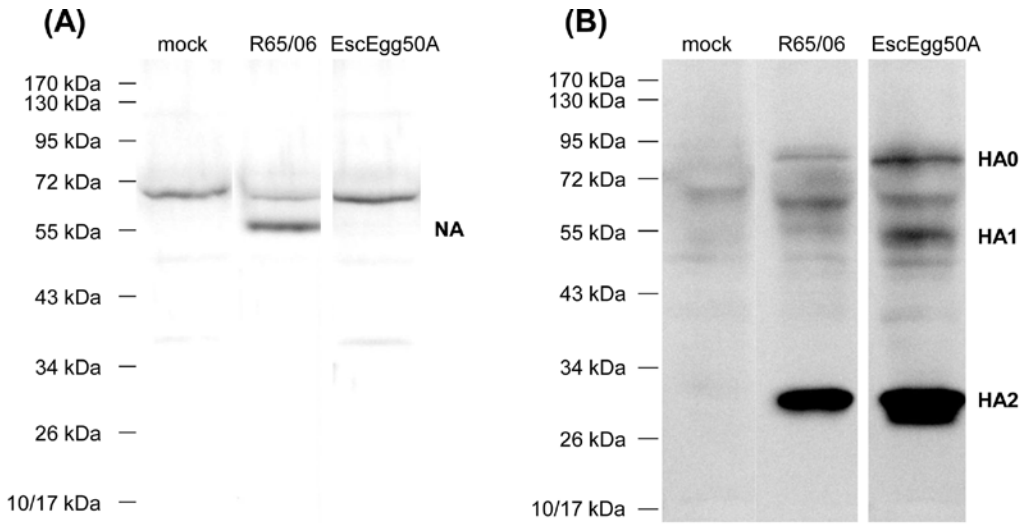

Figure 3: Detection of the hemagglutinin and neuraminidase protein by Western blot analysis. Cells were infected with EscEgg50A or the ancestor virus R65/06 and lysates for Western Blot analysis were collected 48h post infection. Both gels were loaded with equal proteins amounts and run under equal conditions. Molecular masses of the marker proteins are indicated. (A) Detection of the neuraminidase protein. (B) Detection of the hemagglutinin precursor HA0 and its processing products HA1 and HA2.

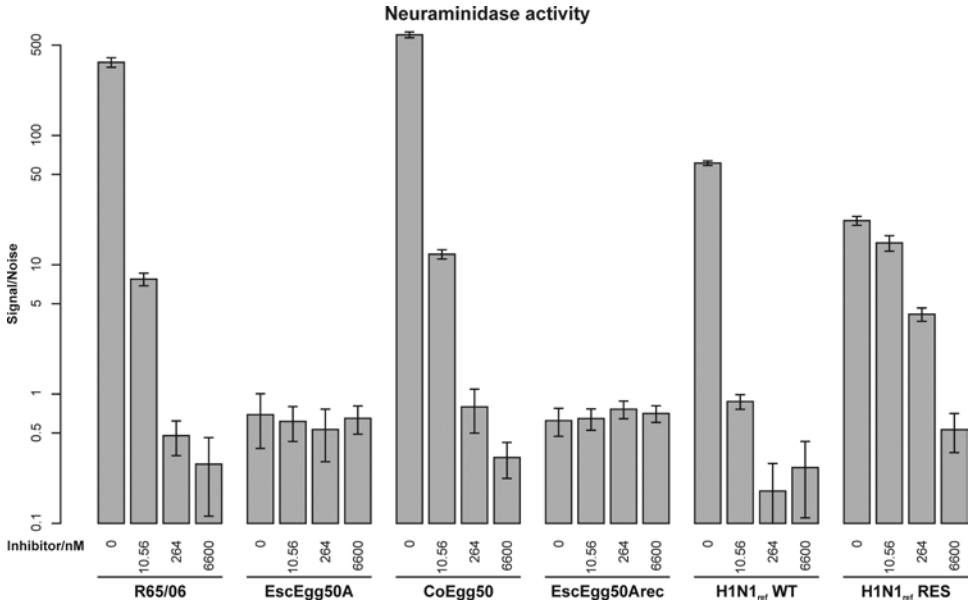

Figure 4: Measurement of neuraminidase activity. Virus preparations of R65/06, CoEgg50, EscEgg50A, EscEgg50rec, and two reference viruses of the subtype H1N1 were adjusted to an equal genome load and tested using the NA-XTD™ Influenza Neuraminidase Assay Kit. Three different concentrations of the neuraminidase inhibitor oseltamivir carboxylate were used. Except for EscEgg50rec (four technical replicates from one biological replicate) signal/noise values are determined from two biological replicates with four technical replicates each. Error bars indicate the standard deviation.

Replication competence of EscEgg50A does not require any compensatory mutations or a functional NA protein, but a polybasic HA cleavage site

A reverse genetics system of HPAIV H5N1 strain R65/06 (19) was used to analyze the role of potential compensatory mutations. While a virus composed of only seven segments could never be generated, the ancestor virus containing the R65/06 segments HA, NP, M, NS1, PA, PB1, and PB2 together with the EscEgg50A NA gene was readily reconstituted (EscEgg50Arec). Furthermore, the measurement of its neuraminidase activity demonstrated the loss of a functional N1 protein (Fig 4). Therefore, none of the 12 additional AA variations within the other segments of EscEgg50A were necessary to enable replication and growth in the absence of a functional neuraminidase, however they do assist to the viral replication (Fig 5). Moreover, we constructed a recombinant virus consisting of 7 segments (HA, NP, M, NS1, PA, PB1, PB2) from the ancestor R65/06 strain combined with the EscEgg50A segment NAATG-, in which the original start codon was silenced. Full replication competence of the NAATG- virus in cell culture demonstrated the non-essential character of the remaining coding information of the truncated segment 6 within EscEgg50A. Furthermore, a recombinant virus expressing EGFP from a minimal segment 6 (118 N-terminal nucleotides and 77 C-terminal nucleotides) was generated (data not shown) confirming the results observed with the NAATG- virus. In order to rule out that compensatory mutations occurred within the HA after transfection, the HA sequence of the rescued virus EscEgg50Arec was determined by classical Sanger sequencing. The HA sequence (nt 9-1704) of the rescued EscEgg50Arec virus was identical to the HA sequence of the ancestor virus R65/06. It has previously been shown for the same H5N1 strain, that exchanging the polybasic HA cleavage site motif RRRKKR/G to the monobasic motif ETR/G (but possessing the competent NA) resulted in nearly identical multi-step titers in the supernatant of MDCK cells in the presence of trypsin (28). Therefore, the ability to replicate and spread from infected cells is not impaired, as long as trypsin is provided.

To evaluate the impact of the polybasic HA cleavage site, we generated a recombinant virus which carried the EscEgg50A NA gene together with a monobasic HA cleavage site. Because a NA-negative monobasic HA virus could not be generated, we suggest, that a polybasic HA protein may be a prerequisite for NA-independent replication of an H5 influenza virus.

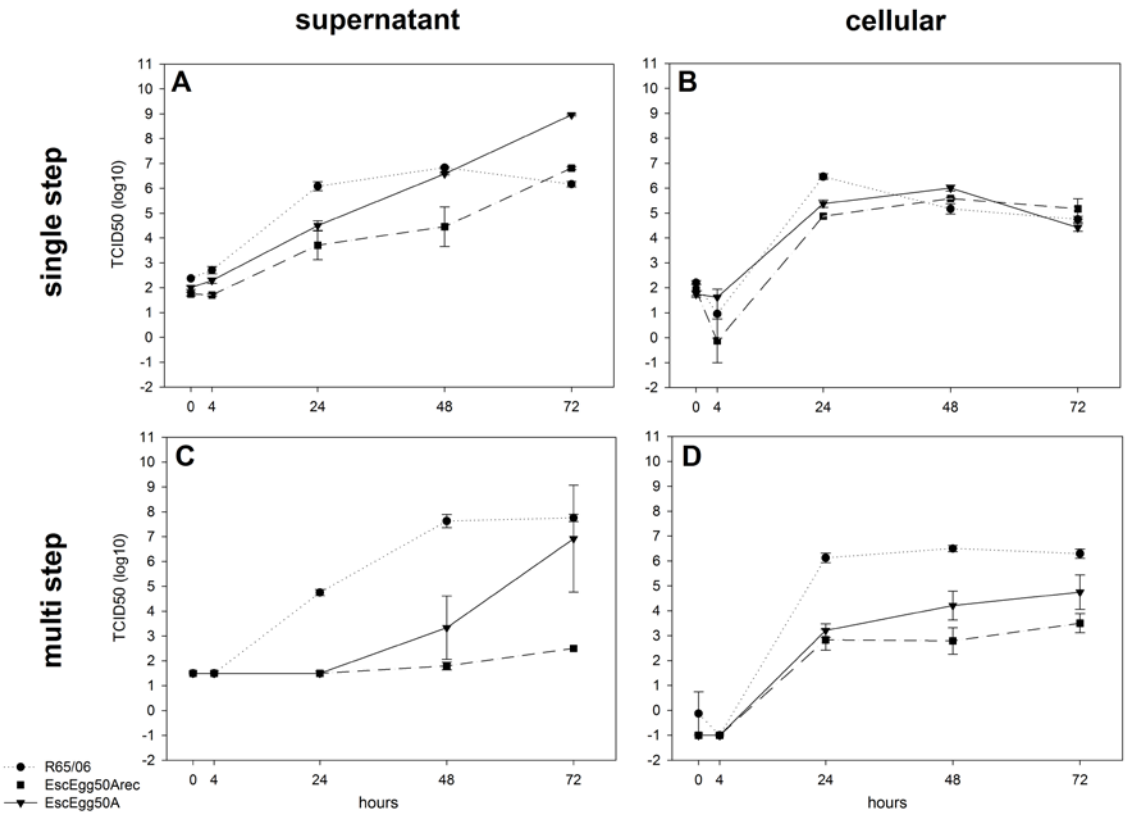

Figure 5: Growth properties of R65/06, EscEgg50A and EscEgg50Arec. For single-step growth kinetics, MDCK-cells were infected at an MOI of 1 (A, B), for multistep analysis MOI 0.01 was used (C, D). Cell culture supernatant (extracellular, A, C) and cellular (cell-associated, B, D) fractions were collected at the indicated time points. Viral titres were determined by titration. The results are mean values of three independent experiments. Error bars indicate SEM.

EscEgg50A and EscEgg50Arec exhibited significantly lower neutralization titers

Viral escape due to the neutralizing activity of the used polyclonal serum was expected as a consequence of the imposed immune pressure. Therefore, the neutralization of the different viruses was quantified by a standard virus neutralization assay. Ancestor virus, CoEgg50, EscEgg50A and a recombinant virus with the ancestor backbone and the EscEgg50A NA protein ("EscEgg50Arec") were tested in comparison. In addition, neutralization tests were carried out with a second polyclonal serum (serum B) from an H5-vaccinated and H5N1-challenged chicken. All tested viruses were neutralized by the passage-serum (serum A) and the second individual serum (serum B), however significantly lower neutralization titers were observed for the group of neuraminidase negative viruses (Fig. 6). Interestingly, applying one-way ANOVA analysis, individual neutralizing titers of the sera against the different viruses did not differ significantly

(Fig 6). These results indicate a more efficient neutralization capacity of all immune sera against viruses encoding a neuraminidase protein.

