IN VIVO EVALUATION OF RECOMBINANT VACCINIA VIRUS MVA DELIVERING ANCESTRAL H9 HEMAGGLUTININ ANTIGEN OF AVIAN INFLUENZA VIRUS

von Jens Michael Becker

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Aus dem Veterinärwissenschaftlichen Department der Tierärztlichen Fakultät
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1. Introduction and Aim of the Study

Avian Influenza (AI) viruses pose a threat to human and animal health and are responsible for potential economic losses. From the waterfowl reservoir, these RNA viruses can be transmitted to domestic poultry and humans, causing illness and death among people as well as mass culling of farm birds worldwide.

This study contributes to increasing the knowledge by evaluating a promising poxvirus-based vector vaccine that carries and expresses an artificial, computationally derived hemagglutinin sequence in order to induce immunity against low pathogenic avian influenza H9N2.

Modified Vaccinia virus Ankara (MVA) based vaccines have been tested in multiple human and animal trials and proved to be a safe and reliable vector system. The ancestral strategy uses a hemagglutinin sequence located at the node of a phylogenetic tree which arranges virus strains according to their evolutionary relationship. Inactivated whole virus vaccines engineered this way have conferred cross-clade protection in the ferret model of influenza A virus infections. In the present study, we aimed at testing the ancestral H9N2 MVA vaccine in the chicken model.

Hereby, we tried to find answers to the following questions: What levels of H9 specific antibody responses are induced in chicken? Do the antibodies elicited by the ancestral H9 antigen cross-react with H9 antigens from other virus strains? Do the induced antibodies confer protection?

2. Review of Literature

2.1 Influenza A Virus

The Orthomyxoviridae family consists of five genera: Influenza A virus is the most widespread genus, infecting a multitude of mammalian and avian species whereas the genera B and C are mostly found in humans. The Isavirus group (fish pathogen) and the Thogotoviruses (tick-borne arbovirus) should be mentioned to complete this overview.

2.1.1 Viral Classification and Structure

Avian Influenza (AI) viruses are enveloped negative-sense, segmented RNA viruses whose genomes encode for at least 16 different proteins. Usually, these proteins are divided into three groups; virus core proteins, matrix layer components, and envelope proteins. The latter, notably the hemagglutinin (HA) and neuraminidase (NA) proteins, key players in cell adhesion and detachment, help to classify

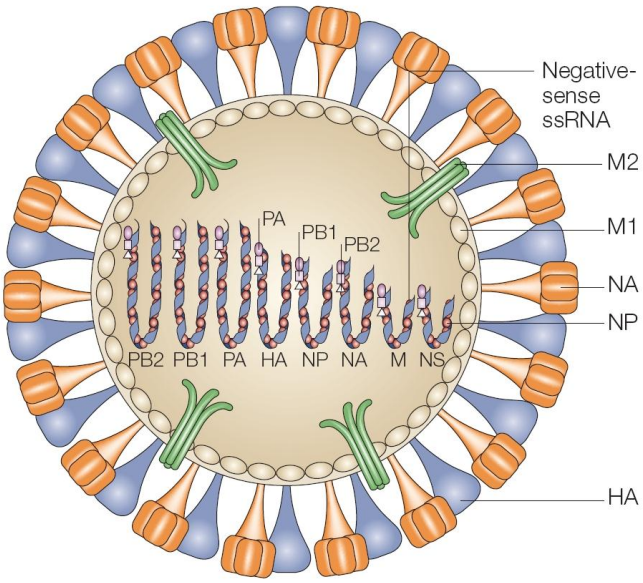


Figure 1: An avian influenza virus in schematic section: The eight gene segments are represented in the center and the orange and blue spikes on the viral membrane represent HA and NA proteins.

influenza A viruses. In birds, there are 16 HA subtypes (H1-H16) and 9 different NA (NA1-9), thus 144 possible combinations of which many could have been found in the field [1-3]. The terms HA and NA also refer to the gene segments that encode for the HA and NA proteins.