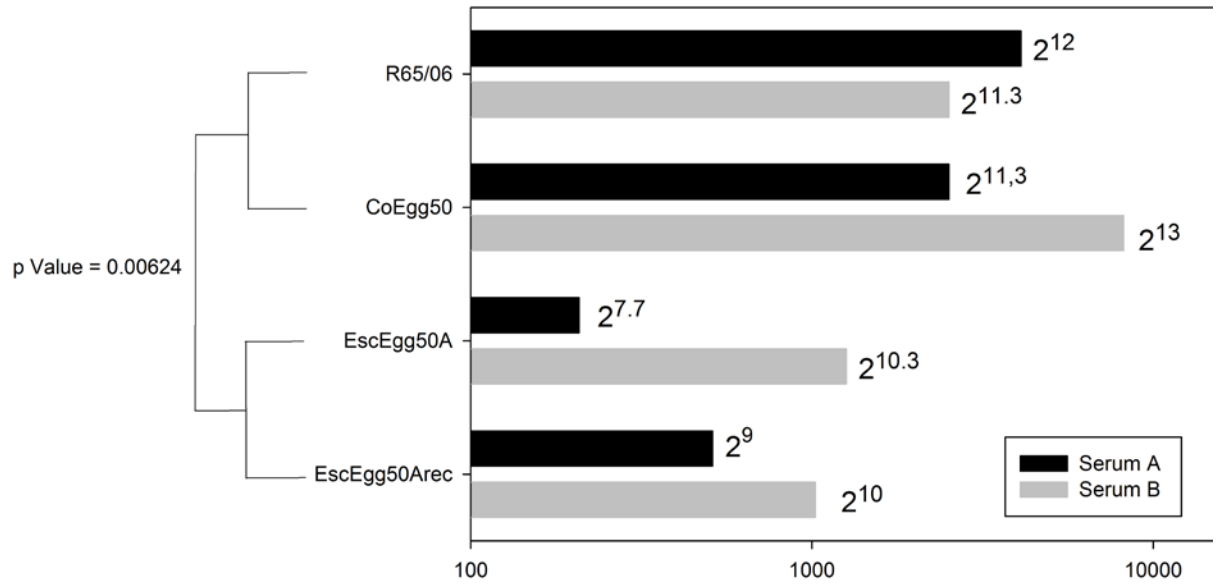

Figure 6: Neutralizing activity of the serum used to generate EscEgg50A serum (A) and a second serum (B) as determined by a standard virus neutralization assay. The numerical values of the neutralizing activity are also displayed as log2 values. Results were statistically evaluated by the one way ANOVA analysis. p-value represent the significance of the serum neutralization of viruses lacking NA activity (EscEgg50A, EscEgg50Arec) in comparison to the viruses possessing NA activity (R65/06, CoEgg50).

Generation of additional H5N1 segment 6 variants (EscEgg50B and EscEgg50C)

To evaluate whether the induction of large deletions and rearrangements of the neuraminidase coding segment is repeatable, we passaged the virus from the 30th passage of R65/06, which was one of the latest passages tested positive for the parental NA and negative for the rearranged NA (Table 3), under the selection pressure of two different chicken sera (sera B and C) and obtained the two additional escape variant viruses EscEgg50B and EscEgg50C. Partial deletion and intra-segmental as well as inter-segmental recombination of segment 6 sequences were detected in the two additional variants (Fig. 1B and C). EscEgg50B exhibited one large deletion of 950 bp within the NA gene (accession EPI383000), while both termini of the segment remained nearly identical to the ancestor sequence (Fig 1, Table 2). The open reading frame

encoded the first 55 AA of the ancestor neuraminidase, followed by three heterologous AA and a stop codon.

The segment 6 sequence of virus EscEgg50C started with 118 nucleotides identical with the ancestor sequence, followed by a short sequence (30 nt) with 100% homology to a sequence from segment 4 of the ancestor virus, followed by sequences from the 3´ terminus of segment 6 again identical to the ancestor virus (Fig. 1, Table 2, accession EPI383001). The AA sequence encoded by EscEgg50C shared 100% homology to the ancestor sequence for the first 32 AA, followed by further 17 heterologous AAs.

Overall, independent truncation and segment shuffling events of segment 6 occurred repeatedly during parallel passaging of an HPAIV H5N1 virus under multiple immunogenic pressures.

	RT-qPCR [C_q-value]			Sequences	
	H5*	N1*	EscEgg50A-NA-specific†	Master sequence [number of nucleotides]	PA-Insertion
Ancestor virus (R65/06)	13.0	15.4	neg	1396	neg
CoEgg50	13.6	12.2	neg	nd	nd
EscEgg30	17.6	20.7	neg	1235	neg
EscEgg35	19.4	22.5	neg	1235	pos
EscEgg36	22.2	25.2	neg	1235	pos
EscEgg37	19.1	neg	21.3	678	pos
EscEgg50	18.5	neg	20.7	678	pos

Table 3: Results of the detection of H5 and native and rearranged N1 sequences by RT-qPCR and sequence length of the master sequence estimated by GS FLX sequencing. Viral RNA detected by duplex RT-qPCR with results presented as quantification cycle (C_q)-values >35 scored as negative.
† Viral RNA detected by EscEgg50A-NA-specific RT-qPCR as described in the material and method section with results are presented as quantification cycle (C_q)-values >35 scored as negative.

The EscEgg50A variant is established after egg passage 36

In order to determine more precisely the passage in which the segment 6 re-arrangement took place, we designed an RT-qPCR assay based specifically on the EscEgg50A segment 6 sequences. With this specific RT-qPCR assay, EscEgg50A virus, the ancestor virus R65/06, and viruses isolated after intermediate passages were analyzed. As a control, every RNA-preparation was tested with H5- and N1-specific duplex RT-qPCR assays in parallel (Table 3). Assessment of the Cq-values demonstrated that sequences specific for the finally deleted and rearranged NA of the EscEgg50A variant first occurred after passage 36 (Table 3). Interestingly, mixed populations of the original and the rearranged sequences were not detected by this sensitive assay (Table 3) suggesting minute frequencies of viral variants. To shed light on the sequence distribution over subsequent passages, segment 6 of the ancestor virus, EscEgg50A, and the intermediate passages were amplified with segment 6 specific primers and subjected to next-generation-sequencing using the GS FLX. Remarkably, already the ancestor population comprised a certain small portion of shortened sequences besides the full-length master sequence (1396 nucleotides); and every successive population retained these minor fractions. The master sequences determined from the 30th passage comprised 1235 nucleotides caused by two short deletions. Within the sequences detected from the 35th and 36th passage, the insertion of the segment 3-derived sequences, i.e. sequence shuffling, could be found in trace amounts, while the master sequences still resembled that one from the 30th passage. From passage 37, the master sequence was identical to the segment 6 sequence of EscEgg50A, being 678 nucleotides long with segment 3 fragments inserted. We therefore concluded that EscEgg50A is a replication-competent virus without neuraminidase activity, having become predominant in the viral population after passage 36.

In-vitro characterization of the NA-deleted variants displaying an altered growth phenotype

In order to examine the effect of the NA-deletion on viral cell-to-cell-spread (ctcs), diameters of 50 plaques for the ancestor virus, the escape variant EscEgg50A and the recombinant EscEgg50Arec were measured and mean diameters and standard deviations were calculated. Values for the parental HPAIV H5N1 strain R65/06 were set to 100 %, and the plaque diameters

observed for the mutant viruses were expressed relative to this value (Fig. 7). Deletion of a functional NA protein resulted in a 93% reduction in plaque diameter (Fig. 7).

Furthermore, hemagglutination using standard protocols could not be demonstrated for any of the NA-negative mutant viruses in contrast to the wild-type (data not shown).

To evaluate the growth characteristics of the EscEgg50A mutant further, viral replication in cell culture was analyzed. EscEgg50A replicated in cell culture in the absence of exogenous sialidase and showed a characteristic growth phenotype of prominent 3-dimensional cloggy structures representing aggregates of infected cells (Fig. 2). In contrast, the parental strain R65/06 induces an influenza typical plaque formation in the cell monolayer. Growth kinetics were determined for the ancestor virus R65/06, the escape variant EscEgg50A and a reconstituted recombinant virus EscEgg50Arec in one-step and multi-step assays. After inoculation using an MOI of 1, a growth delay of the EscEgg50A and EscEgg50Arec variants until 48h post infection was observed for the cell culture supernatant, i.e. released virus, and viral titers at 72 h post infection exhibited similar values as the wild-type HPAIV H5N1 (MOI 1, Fig. 5 A). Multistep kinetics revealed a much more prominent delay of viral growth, especially for EscEgg50Arec (Fig. 5 C). Since EscEgg50Arec, which consists of the rearranged NA in the background of the parental virus R65/06, seem to have an impaired spread, we speculate that additional aa substitutions in EscEgg50A may contribute to the virus spread, which are not present in EscEgg40Arec (Table 1). Viral titers from cell lysates showed no marked replication differences (MOI 1, Fig.5 B), or were reduced by 10fold (MOI 0.01 EscEgg50A) or 400fold (MOI 0.01 EscEgg50Arec, Fig 5 D), respectively. Taken together, our data demonstrate a clear effect of the used MOI which is consistent with the observed markedly reduced cell-to-cell-spread ability of the NA-deleted variants. In addition, virus release might be delayed, but is not markedly influenced by the NA-deletion at high MOIs.

In order to examine the effect of exogenous sialidase onto the growth characteristics, we cultured the ancestor virus R65/06, the escape variant EscEgg50A and the recombinant virus EscEgg50Arec in the presence of Clostridium perfringens neuraminidase. Interestingly, supplementation with that bacterial sialidase resulted in significantly improved viral titers for the NA-negative variants as well as for the ancestor virus (Fig. 8A). However, that titer increase

was more pronounced for the NA-negative viruses tested: EscEgg50A 1600fold and for the recombinant virus EscEgg50Arec 870fold (Fig. 8B). Therefore, the used bacterial sialidase was able to compensate the growth deficiency of the NA-negative viruses.

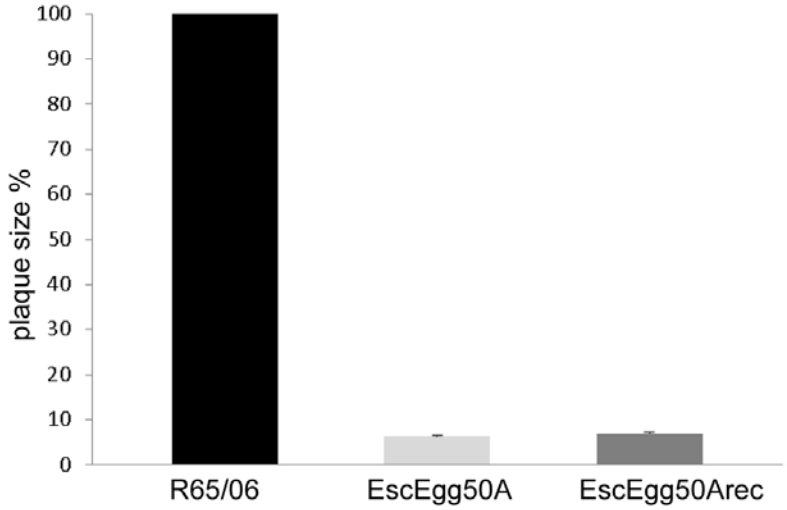

Figure 7: Plaque sizes of R65/06, EscEgg50A and EscEgg50Arec. MDCK cells in six-well plates were infected at an MOI of 0.01 and overlaid with agarose for 24 h. The plaque diameters of 50 randomly selected plaques were determined. The average diameter of plaques formed by the wild type strain R65/06 was set to 100 %. Error bars indicate SEM.

	NP Elisa‡	HA5 Elisa‡	NA1 Elisa‡	Challenge survivors
EscEgg50A†	4/10	3/10	0/10	3/10
EscEgg50B	5/10	4/10	0/10	6/10
EscEgg50C	2/10	1/10	0/10	1/10

Table 4: Serology after oronasal application of EscEgg50A, B, or C
† 14 days after inoculation and before challenge infection
‡ positive/total tested

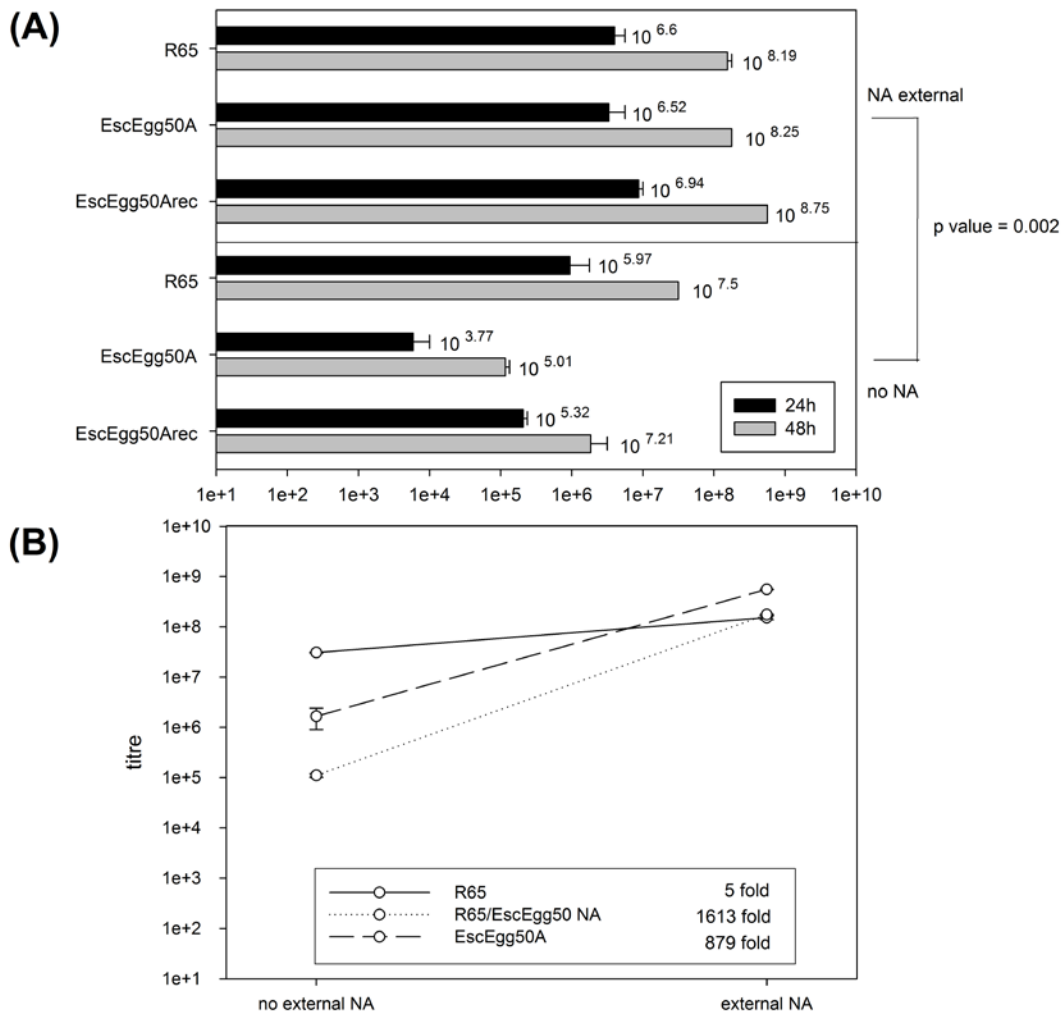

Figure 8: Viral titers after supplementation of external bacterial sialidase. MDCK-cells incubated with and without bacterial neuraminidase were infected at an MOI of 0.1 with R65/06, EscEgg50A and EscEgg50Arec. Cell culture supernatant was collected after 24h and 48h incubation time and viral titers were determined by titration (A). The increase of viral titers effected by the bacterial neuraminidase is depicted in (B). The results are mean values of two independent experiments. Graphs and statistical analyses were performed using SigmaPlot (Windows Version 11.0; Build11.2.0.5; Systat Software Inc.). The p-value of different titers from virus cultured with or without external neuraminidase was determined using the analyses of variance by Kruskal-Wallis.

The neuraminidase negative variants are fully attenuated

To assess changes in virulence of EscEgg50A, EscEgg50B, EscEgg50C, or CoEgg50, we determined the intravenous pathogenicity index (IVPI) in chicken (22). With an IVPI of 2.97, the ancestor virus is classified as highly pathogenic (29) meaning that every animal succumbed to the disease within three days. The control virus CoEgg50 was also demonstrated to be an HPAIV with a very close IVPI of 2.55. In contrast, the IVPIs of all three NA-negative variants, EscEgg50A, EscEgg50B, and EscEgg50C, were 0, i.e. none of the chickens became sick. Therefore, despite the presence of the unchanged polybasic HA cleavage site, being the major molecular marker of HPAIV, the EscEgg50A, EscEgg50B, and EscEgg50C mutants are unequivocally classified as low-pathogenic. Almost all chicken sera (27 out of 30 animals) were scored positive by NP- and H5-antibody ELISA indicating occult infection whereas all those individual sera were tested negative for N1-specific antibodies.

To simulate natural infection via the respiratory tract, groups of ten chickens were infected oronasally with the three different EscEgg50 viruses. None of those birds showed any clinical symptoms. In contrast, all CoEgg50-infected animals succumbed to death within six days. Four out of ten EscEgg50A-inoculated chickens had a positive antibody reaction in an NP-specific ELISA, and 3 of them were also positive in an H5-specific antibody ELISA (Table 4). Five chickens inoculated with EscEgg50B seroconverted against NP, and 4 of them reacted also against H5. From the group of chickens inoculated with the EscEgg50C variant only two seroconverted against NP and one of these scored also positive within the H5-Elisa (Table 4). Fourteen days after inoculation of EscEgg50 mutants, all chickens were oronasally challenged with a lethal dose of the ancestor virus, the HPAIV R65/06. Remarkably, all chicken scoring positive for H5 antibodies survived the challenge infection asymptomatically, while all other animals died (Table 4).

Those results suggest a reduced infection efficacy of the EscEgg50 mutants via the oronasal route. Overall, the EscEgg50 viruses exhibit an apathogenic phenotype in chickens of 6 weeks of age.

3.5. Discussion

Continuous circulation of HPAIV H5N1 viruses in poultry and wild birds with repeated spillover to humans is reported from South-East-Asian countries and Egypt despite extensive vaccination campaigns or eradication programs in place. Antigenic drift variants have arisen in immunized, not fully protected animals and hamper vaccine-based eradication strategies. In this study, we aimed to model influenza A virus immunoescape closer to the in-vivo situation by egg-passaging an H5N1 HPAIV under the more authentic multifarious selection pressure of a polyclonal serum from individual chickens, since cell culture systems may not simulate the real situation of vaccinated flock herds with thousands of birds. However, to assess the enormous genetic plasticity of influenza viruses, we intended to implement an antigenic drift model based on repeated passaging in the presence of polyclonal immune-sera in embryonated egg culture. During egg passages, immunogenic pressure results in emergence of progeny viruses with mutations with an altered antigenic pattern (30). In vitro selection in presence of monoclonal antibodies and polyclonal (rabbit or mice derived) antisera, was utilized to identify several antigenic epitopes of the hemagglutinin (8, 31-34) and the neuraminidase (35). Using polyclonal chicken sera in a cell culture system (10), we recently obtained escape variants whose variations reflect immunoescape beyond the major antigenic HA epitopes affecting several viral proteins. Surprisingly, our repeated long-term passaging experiments resulted in the NA-deleted virus variants derived from HPAIV H5N1, hitherto never described before.

Although the sera used for selection showed HI-titers similar to sera from field studies or similar experiments (12,13), there were no relevant changes in the HA sequence. Despite the dogma that antibodies against the neuraminidase were non-neutralizing and of lower relevance (36), the changes observed within segment 6 were significant and a functional NA protein was no longer expressed. On the other hand, the neutralization data suggest some effect of the observed segment 6 changes on the neutralizing capacity of the tested polyclonal antisera. Repeated passaging resulted in similar, but different NA variants. One possible explanation for the emergence of NA-deleted virus variants during passaging is an indirect effect onto the

neuraminidase gene, since neutralization might be circumvented by limiting the release of free floating virions but spreading instead via cell-to-cell-spread (37).

Furthermore, since the EscEgg50A virus evolved only one AA substitution within the HA sequence after 50 egg passages, a stabilization of the HA sequence and some kind of immunoevasion due to the loss of the corresponding neuraminidase protein has to be taken into account. The minor role of the HA variation is further proven by the not markedly different neutralizing data for the EscEgg50Arec virus, which has the same HA segment as the ancestor virus. Furthermore, the antisera A, B, and C were each able to efficiently select the three different neuraminidase-negative virus variants EscEgg50A, B, and C. It could be also demonstrated that a direct inhibition of the neuraminidase function by antibody-positive sera is possible as demonstrated with a neuraminidase assay. However, the N1-specific antibody ELISA scored negative for the sera tested (B and C), thus rendering high amounts of N1-specific antibodies unlikely, but does not exclude neuraminidase antibodies at lower titers because the ELISA used is directed against a single epitope only. Therefore, the exact selection mechanism remains unclear, but the role of possible factors like neuraminidase-specific antibodies will be further investigated in future studies.

The H5-virus variants we detected after 50 egg passages under pressure with an antiserum are unique and are focused on segment 6. Extensive deletions and rearrangements were ascertained exclusively in the segment 6. Interestingly, the new segments 6 resulted from complex sequence shuffling and two out of three variants had insertions of a very short sequence fragment originating from other segments. Therefore, recombination events are an underestimated mechanism of sequence variation in influenza viruses (38). Characterization of the novel variants confirmed the complete loss of the neuraminidase protein and of any neuraminidase activity. Recombinant viruses with a deleted ATG sequence or the insertion of EGFP proved that even the N-terminal residual peptide encoded by the truncated segment 6 of the EscEgg50A virus is not essential for virus replication.

In previous reports, deletion of major parts of segment 6 was seen only after virus passaging in the presence of an exogenous sialidase (39-41) or after passaging of H3N2 viruses on MDCK cells (40). However, in the latter case, and in contrast to our NA-deleted variants, attempts to isolate

a neuraminidase negative virus strain were not successful and the authors therefore suggested that full-length segment 6 remained in the virus population at a lower frequency. A complete loss of a neuraminidase encoding segment or function is deemed impossible (42) with the only example of a human H3 isolate which was claimed to lack the complete segment 6 and addressed as seven-segmented Influenza A virus (43). Alternatively, growth of neuraminidase negative influenza A virus without supplementation of exogenous sialidase was demonstrated in vitro and in mice only if the loss of neuraminidase activity was accompanied by mutations around the HA receptor-binding pocket that lower avidity for receptors (44, 45). Since a recombinant virus composed of 7 ancestor virus R65/06-specific segments in combination with the segment 6 of the escape variant EscEgg50A was generated by reverse genetics, the role of compensatory mutations is negligible here. Replication competence could be demonstrated and the HA sequences did not indicate any compensatory mutations. Therefore, replication competence despite a truncated segment 6 is achievable without any additional compensatory changes in the HA protein in the presence of a polybasic cleavage site. Thus, the NA of HPAIV H5N1 may not be essential for virus replication and assembly but is necessary for efficient cell-to-cell-spread and growth at low MOIs.

The neuraminidase deletion apparently provided an advantage as the viral population shifted to neuraminidase-truncated viruses on embryonated eggs as was demonstrated by predominance of the EscEgg50A-specific sequences in the viral population from passage 37 on (Table 3). This is in accordance to inter-segmental recombination events within segment 6 after passaging a segment 6 stalk deletion mutant in egg culture, where recombination events occured within one passage step (38). Sequence variation of segment 6 could be verified as early as passage 30, leading to a truncated form of segment 6. In addition, rigorously shortened versions of segment 6 could be detected in every passage. Since under von Magnus conditions, influenza viruses are known to produce defective interfering (DI) particles carrying mostly a large deletion in the P genes (46) and maintained in the population by co-replication. However, it is a novel finding that such a DI-segment can replace the full-length counterpart entirely as demonstrated by reconstitution of a recombinant EscEgg50A. Mechanistically, such sequence rearrangements were postulated to be due to viral polymerase jumping across the ends of hairpin RNA

structures (42), which might be to some extent the underlying reason for the observed truncation, shuffling, and recombination events within segment 6.