Whereas clinical isolates often appear as filamentous particles (up to several microns in length), laboratory passaged viruses mostly show a spherical phenotype of 80 to 120nm [1].

AI viruses contain eight gene segments of different sizes, named for the major protein they encode (HA, NA, PB1, PB2…) as shown in the image above. Briefly, the most important viral proteins and their major functions will be presented.

2.1.2 Major Influenza A Virus Proteins

Envelope Proteins– [1] – Hemagglutinin (HA)

HA is an envelope protein that covers approximately 80% of the outer surface of a virus particle. This homotrimer plays a crucial role in cell fusion and intrusion as it has a high level of specificity for binding to sialic acids on the host cell surface. After endocytosis into the cytoplasm, which is a common method for material to enter the cell, the endosome is transformed into a lysosome by acidification. Triggered by this pH change, the HA molecule splits into two subunits (HA1 and HA2) and plants the fusion peptide, a part of HA2, into the endosome's membrane. The viral and host cell membranes merge and ribo nucleo proteins (RNP) leave the virion, heading towards the nucleus. However, the splitting of HA molecules works only when the HA had been cleaved by cellular enzymes during the virus' prior assembly, when leaving the former host cell. Since only certain tissues are equipped with adequate proteases, only those tissues can produce infectious particles. In humans, influenza viruses are specific to the upper respiratory tract due to the local cells that synthetize the suitable enzymes to cleave the precursor HA molecule. In contrast, highly pathogenic avian influenza viruses (HPAI) display cleavage sites composed of multiple basic amino acids, making them accessible for furin or other ubiquitous proteases that can be found in most cells of the body. This implies that infectious HPAI virus is produced in many different

tissues, including skeletal muscle, pancreas, heart, intestines, kidney and brain; thus harming essential organs and causing severe symptoms.

[2] – Neuramindase (NA)

Being four times less numerous than HA, the highly conserved neuraminidase (NA) covers about 17% of the viral surface and rises out of the lipid particle membrane like a mushroom among the hemagglutinins. With its tetrameric shape, this enzyme catalyzes the cleavage of sialic acids from cellular glycan structures as glycolipids and glycoproteins which is imperative both for viral spread and for the release of viral progeny. Although HA binds to sialic acids (also called neuraminic acids) to invade the host cell membrane, recently produced viruses need to be released in order to infect surrounding cells. Therefore, both surface proteins act in concert [4] to first attach and penetrate, then to detach from the host cell. NA helps the virus spread in respiratory mucosal membrane by cleaving sialic acid residues from the mucins of the respiratory tract and helps to avoid viral clustering. Moreover, NA may also be able to strengthen HA's hemagglutinating activity as it removes sialic acids from oligosaccharides that are found in close vicinity to the binding site of HA [5].

[3] – M2

M2 is a transmembrane protein which provides passage for protons during early and late stages of virus replication. This minor component (approx. 20 molecules/particle) is activated by the low pH (~6) in the lysosome where it opens its 'tryptophan-gates' [6] and conducts protons through the viral envelope into its interior, where the pH decreases to ~5. As mentioned above, this step is crucial for the conformational change of HA before membrane confluence can occur. It is believed that the pH drop enables the dissociation of the matrix protein (M1) from the viral nucleoproteins to enable them to be transported into the nucleus for mRNA production. Furthermore, M2 proton channel proteins in the membrane of the trans-Golgi network equilibrate cellular and Golgi lumen pH preventing precocious cleavage of HA on their way to the host cell surface [7].