Like previously described NA variants (40), EscEgg50A segment 6 retained the sequences encoding the cytoplasmic tail together with the transmembrane region of the NA protein, and in addition, the non-coding sequences at both the 3’ and the 5’ segment ends, required for efficient incorporation of the viral genome into budding particles (47).

One possible source of a sialidase enzyme in our experiment could have been the embryonated egg itself (48). However, the control virus, also passaged fifty times in egg culture, showed no variation in the NA-sequence implying that negative selection by the polyclonal serum was necessary to force the NA deletion. Furthermore, EscEgg50A, -B, and -C viruses replicate in cell cultures without any exogenous neuraminidases refuting a determining role of the egg neuraminidases for generation of the NA-truncated mutants.

Growth analysis of the NA-deleted viruses demonstrated a delay of replication, but viral titers achieved after 72h from MOI 1 infection were clearly in the range of the ancestor virus of about 108 TCID50/ml. Despite of high viral titers within the supernatant, the NA-negative viruses displayed in vitro two marked differences in comparison to the ancestor virus: they exhibited no hemagglutinating activity at all, and viral cell-to-cell-spread (ctcs) was drastically reduced. NA-negative viruses are known to lose the hemaglutinating activity (38, 49), and the lack of desialysation of the HA-protein (50) likely explains this phenomenon. In addition, the functions of HA and NA have to be orchestrated (38), and therefore, NA-negative viruses may reduce HA binding affinity. Whether the impairment of ctcs is due to impaired HA-NA cooperation, remains an open question. An NA-effect on plaque sizes was observed earlier (51). The altered plaque phenotypes in cell culture exhibiting cloggy like structures of tightly agglomerated cells were likely a result of the impaired ctcs where plaques are of a very small size.

Despite the multibasic cleavage site within the HA protein, which is typical for highly pathogenic viruses, EscEgg50A exhibited an apathogenic phenotype in vivo. The deletion of the neuraminidase obviously led to complete attenuation of the virus in 6 weeks old chickens; furthermore, oronasal infection was not successful in the majority of the inoculated animals.

As one proposed function of the neuraminidase protein in the airways is cleavage of complex substrates (mucins) to mediate access to target cells and virus release from infected cells (52), the markedly reduced infectiousness of the EscEgg50 variants in the oronasal-infection model seems reasonable. Furthermore, the drastically reduced ctcs corresponds to the in vivo phenotype of the NA-deleted viruses. We therefore conclude that the neuraminidase protein is not essential for in vitro growth of HPAIV H5N1-derived viruses, but is required for host entry and probably spread within the host, and is therefore a substantial virulence factor in vivo. Overall, such attenuated H5N1 viruses carrying large deletions in their NA gene segments may serve as unique tool to study the role of the neuraminidase for virus assembly, growth, and pathogenesis.

Furthermore, the deletion of the neuraminidase provides a new approach for attenuated life vaccines. Provided that wild-type virus infections cause a serological response to the NA detectable in vaccinated animals, an NA-deficient virus would be a perfect vaccine candidate, enabling the “differentiating infected from vaccinated animals” (DIVA) concept. The present limitation of such a vaccine strain would be still the highly pathogenic genotype of the HA-segment, which is essential for efficient growth of the NA-deleted H5-viruses. This HA gene might be delivered to circulating low-pathogenic viruses, generating a novel highly pathogenic strain. Therefore, the essential requirement of a polybasic HA cleavage site for efficient growth of NA-deleted H5-viruses remains to be studied further. A future milestone would be the construction of high-producer NA-deleted H5 or H7 strains without polybasic HA cleavage site for usage as attenuated live vaccines with high safety.

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4. A model for early onset of protection against lethal challenge with highly pathogenic H5N1 influenza virus

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Vaccine **JVAC-D-13-01881R1**

4.1. Abstract

Highly pathogenic avian influenza viruses (HPAIV) H5N1 sporadically cause severe disease in humans and involve the risk of inducing a pandemic by gaining the ability for human-to-human transmission. In naïve poultry, primarily gallinaceous birds, the virus induces fatal disease and the used inactivated vaccines occasionally are unable to provide efficient and early onset of protection. Therefore, optimized vaccines must be developed and evaluated in model systems. In our study, we tested a novel H5 neuraminidase-deleted influenza A virus variant to analyze the induction of a very early onset of immunity. Ferrets, mice and chickens were each immunized with a single vaccine dose seven, three and one day before lethal challenge infection, respectively. Sound protection was conferred in 100 % of animals immunized seven days prior to challenge infection. In these animals, no clinical signs were observed, and no challenge virus RNA was detected by real-time RT-PCR analyses of swabs, nasal washings, and organ samples. Moreover, the attenuated modified-live virus variant protected all chickens, mice, and ferrets as early as three days after vaccination against severe clinical signs. Chickens and ferrets developed hemagglutinin-specific antibodies after seven days, but no neuraminidase-specific antibodies, making this kind of neuraminidase-negative strain suitable for the DIVA ("differentiating vaccinated from infected animals") strategy.

4.2. Introduction

Highly pathogenic avian influenza virus (HPAIV) of subtype H5N1 continues to pose a serious threat to poultry in several Asian and African countries. Huge economic losses are due to high mortality rates in infected gallinaceous birds as well as harsh restriction measures including stand-still and stamping out campaigns. In addition, spill-over transmission of HPAIV H5N1 from animals to humans caused more than 600 human infections worldwide with a case fatality rate of nearly 60% (WHO update April 2013). Therefore, control and eradication of HPAIV H5N1 is of utmost importance, even more so since the risk of an evolving highly pathogenic human-to-human transmissible H5N1 virus [1] would be reduced.

Ultimate eradication of viral diseases such as terrestrial rabies in Western Europe or Rinderpest on a global level was achieved using effective live vaccine preparations for veterinary use. Since the host reservoir of avian influenza viruses, aquatic wild birds, cannot be drained [2], eradication of endemic circulating influenza viruses or sporadic outbreaks in poultry holdings via stamping out strategy and vaccination campaigns was successful e.g. in Mexico [2], and should in principle be possible. Nevertheless H5N2 LPAIV strains are circulating in Mexico until now and vaccine campaigns lead to antiviral drift in field viruses. In summary, none of the vaccine candidates against influenza infection combines the postulated characteristics of a perfect vaccine, such as long-lived immunity, needle-free administration, single-dose protection and especially the early onset of immunity and protection against clinical signs and virus shedding [3].

In the veterinary field, and especially in the poultry sector, mainly inactivated preparations are used for influenza virus vaccination, and only a minority of vaccines is based on modified live vector vaccines originating from recombinant fowl pox, Newcastle Disease [4] or Turkey herpesvirus (HVT) [5]. In addition, most of these preparations are recommended for a prime-and-boost application which is difficult to achieve in target species with a very high population turn-over rate such as broilers.

The majority of human seasonal influenza vaccines are based on purified HA and NA formulations of currently circulating influenza A and B virus strains. Due to antigenic drift of the viruses these vaccines may have to be re-adjusted on an annual basis according to the recommendations of a panel of WHO experts [6]. This ensures an optimal antigenic match between vaccine field viruses, which is a prerequisite for high vaccine efficacy.

In contrast, besides a humoral immune response modified live influenza vaccines also stimulate an enhanced mucosal and cell-mediated immunity. They can also induce immunological cross reactions against heterologous strains [7, 8].

A key issue for an optimized vaccine against avian influenza in poultry and humans would be the fast induction of an early protection. So far, attempts to induce an early protection in the mouse model have been successful with live virus vaccines [9] or live vector vaccines [10]. Interference induced by the vaccine virus [9] as well as antiviral cytokine reactions followed by adaptive immune responses are discussed to synergistically mediate early protection [9, 10]. Modified live influenza vaccine preparations also present an opportunity for prepandemic vaccines. Several authors have demonstrated the general efficacy in animal models [11, 12].

Here, we focused on studies to induce a very early onset of protection against HPAIV H5N1 infection using a single application of a novel replication-competent H5 neuraminidase deletion mutant virus ("EscEgg50A")[13] in chickens, mice and ferrets.

4.3. Materials and Methods

Viruses:

The HPAIV neuraminidase-deleted mutant ("EscEgg50A") [13] was used for immunization experiments. As described by Kalthoff et al. [13] the EscEgg50A mutant is a NA-deleted H5 virus, arisen from 50 passages in 9- to 11-day-old embryonated egg culture together with an antiserum. The serum originated from chickens immunized with an H5N2 vaccine. Compared to the ancestor virus, the mutant lost the complete NA encoding region, the protein and the enzymatic activity, but only 12 amino acid substitutions were detected in the other segments. The virus is highly attenuated in chickens and apathogenic for 6-week-old individuals, although

the multibasic cleavage site of the HA segment is still present. Application doses and routes are described in the section “animals”.

Each challenge infection was performed by oronasal (o.n.) application of a lethal dose of 1 x 10^6 $TCID_{50}$ of the HPAIV strain A/Cygnus cygnus/Germany/R65/2006 (“R65”) to each individual animal. Both viruses are related to clade 2.2.2 [14].

Viruses were propagated in 9- to 11-day-old embryonated egg culture without further supplements.

Animals:

All groups of animals were given standardized names: “species / infection route / days before challenge infection”.

30 specific-pathogen-free six-week-old chickens (Lohmann Tierzucht, Cuxhaven, Germany) were immunized i.m. with a single dose of 1 x $10^{5.5}$ $TCID_{50}$ applying the same group scheme as for the mice with ten individual birds per group. The non-immunized control group consisted of four animals.

Six-week-old female BALB/c mice were purchased from Charles River (Charles River Breeding Laboratories, Sulzfeld, Germany) and kept in an Isocage system (Tecniplast, Buguggiate, Italy). The mice were immunized once with a dose of 1 x $10^{4.5}$ $TCID_{50}$ intraperitoneally (i.p.) or 1 x $10^{3.5}$ $TCID_{50}$ intramuscularly (i.m.) or o.n., respectively, seven days, three days or one day before challenge infection. One group per administration route served as “R65” infection control. Additionally, four mice were euthanized one day after o.n. immunization and four individuals were euthanized three days after immunization to estimate both, the immune response and possible viremia induced by the NA-deleted virus variant.

Six 9-week-old ferrets (in-house breeding program, Friedrich-Loeffler-Institut, free of influenza virus antibodies) were immunized by intranasal application of a single shot of 1 x $10^{4.5}$ $TCID_{50}$ NA-deleted “EscEgg50A” H5-strain. One animal served as negative environment control, while three animals were challenged without prior immunization, serving as infection control ferrets.

Mice and ferrets were immunized and infected under isoflurane anesthesia (CP-Pharma Handelsgesellschaft mbH, Burgdorf, Germany). During ten days after challenge, the animals

were checked daily for clinical signs. In addition, the individual body weight of the mice was measured. Any severe signs, especially loss of > 25 % of body weight (mice) or exhibition of neurological signs were endpoint-criteria and affected animals were euthanized.

All animal experiments were conducted in biosafety level 3+ containment facilities at the FLI and were carried out in accordance with the German Animal Welfare Act, approved by the Committee on the Ethics of Animal Experiments of the Federal State of Mecklenburg-Western Pomerania (registration and approval number LALLF MV/TSD/7221.3-1.1-003/07).

Samples:

Combined oropharyngeal/cloacal swab samples (Bakteriette, EM-TE Vertrieb, Hamburg, Germany) from chickens were taken daily. For this purpose, one swab was used first orally and then cloacally. Swabs were suspended in 2 ml of Dulbecco's modified Eagle medium supplemented with 5 % fetal bovine serum, enrofloxacin 1 mg/ml, gentamicin 0.05 mg/ml, and lincomycin 1 mg/ml.

Nasal lavages using phosphate-buffered saline were taken every second day from individual ferrets under isoflurane anaesthesia, starting one day after immunization or infection, respectively.

At the end of the experiment lung and brain tissue samples were collected from all animals and suspended in 1 ml of medium supplemented with 5 % fetal bovine serum and Penicillin and Streptomycin (PenStrep® Gibco). One stainless steel bead (∅ 5mm) per organ sample was added and samples were homogenized in a 2ml collection tube for 2min in a TissueLyser instrument (Qiagen, Hilden, Germany). After centrifugation, RNA was extracted from supernatant using the Nucleic Acid and Nucleospin 96 RNA Tissue kit (Macherey-Nagel, Düren, Germany) on a Freedom Evo robotic platform instrument (Tecan, Crailsheim, Germany).

Viral RNA load was determined by real-time reverse transcription polymerase chain reaction (real-time RT-PCR). Amplification of a target sequence specific for the vaccine virus "EscEgg50A" was performed as described previously [13], while N1 and H5 specific amplification detection was performed after challenge infection [13]. For all sample matrices, analyses were performed over a total of 42 PCR cycles without additional cut-off.

Virus isolation was achieved on Madin–Darby canine kidney cells (MDCK, Collection of Cell Lines in Veterinary Medicine CCLV-RIE-1061) seeded in six-well plates (Costar® 6 Well Clear TC-Treated Multiple Well Plates, Corning Life Science, Wiesbaden, Germany). Cells were inoculated with 100 µl supernatant of the organ samples from the mouse experiment.

Serology:

Blood samples from chickens were collected via the ulnar vein, from mice during the euthanasia process, and from ferrets via the saphenous vein. Specimens were collected in Microtainer ® Brad Tubes (BD, Franklin Lakes, NJ, USA), in Monovette (Sarstedt, Nuembrecht, Germany), or Multivette R600® tubes (Sarstedt), respectively - and processed further according to the manufacturer's protocols. After inactivation at 56° C for 30 min, all sera were tested for nucleoprotein (NP), N1 and H5 specific antibodies using commercial ELISA assays (ID Screen® Influenza A Antibody Competition ELISA kit, ID Screen® Influenza N1 Antibody Competition ELISA kit, ID Screen® Influenza H5 Antibody Competition ELISA kit, all by ID-vet, Montpellier, France). Due to the limited volume of serum available from mice, these samples were only tested for the presence of NP-specific antibodies.

Hemagglutination inhibition assays (HI) were conducted as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [15]. The ancestor virus "R65" was used as antigen.

Statistical analyzes:

Statistical analyses (Fisher exact test) of the real-time RT-PCR data were performed using the R software package [16].

4.4. Results

Survival and clinical signs

Within ten days after immunization with the NA-deleted "EscEgg50A" H5-strain none of the animals exhibited any side effects and the strain proved to be apathogenic in the tested animals.

All animals of group “mice/i.p./1 dpi/3 dpi/7 dpi” survived the challenge infection, while all animals of group “mice/i.m./1 dpi/”, two animals of group “mice/i.m./3 dpi” and one animal of group “mice/i.m./7 dpi” succumbed to the challenge infection. “Mice/o.n./3 dpi/7 dpi” survived, whereas all mice of group “mice/o.n../1 dpi” died or had to be euthanized (Table 1). Two of the i.p. immunized mice showed only mild clinical signs including rough fur, mild depression, mucoid rhinorrhea and body weight loss of < 20 %. However, these animals recovered rapidly after four days. In contrast, animals which died or had to be euthanized and the non-immunized control group showed characteristic clinical signs including loss of body weight exceeding 20 % and neurological signs.

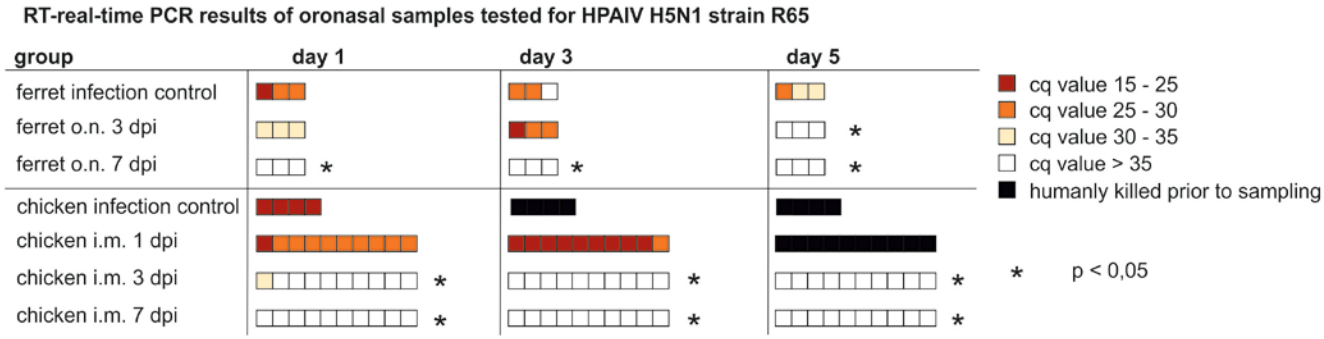

Figure 1: Real-time RT-PCR of oronasal samples tested for HPAIV H5N1 strain “R65”. Different colors show the range of Cq values. Each box represents one sample, i.e. animal. Data were tested for significant differences to the infection control groups with the Fisher exact test and a confidence level of 95 %. Groups without asterisks showed no significant differences in the statistical analyses.

groups	survival	clinical signs	NP antibodies b.c. - p.c.	H5 antibodies b.c. - p.c.	N1 antibodies b.c. - p.c.	RNA load organ	viral Excretion
ferret infection control	0/3	3/3	0/3 - 2/3	0/3 - 0/3	0/3 - 1/3	3/3	3/3
ferret o.n. 3 dpi	3/3	3/3	0/3 - 3/3	0/3 - 0/3	0/3 - 1/3	0/3	3/3
ferret o.n. 7 dpi	3/3	0/3	3/3 - 3/3	0/3 - 0/3	0/3 - 0/3	0/3	0/3
chicken infection control	0/4	4/4	0/4 - nd	0/4 - nd	nd - nd	4/4	4/4
chicken i.m. 1 dpi	0/10	10/10	0/10 - nd	0/10 - nd	0/10 - nd	10/10	10/10
chicken i.m. 3 dpi	10/10	0/10	1/10 - nd	0/10 - 9/10	0/10 - 0/10	6/10	1/10
chicken i.m. 7 dpi	10/10	0/10	10/10 - nd	10/10 - 10/10	0/10 - 0/10	0/10	0/10
BALB/c mice infection control	0/10	10/10	nd - nd	nd - nd	nd - nd	5/10	nd
BALB/c mice i.m. 1 dpi	0/3	3/3	nd - nd	nd - nd	nd - nd	2/3	nd
BALB/c mice i.p. 1 dpi	3/3	3/3	0/3 - 0/3	nd - nd	nd - nd	0/3	nd
BALB/c mice o.n. 1 dpi	4/4	0/4	nd - nd	nd - nd	nd - nd	3/4	nd
BALB/c mice i.m. 3 dpi	1/3	2/3	nd - nd	nd - nd	nd - nd	1/3	nd
BALB/c mice i.p. 3 dpi	3/3	3/3	0/3 - 0/3	nd - nd	nd - nd	0/3	nd
BALB/c mice o.n. 3 dpi	4/4	4/4	nd - nd	nd - nd	nd - nd	0/4	nd
BALB/c mice i.m. 7 dpi	2/3	1/3	nd - nd	nd - nd	nd - nd	0/3	nd
BALB/c mice i.p. 7 dpi	3/3	1/3	2/3 - 3/3	nd - nd	nd - nd	0/3	nd
BALB/c mice o.n. 7 dpi	4/4	0/4	nd - nd	nd - nd	nd - nd	0/4	nd

Table 1:

Summary of experimental results. Animals are listed as “number of animals positive/total number of animals in the trial”. Serum samples were harvested and tested for antibody response before challenge infection (b.c.) and after (p.c.). “nd” means no data available.

All chickens of group “chicken/i.m./3 dpi/7 dpi” survived the infection. Nevertheless, all animals in group “chicken/i.m./1 dpi” died or had to be euthanized after 4 days, one day later compared to the challenge infection control group. All individuals of group “chicken/i.m./3 dpi“ showed mild depression on two days post challenge infection (dpc), but were inconspicuous afterwards. The group “chicken/i.m./7 dpi” did not show any clinical signs at any time point (Table 1). All diseased animals exhibited severe depression, ruffled feathers, ataxia, pin-point hemorrhages on the feet and shanks and edema.

All immunized ferrets survived the challenge infection. In contrast, control animals exhibited clinical signs [17] e.g. lack of appetite, apathy or fever until 5 dpc, and had to be euthanized. Among the immunized animals, only the group “ferret/o.n./3 dpi” showed mild depression for three days after challenge infection, whereas all animals of group “ferret/o.n./7 dpi” stayed completely healthy (Table 1).

Analysis of swab and nasal washing samples

All swab samples from chickens as well as the washing samples from ferrets tested for the strain used for immunization (NA-deleted EscEgg50 A) viral RNA load scored negative.

Challenge virus RNA could be detected in the swab samples of group “chicken/i.m./1 dpi” and the control animals with cycle of threshold (Cq) values between 18.5 and 30.7, whereas Cq values in group “chicken/i.m./3 dpi” were markedly higher and ranged from 32.2 to 39.7 (Figure 1). Challenge virus RNA could not be detected in samples of the group “chicken/i.m./7 dpi”. Similarly, samples from group “ferret/o.n./7 dpi” scored negative for viral RNA. In contrast, in samples of ferret group “ferret/o.n./3 dpi” viral RNA was detected until three days after challenge infection (Figure 1).

Analysis of organ samples

The real-time RT-PCR analysis of organ samples collected from surviving mice, chickens as well as ferrets nine days after challenge infection showed no vaccine virus “EscEgg50A”-specific RNA loads. In contrast, organ samples taken from mice one and three days after vaccination showed

viral growth in MDCK cell cultures with a titer of $10^{4.7}$ $TCID_{50}$/ml of the NA-deleted virus (Figure 2).

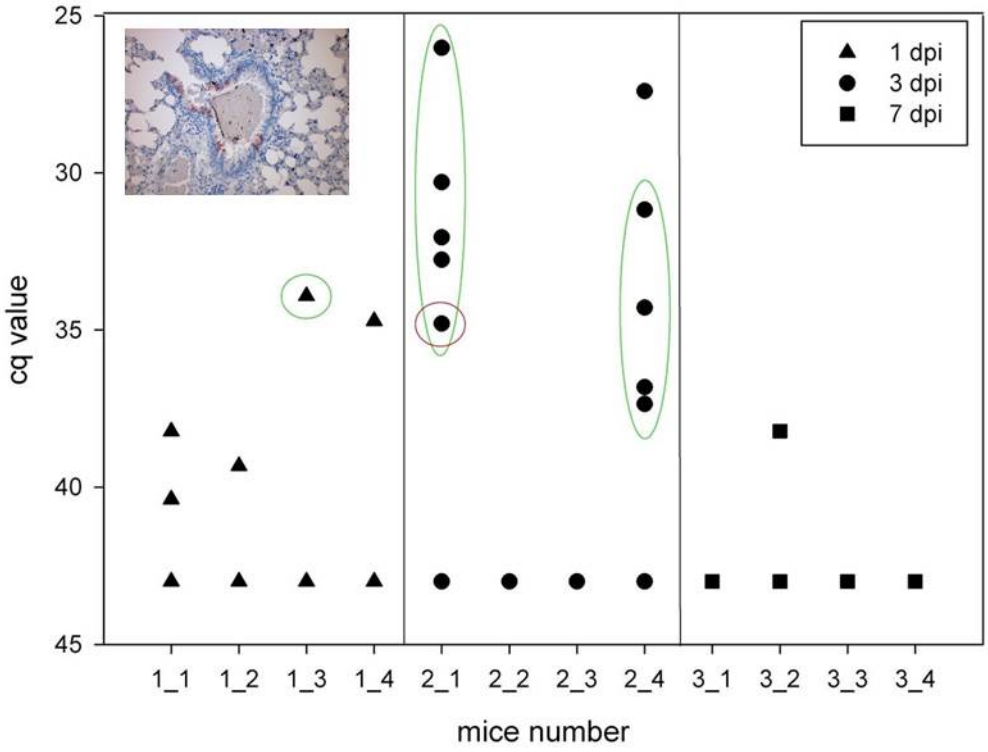

Figure 2: Real-time RT-PCR of organ samples tested for "EscEgg50A". Mice were immunized once with the "EscEgg50A"-mutant and euthanized after one, three or seven days (1,3,7 dpi). The green circles indicate samples positive for viral replication in MDCK cells after two passages. The red circle 3 dpi of mouse 2_1 indicates the histopathology positive tested sample for Influenza NP, as shown in the picture top left.

HPAIV H5N1 "R65" viral genome loads detected from brain and lung from mice immunized three and seven days before challenge infection were markedly reduced (one animal) or scored negative (19 mice; Figure 3). Samples from group "mice/i.m./o.n./1 dpi" scored positive, while i.p. immunized mice still scored negative. The challenge infection control mice had to be euthanized early or died rapidly; therefore, challenge virus RNA was not detected in every single organ sample as shown in Figure 3. From organ samples of chickens that had to be euthanized or succumbed to the disease during the experiment, high challenge virus RNA loads, especially in the brain, could be detect. In contrast, the Cq values of the organ samples of the groups "chicken/i.m./3 dpi" scored significantly higher (Cq values > 30). In summary, samples from 4 of

10 animals from group “chicken/i.m./3 dpi” and from 10 of 10 animals from group “chicken/i.m./7 dpi” scored negative (Figure 3). Finally, there was no evidence of any viral RNA loads in the organ samples of the immunized ferrets, while low Cq values indicating very high viral genome loads could be found in organ samples of the challenge infection controls (Figure 3).

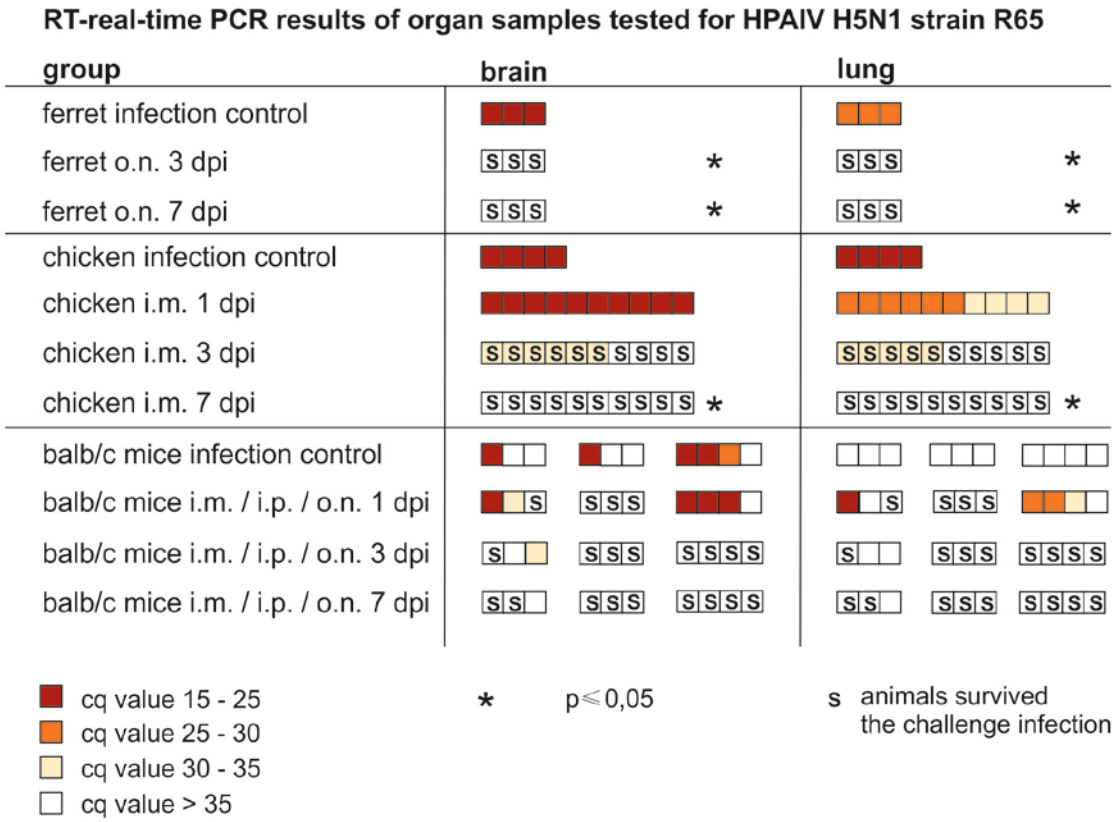

Figure 3: Real-time RT-PCR of organ samples tested for HPAIV strain “R65”. Different colors show the range of Cq-values. One small box represents one sample, i.e. animal. Data were tested for significant differences to the infection control groups with the Fisher exact test and a confidence level of 95 %. Groups without asterisks showed no significant differences in the statistical analyses.

Serology

Prior to the experiments, sera from all chickens and ferrets were confirmed as influenza negative using an NP-antibody ELISA.

All serum samples from “ferrets/o.n./1 dpi/ 3 dpi” and “chicken/i.m./1 dpi” were negative in the influenza NP-ELISA before challenge infection (see table 1 and figure 4). In blood samples from

the animals of group “chicken/i.m./7 dpi” and “ferrets/o.n./7 dpi”, an NP-specific antibody response could be detected. All chicken samples were also tested for H5-specific antibodies, and the results were comparable to the results of the NP-ELISA system (Figure 4). Additionally, in these samples no NA-specific antibodies could be detected. Interestingly, also ten days after challenge infection no NA-specific antibodies could be detected, but H5-specific antibody titers were present. Additionally, the ferret sera scored negative in the H5-ELISA both before and after challenge infection (table 1)**.**

The results of the HI analyses of the chicken samples confirmed the ELISA results. In the groups “chicken/i.m./1 dpi/3 dpi” as well as in the infection control group no HI titer could be detected before challenge infection, whereas the titer of group “chicken/i.m./7 dpi” ranged between 1:128 and 1:256. Due to small sample volumes, sera from ferrets and mice, collected after challenge infection, could not be analyzed in the HI assay.

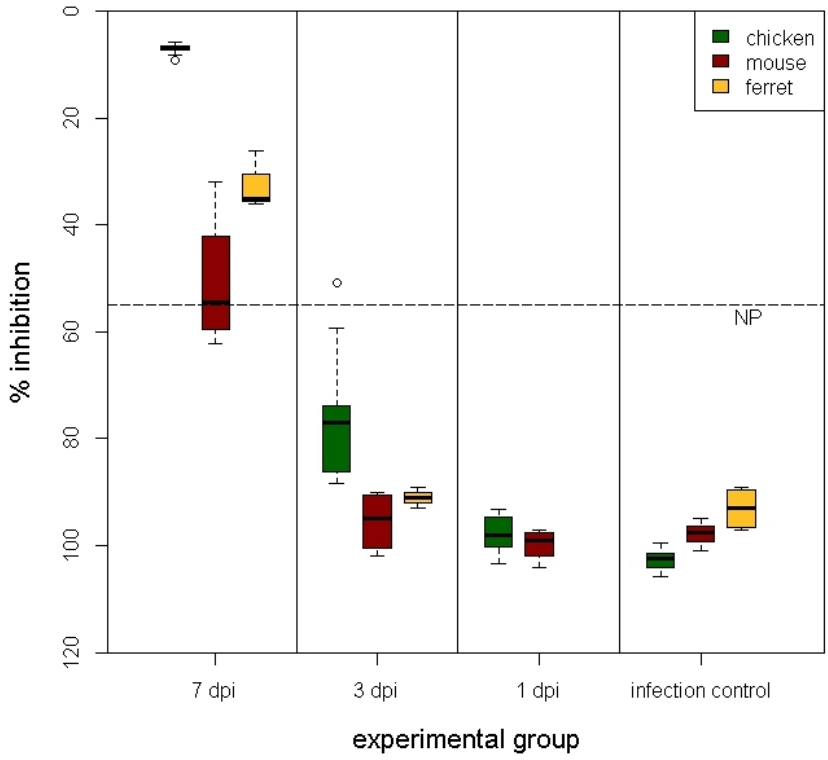

Figure 3: NP-specific antibody responses in chicken, mice and ferrets seven, three and one day after immunization with the “EscEgg50A” mutant. The dashed line shows the cutoff of the influenza A Antibody Competition ELISA. All values below the dashed line are considered negative for avian influenza NP antibodies.

4.5. Discussion

Hemagglutinin and neuraminidase surface glycoproteins of Influenza A viruses are major immunogenic factors of influenza vaccines. The HA protein is of utmost importance for the induction of a protective immune response as shown in several animal model studies [18, 19], and is responsible for the induction of neutralizing antibodies [20]. As shown by previous studies, NA-lacking influenza virus mutants exhibited a stable replication in cell culture and were highly attenuated in the used animal models [19, 21]. However, there were no H5-NA-lacking-variants available before and no animal experiments in chickens were performed. Therefore, the novel NA-lacking virus variant "EscEgg50A" [13] was used as a modified live influenza vaccine model, and the study focused on the induction of a very early onset of protection. A single-shot, orally applicable vaccine, usable for avian as well as mammalian species – inducing solid protection against virus replication and shedding – would be a perfect tool to limit the spread of an influenza virus infection and would allow to control efficiently even severe outbreak situations as frequently witnessed during the on-going HPAIV H5N1 epizootic.

In the sector of veterinary vaccines, several attenuated H5N1 modified live influenza vaccine constructs were shown to confer protection against clinical disease and to reduce viral load in organs or excretion samples, also after challenge infection using a heterologous virus strain; however, vaccination protocols were performed with a challenge infection at least three weeks after immunization [9, 11, 21, 22]. More recently, HVT-vectored H5 vaccines were shown to confer solid protection against infection with divergent HPAI strains, but again, challenge was carried out at the earliest 21 days post vaccination [5]. Recombinant and adapted live H5N1 influenza vaccines tested in the backbone system of FluMist^{TM}, an approach derived from human medicine, are immunogenic for mice as well ferrets, nevertheless, viral RNA detection could be achieved after single intranasal vaccination and challenge four weeks post immunization. Thus, viral spreading might be still possible and a very early onset of immunity cannot be expected [11].

In our study, we were able to protect mice, ferrets as well as chickens from morbidity only three days after single-shot immunization with “EscEgg50A” H5 virus, although there were no detectable NP-specific antibodies. This is in accordance with previous work by Seo and Webster [9, 23]. They showed that CD8+ T-lymphocytes and TLR3 and TLR7 signaling pathways are more important for early protection against the disease than B lymphocytes. These early responses can only be seen with live attenuated vaccines and are thus a major advantage over inactivated preparations that act mainly through humoral response [4, 5, 6]. Although far from being understood, especially in the avian host, strong innate immune responses including interferons as well as viral interference are among the factors held responsible for these early protection phenomena. Especially in the early phase after vaccination, the inactivated vaccines do not prevent infection and transmission [4, 5, 6] and may facilitate antigenic drift of virus strains under field conditions. Thus, field applicability is severely hampered. Nevertheless, complete protection without detection of challenge virus RNA in lung tissues as well as swab and lavage samples (Figure 1 and 3) was in our experiments only achieved in those animals that showed a clear seroconversion against influenza virus NP protein proving the importance of humoral responses for solid protection.

In terms of application in the field, non-invasive vaccine administration would be beneficial. Interestingly, chickens oronasally immunized with the “EscEgg50A” H5-strain were not fully protected against challenge infection [13]. One possible reason might be differences in the receptor repertoire and the mucous barriers of chickens, ferrets and mice as well as different susceptibilities of the mucous membranes of these species. Furthermore, it has to be taken into account that the inoculated vaccine virus was administered to chickens without anaethesia, while ferrets and mice in our study were inoculated under isoflurane, which might lead to deeper penetration of the vaccine inoculum.

In summary, the NA-deleted virus variant tested here efficiently protected all animals very early after a single-shot application and thus represents an interesting model for early protection against HPAIV. However, in terms of a true vaccine candidate, a major drawback is the polybasic cleavage site within the HA protein, which determines HA as a protein of the highly pathogenic phenotype [13]. The possibility of reassortment events between the vaccine virus strain and

circulating field viruses is discussed as a possible side-effect of modified live influenza vaccines. While this is not a relevant problem for seasonal influenza, as seasonal modified live influenza vaccine cannot donate novel HA and NA genes [24], usage of prepandemic modified live influenza vaccines beyond an endemic region is not recommended in order to avoid the introduction of a “new HA” [12]. To overcome this problem, future studies will especially focus on the generation of further H5 and H7 influenza viruses lacking NA expression combined with a HA cleavage site derived from low pathogenic AIV. However, Kalthoff et al. [13] showed that NA-deleted mutants with HA monobasic cleavage sites do not replicate in cell culture. Therefore, the role of adaptive mutations in the different genome segments as well as cell-culture adapted influenza viruses as possible backbones have to be studied. Furthermore, NA secreting cell lines or external NA could be used and give the virus an initial aid. Adding a neuraminidase activity could be a promising approach especially for the o.n. application route for chickens. In terms of a field application in very young animals, especially one-day-old chickens, their immature immune system and interference with maternally derived antibodies should be taken into account. Here, further studies are needed, especially with regard to apathogenicity and immunogenicity.

To the best of our knowledge, this is the first time that three different species were shown to be fully protected against the clinical outcome of an HPAIV infection by a single dose of an experimental modified live influenza vaccine three days after immunization. Furthermore, the vaccine virus preparation not only decreased the shedding of HPAIV in challenged chickens, but also prevented it in ferrets, and accomplished a very complete immunity in chickens, ferrets and mice seven days post immunization. The here described model therefore shows that a very early onset of protection is possible, and the NA-deleted mutant may be a promising live vaccine candidate for broiler flocks, which are relevant for HPAIV H5N1 spread in endemic regions but are slaughtered early, e.g. at an age of five weeks. Furthermore it could be used as an “emergency vaccine” in addition or alternatively to the pre-emptive culling strategies.

Notes:

The authors state that they have no conflicts of interest.

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Contributors:

SR, DK and MB designed the study, SR carried out data analyses and statistics. DK and MB checked data extraction. All authors contributed to both protocol, final report and wrote the manuscript.

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5. Discussion

Influenza A viruses have an impressive genetic plasticity and variability. Antigenic shift due to the reassortment of genome segments is the most drastic possibility for adaptation or escape. However, also antigenic drift of the viral quasispecies population within an individual host and moreover within a population of individual hosts is a major evolutionary mechanism of Influenza A viruses (107). With the accumulation of adaptive mutations based on the error prone viral polymerase the viral quasispecies evolves rapidly. This mechanism is underlying the development of viral escape mutants especially in immunized host populations. Immunity, that is not able to block the infection chain within a host population efficiently, serves as evolutionary hurdle that is compensated by antigenic drift based adaptation.

While these principles are well known, scientific approaches to simulate the scenario of an Influenza A infection of an immunized but not protected population are limited. In previous studies, the passaging of HPAIV H5N1 on cell culture in the presence of an antiserum resulted in escape variants with numerous mutations especially in the HA-encoding segment (29). Nevertheless, those mutants also lost their virulence despite a conserved polybasic cleavage site in the HA and were no longer highly pathogenic (29). Therefore, in this study embryonated eggs were used as a growth system, which is closer related to in vivo replication, and the newly generated viruses are discussed here.

Generation and in vitro characterization of a novel neuraminidase-negative H5N1 variant

Passaging HPAIV A/swan/Germany/R65/2006 (H5N1) in embryonated chicken eggs by application of an immunogenic pressure with a polyclonal antiserum was performed in order to mimic the natural evolution of H5N1 viruses in inadequately vaccinated poultry flocks. Using this relevant in vitro culture system, a novel escape mutant virus - “EscEgg50A“ - was isolated successfully, and a control virus passaged in the same system without adaptive pressure was generated in parallel and named as “CoEgg50”.

The general applicability of the used in vitro system for studying escape mechanism could be demonstrated by the fact that the resulting escape mutant was neutralized by the relevant antiserum to a lesser extent compared with the control virus.

While immune pressure normally leads to a series of adaptive mutations, primarily within the antigenic globular head of the HA protein (29), unexpectedly, the here described novel mutant “EscEgg50A” showed only one amino acid substitution in the HA protein located in a position not related to antigenicity. In contrast, the control virus “CoEgg50” exhibited substitutions of four amino acids within the HA, representing the background level of genetic variation due to polymerase errors (64). Further ten amino acid mutation events were detected in both egg-passaged viruses distributed throughout the genome. Most interestingly, sequence analyses of EscEgg50A showed major deletions and rearrangements together with a nucleotide shuffle between different segments in its neuraminidase encoding segment 6. On the contrary, no substitutions at all were detected within the segment 6 of “CoEgg50”. Repeated passaging experiments under similar conditions resulted in two further virus isolates: “EscEgg50B” and “EscEgg50C”. The analysis of segment 6 of “EscEgg50B” and “EscEgg50C” revealed again major sequence deletions and a partly segment recombination event within segment 6.

Therefore, antiserum-induced rearrangements of segment 6 are a non-singular phenomenon. Further characterization of “EscEgg50A” demonstrated the loss of the complete neuraminidase protein and hence the respective enzymatic activity. However, the “EscEgg50A” mutant was able to replicate in cell culture as well as in embryonated chicken eggs without supplementation of sialidase activity. This is in contrast to other reported NA-deleted viruses, which had to be supported by a helper virus or by an externally supplied neuraminidase to allow efficient growth (48, 117). In order to identify the serum component responsible for the adaptive mutations identified, the used serum was tested for N1-specific antibodies and neuraminidase inhibitory functions. While the antibody-mediated input was neglectable, an inhibitory effect onto neuraminidase activity by antisera in general was demonstrated. These inhibitory effects are possibly responsible for the loss of the neuraminidase protein within the different here described NA-deleted variants. This is in accordance to experiments which applied

neuraminidase inhibitors in vitro during influenza A passaging, which could result in the occurrence of neuraminidase negative viruses (48). However, also in most of these cases, externally supplemented neuraminidase activity was necessary for viral growth (117).

By the generation of recombinant viruses using reverse genetics, it could be shown that no additional mutations in further segments were essential for replication without any neuraminidase activity, but clearly revealed that the polybasic cleavage site of the HA protein e.g. of EscEgg50A is essential for successful transfection and subsequent generation of virus progeny. Furthermore, the different NA-deleted virus variants demonstrate, that eight segments have to be present, and that a minimum composition of segment 6 (e.g. the non-coding 3´and 5´sequences) has to be present for the generation of replicable viral particles as was also demonstrated by others (48, 87).

NA-deleted mutants, but not of subtype H5, were also described by several authors, but all these viruses were reliant upon the supplementation of external neuraminidase or alternative modification in the HA encoding segment for efficient replication and growth (48, 87). By these results the dependency upon an orchestrated relationship between the functions of both viral glycoproteins was verified (117). While our recombinant virus “EscEgg50Arec”, exhibiting an unaltered ancestor HA in combination with a truncated segment 6, was viable, modifications of the HA beyond sequence variations are likely responsible for an altered receptor avidity (117). Indeed, a non-desialized HA protein (due to a missing neuraminidase activity) seems to be reasonable, and the modified receptor avidity was e.g. demonstrated by the non-hemagglutinating nature of the NA-negative viruses (93). Despite this varied receptor avidity, our “EscEgg50A” virus replicated to virus titers comparable to ancestor virus titers at least after inoculation with a high MOI. To the best of our knowledge, this is the first description of an HPAI H5N1 virus that demonstrated this kind of complete NA-independent replication capacity. However, the investigation of the viral plaque sizes, applying a semisolid medium, revealed a tremendous reduction of plaque sizes of the NA-negative virus. A massive impairment of the cell-to-cell-spread (ctcs) could be deduced from these results for EscEgg50A. The impact of the neuraminidase onto the plaque sizes was already reported (53), but the exact mechanism

behind this phenomenon remains to be elucidated. For the future, studies are planned to investigate the ctcs of influenza viruses also in polarized epithelial cells in order to evaluate the effect in a system closer to the natural environmental system.

The evaluation of the intravenous pathogenicity index (IVPI) of the generated NA-negative viruses categorized the tested viruses, with a large deletion of segment 6, as low pathogenic for chickens. Despite the polybasic cleavage site within the HA-sequence, the viruses did not induce any clinical disease. However, as demonstrated by seroconversion of the inoculated birds, the virus replicated after intravenous application. In contrast, after oronasal application of "EscEgg50A", only a minority of the inoculated chickens showed seroconversion, indicating that the immune system of the host did not react with the viruses. Most probably the reason behind this is that one function of the neuraminidase protein is cleavage of complex substrates such as mucin of the respiratory tract (84). Therefore, NA-negative viruses might be inefficient in getting access to susceptible cells by being trapped within the mucin material. The new NA-deleted virus allows now for the first time to study this function separately e.g. in in vitro mucin infection models (39, 55).

From our results we concluded, that the neuraminidase of HPAIV H5N1 is not essential for virus replication in both cell culture and embryonated chicken eggs. However, the neuraminidase is of importance for ctcs, host entry and most likely also an efficient spread within the host. Therefore, the neuraminidase protein is a considerable pathogenicity factor despite the fact that it is not essential in HPAIV H5N1 for high titer virus growth. The generally accepted viral neuraminidase function of enabling the release of infectious virions from the cell membrane by cleavage of sialic groups (1, 70) is not the only major feature of influenza NA proteins. This finding is unexpected and obviously tightly connected to the polybasic HA-cleavage site of HPAIV H5 (34).

The here demonstrated marked attenuation of the neuraminidase-negative "EscEgg50A" variant together with its negative selection marker potential suggests that this virus might be a suitable differentiation of vaccinated from infected animals (DIVA) vaccine candidate virus.

The neuraminidase-negative new H5-variant as possible modified live marker vaccine candidate

In order to evaluate the modified live vaccine properties of the "EscEgg50A" H5 virus, we performed a series of immunization-challenge experiments in three different species. Since onset of immunity is one of the major problems of Influenza A virus vaccines, especially the capacity of our modified live virus to induce an early onset of immunity against a lethal H5 challenge infection was analyzed. A recently described fowlpox virus-based live vector vaccine (18) was able to induce HI antibody titers in 4-week old chickens and Seo and Webster (86) have shown protection of chickens immunized with H9N2 after only three days. Rauw et al. (78) found that serum from 1-day old chickens immunized with a live Newcastle disease vaccine showed a high HI titer after 2 days. As described, there are only very few studies about an early onset of immunity against avian influenza viruses in animals (19, 86, 118).

We therefore immunized ferrets, mice and chickens with the EscEgg50A mutant intramuscularly or oronasally one, three or seven days before challenge infection. None adverse effects were observed after immunization, and while animals immunized one day before challenge infection still succumbed to the disease, the NA-negative mutant virus protected all the animals immunized three and seven days before challenge infection against both death and clinical signs. In addition, all animals challenged seven days post immunization did even not shed any challenge virus, proving a very high level of immunity and protection. This kind of "sterile immunity" can only be reached with inactivated vaccine preparations after several administrations and not in all cases (98). Furthermore, even if clinical protection can be reached, virus shedding and low level replication are observed which are major reasons for antigenic drift (8, 98) as also discussed before.

The here described highly efficient and very swift immune response to "EscEgg50A" can be mainly explained by two reasons. Firstly, an early immune response can mainly be guaranteed by live vaccines since they can induce a strong response of both the cellular as well as humoral immunity (25, 35), and secondly it has been described before, that the HA-protein is the main

immunogenic determinant for protection against the influenza A viruses (63). The here tested "EscEgg50A" strain contains both features and is therefore a promising candidate for "emergency vaccines" even for the use in broiler flocks, which are slaughtered very early making vaccination with conventional vaccines not feasible. Furthermore, the NA-lacking mutant permits a DIVA concept in combination with a suitable neuraminidase antibody detection system like ELISA or indirect immunofluorescence with recombinant baculovirus-expressed antigens (26, 94). This type of differentiation of infected from vaccinated animals can be also used for inactivated vaccine (2, 94) with heterotypic neuraminidase combinations. Chickens vaccinated with an H5N9 inactivated vaccine can for example be differentiated from an H5N1 infection by using N1- and N9-specific test systems (57). The NA-deleted live virus mutant EscEgg50A would allow even an easier differentiation, since the lack of any neuraminidase response enables the use any neuraminidase type for differentiation without any problems of a cross reactivity with vaccine-derived neuraminidase-specific antibodies.

Additional research has to be done in the future for the following major issues: The "EscEgg50A" mutant still exhibits a polybasic cleavage site within the HA-segment. Theoretically, reassortment events with wild type strains are possible which can donate "new" HA segments, in the case of "EscEgg50A" with the HPAIV cleavage site, to the circulating AIV strains and induce a potentially dangerous evolutionary situation. Hence a most promising 2nd generation vaccine candidate would be a NA-negative virus with a "safe" monobasic cleavage site. This might be achievable by implementing further changes in other segments, for example the polymerase complex or applying external NA-activity. These arrangements could further improve the vaccine candidate making the use e.g. as an emergency vaccine in the near future more likely.

Secondly, chickens could be only be efficiently immunized by the intramuscular route, but not oronasally. One reason is probably the lack of a neuraminidase activity, since it is described that the enzyme cleaved the mucin of the respiratory tract (12) or the application under Isofluran narcosis of mice and ferrets induced deeper breathing, and it is described that the upper respiratory tract contains less mucin (47, 79) which would allow efficient attachment of the

vaccine virus. In addition, lungs of mammals and birds possess different receptor repertoires and it can not be excluded that viral binding affinity is also an important factor (82).

In any case, the here presented modified live vaccine candidate is an impressive model for the induction of a very early onset of immunity, demonstrating the possibility of efficient immunization against lethal challenge infection even 3 days before challenge infection. Our data therefore are a promising first step in the direction of a new generation of novel highly efficient early onset emergency vaccines.

Future studies will show pathogenicity and immune response also in very young animals, especially one day old chicken, since this age is very convenient and important for immunisation procedures. Furthermore it has to be tested if maternally antibodies inhibit immune response to the vaccine virus.

6. Summary

Since about one decade, highly pathogenic avian Influenza viruses (HPAIV) of subtype H5N1 have been extensively circulating in poultry as well as wild birds particularly in Asian and African countries. Control campaigns with inadequate or inefficient vaccines, as well as poultry movement and wild bird migration, contributed to viral spread, reassortment with other circulating avian influenza strains, and antigenic drift. However, to date, no emergency vaccine is available, that allows sufficient and swift protection against HPAIV H5N1, blocks viral shedding and is easy to administer.

The main objective of the work described here was the *in-vitro* and *in-vivo* characterization of a novel neuraminidase-negative variant of HPAIV H5N1 and the use as a modified life vaccine virus model for the early onset of protection.

In the initial step, a highly pathogenic H5N1 isolate of clade 2.1 (A/swan/Germany/R65/2006) was passaged fifty times in embryonated chicken eggs together with polyclonal antisera. After 50 passages, a virus escaping the antisera was isolated and named “EscEgg50A”. Surprisingly, the major observed changes were severe deletions and a segment shuffling within the neuraminidase (NA) encoding segment 6, resulting in NA-deletion and the absence of any neuraminidase activity.

Despite NA-deletion, high titer virus growth in cell cultures and embryonated chicken eggs, without helper viruses or supplementary neuraminidase activity, could be observed, and a functional copy of the novel “EscEgg50A” mutant was constructed by using reverse genetics. However, propagation of the NA-deleted “EscEgg50A” strain in cell culture revealed significantly smaller plaque sizes and a delayed growth in relation to the ancestor virus. Infection studies in chickens showed apathogenicity, and neither spreading nor transmission of “EscEgg50A” were observed. Nevertheless, high antibody titers could be achieved following intramuscular application, whereas oronasal infection revealed sero-conversion in only a portion of the inoculated chickens.

Apathogenicity as well as immunogenicity of “EscEgg50A” prompted to investigate whether the mutant is a suitable modified live vaccine model candidate for early onset of immunity. Balb/C mice, ferrets and chickens were therefore immunized intramascularly or oronasaly one, three or seven days before lethal HPAIV H5N1 challenge infection. It could be shown that all animals which had been immunized seven days before challenge infection were not only protected against clinical signs, but also against both challenge virus shedding and transmission. Additionally, animals immunized three days before challenge infection did not show any clinical sign, but viral RNA could be found in organs as well as swabs or nasal washing samples. The new H5 deletion mutant also allowed differentiation of vaccinated from infected animals, since immunized animals showed only antibodies against the hemagglutinin and the nucleoprotein, whereas infected ones could develop neuraminidase-specific antibodies. Nevertheless, presence of the polybasic hemagglutinin cleavage site in those H5-viruses implies the risk of generating new HPAIV after possible reassortments with circulating wild type strains.

In conclusion, a first viable NA-negative H5-influenza virus was established and characterized, which can be used for further research e.g. about the role of NA for avian influenza viruses and for early onset of immunity studies. Future studies will e.g. concentrate on the construction and characterization of novel NA-deleted viruses of the subtypes H5 and H7 for in vitro studies and optimized marker vaccine candidates for oro-nasal application.

7. Zusammenfassung

Hochpathogene Aviäre Influenzaviren (HPAIV) des Subtypes H5N1 zirkulieren seit den ersten Ausbrüchen vor mehr als 10 Jahren in Wirtschaftsgeflügel und Wildvögeln, vor allem in Asien und Afrika. Vakzinekampagnien mit ungeeigneten oder unwirksamen Impfstoffen sowie Transporte von infiziertem Geflügel, sowie Wildvogelzüge führten nicht nur zur Verbreitung der Viren, sondern auch zu Reassortierungen mit anderen zirkulierenden Influenzaviren und zur antigenetischen Drift. Bis heute ist kein Impfstoff verfügbar der ausreichenden und schnellen Schutz vor einer HPAIV H5N1 Infektion, sowie der Ausscheidung bietet, genauso wie eine einfache Applikation ermöglicht und sowohl im Säugetier, als auch Vogel einsetzbar ist. Im Falle eines Ausbruches wäre eine solche Vaccine das perfekte Werkzeug, um eine Pandemie zu verhindern.

Die in dieser Dissertation vorgestellten Arbeiten zeigen die in-vitro und in-vivo Charakterisierung und einer Neuraminidase-negativen H5N1 Mutante, so wie den Einsatz als Impfstoffmodel zur Untersuchung der Frühimmunisierung.

Ein hochpathogenes H5N1 Isolat des Stammes 2.1 (A/swan/Germany/R65/2006) wurde fünfzigmal im embryonierten Hühnerei passagiert. Den Passagen wurde bei jedem Durchgang polybasisches Hühnerserum mit Antikörper gegen H5 und N2 beigemischt, um einen selektiven Druck auf das Virus zu erzeugen. Die daraus resultierende Mutante zeigte überaschenderweise nicht die erwarteten Veränderungen und Anpassungen im Haemagglutinin, sondern Deletionen und „Segment-shuffling“ im Segment 6, welches für das Neuraminidaseprotein kodiert. Des Weiteren konnte keine Neuraminidaseaktivität mehr nachgewiesen werden.

Die Deletionen führten zu Veränderungen im Wachstumsverhalten des Virus und der Pathogenität im Tier. So konnte hier gezeigt werden, dass die Mutante ohne Zugabe von externer Neuraminidase oder Adaptation des Haemagglutinins in Zellkultur und Ei hohe Infektionstiter erreichen kann. Im Vergleich zum Ursprungsvirus aber Wachstumsdefizite aufweist, die sich in kleineren Plaques und langsamerem Wachstum auf Zellkultur zeigen.

Infektionsversuche im Huhn zeigten, dass das Virus weder Klinik auslöst, noch ausgeschieden oder übertragen wird, aber eine gute Immunantwort induziert. Hohe Antikörper konnten nur erreicht werden, wenn Hühner intramuskulär infiziert wurden, wohingegen eine Applikation über den oronasalen Weg nicht bei allen Tieren gelang.

Die gute Immunantwort und Apathogenität des Virus machten es im weiteren Verlauf zu einem geeigneten Kandidaten für Frühimmunisierungsversuche im Säugetier- und Vogelmodell. So wurden Balb/C Mäuse, Frettchen und Hühner ein, drei und sieben Tage vor einer H5N1 Belastungsinfektion mit der H5N1 Mutante intramuskulär oder intranasal immunisiert. Dabei konnte ein 100% Schutz vor klinischen Symptomen und der Ausscheidung des Challenge-Virus nach nur 7 Tagen gezeigt werden. Darüber hinaus waren die Tiere vor Klinik bereits drei Tage nach der Immunisierung geschützt, wobei aber virale RNA in oronasalen Proben und auch den Organen nachgewiesen werden konnte.

Die Neuraminidasedeletion der Mutante ermöglichte außerdem eine Unterscheidung von immunisierten zu infizierten Tieren, da erstere im ELISA NP- aber keine NA-Antikörper zeigten, wohingegen infizierte Tiere Antikörper gegen beide Proteine bildeten.

In Zukunft könnten NA negative Influenzaviren zusätzlich oder als Alternative zu den gängigen stamping out Strategien eingesetzt werden. Dafür aber sind weiter Untersuchungen essentiell. Die hochpathogene Spaltstelle im HA von „EscEgg50A“ impliziert das Risiko mit zirkulierenden AI Stämmen zu reassortieren und ist daher für den Einsatz zum Beispiel in Zuchtherden ungeeignet. Darüber hinaus ist das Wissen über das NA Protein und seine Funktionsweise sehr lückenhaft und NA-negative Mutanten könnten für zukünftige Untersuchungen genutzt werden.

8. Abbreviation

Cq	Cycle of threshold
ctcs	Cell-to-cell-spread
DI	Defective interfering
DIVA	Differentiating infected from vaccinated animals
dpc	Days post challenge
FLI	Friedrich-Loeffler-Institut
HA	Haemagglutinin
HI	Haemagglutinition inhibition tes
HPAIV	Highly pathogenic avian influenza virus
HVT	Turkey Herpesvirus
i.m.	Intramascularly
i.p.	Intraperitoneally
IAV	Influenza A virus
IVPI	Intravenous pathogenecity index
LPAIV	Low pathogenic avian influenza virus
M1	Matrix protein 1
M2	Matrix protein 2
MOI	Multiplicity of infection
mRNA	Cellular messenger RNA
NA	Neuraminidase
NP	Nucleoprotein
NS2	Non-structural protein 2
nt	Nucleotides
o.n.	Oronasal
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PBS	Phosphate-buffered salin
PCR	Polymerase chain reaction
RNP	Ribonucleoprotein complex
RT	Reverse transcription
SPF	Specific pathogen free
TCID	Tissue infectios dose
VNT	Virus neutralization test
vRNA	Viral RNA
WHO	World Health Organization

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