Matrix Layer – [4] – Matrix Protein M1

Right underneath the lipid bilayer that forms the envelope, the most abundant virus protein M1 is arranged as a helical net structure, providing support and structure for the viral envelope [8]. It links the cytoplasmatical tails of HA, NA and M2 on the outer side to viral RNA (vRNA) and nucleoproteins in the form of the ribonucleoprotein (RNP) on the inner side. RNP can only be transported to the nucleus when released by M1 [9]. Subsequently, M1 is involved in the transport of RNP to the cell surface as well as in transcription and replication of vRNA. It might be involved in virus budding and design of progeny as it may influence membrane curvature in budding processes and therefore determine if newly produced virions are filamentous or spherical [8, 9]. To what extend M1 collaborates with HA, the initiator of budding, and if it mediates the recruitment of proteins to finish viral assembly remains to be investigated.

Virus Core – [5,6,7] – Polymerase Complex PB1, PB2, PA

Consisting of three non-congruent proteins PB1, PB2, and PA, the viral RNA-dependent RNA-polymerase replicates the viral genome and transcribes messenger RNA in the nucleus of an infected cell. It is highly host cell specific but has no proofreading activity, resulting in frequent errors in the new nucleotide sequence and in production of viral mutants on a regular base. Together with the nucleoprotein (NP) and the vRNA, the polymerase forms the ribonucleoprotein RNP, the major functional unit of the viral core. The replication works via an intermediate step, a positive-sense complementary RNA (cRNA) which then is transformed into the negative-sense vRNA. Transcribed mRNA, however, requires several modifications: PB1 cleaves an oligonucleotide sequence from the 5'-head of a host cell precursor mRNA and uses it as primer to induce transcription of vRNA, a process called "cap-snatching". In addition to the cap, the polymerase creates a poly(A) sequence as a tail and therefore finishes the new mRNA that resembles the one which occurs naturally in the host cell: positive-sense, capped, polyadenylated [10].

[8] – Nucleoprotein (NP)

Shaped like a banana, nucleoproteins bind both individually and clustered to the

phosphate backbone of viral RNA as well as to PB1 and PB2 of the polymerase complex. Primarily, it is a structural RNA-binding protein but it interacts with cellular molecules as well. There is little or no specificity to nucleotide sequences but a high affinity to RNA strands, making the three of them constant companions during the viral life and replication cycle. One of the major tasks of NP is to hold the RNA in a convenient position for the polymerase to work correctly or for packing new virions. NP-cell-interactions are very diverse; although underlying mechanisms have not been described, NP collaborates with the nuclear import and export apparatus, it binds to actin filaments and to nuclear RNA helicase [11].

[9] – Nonstructural Protein 1 (NS1)

The NS1 protein is involved in the suppression of the host immune response that eliminates the virus. It prevents polyadenylation of cellular mRNA. This means that there are fewer mature, functional RNA molecules that can be translated into proteins [12]. Typical targets of NS1 are mRNAs that serve as a basis for interferon production. It is possible that the virulence changes dramatically through a single point mutation in the nucleotide sequence of NS1 genes as shown for a HPAI H5N1 virus that completely lost its NS1 characteristics by replacing glutamic acid with aspartic acid at position 92 [13].

[10] – Nuclear Export Protein – NEP (also referred to as NS2)

Formerly, the Nuclear Export Protein (NEP) was thought to be crucial only for the export of new RNP complexes. These complexes are formed in infected host cell nuclei and then transported to the periphery where they merge with the cell membrane to be released as viral progeny. However, there has been proven evidence that NEP interferes with a cellular ATPase and, by doing so, contributes to the viral budding process. The NEP also regulates the amount of vRNA and mRNA that accumulates in the cell. Similar to one of the effects of NS1, NEP can influence the degree of pathogenicity of a virus particle as it has an impact on the balance of replication and translation [14].

2.1.3 Viral Reproduction Cycle

AI viruses need live cells to replicate. Figure 2 shows briefly, which steps lay between the first attachment and the release of viral progeny. Matsuoka et al published a much more detailed map of the AI virus life cycle, indicating how complex viral reproduction is in reality [15].

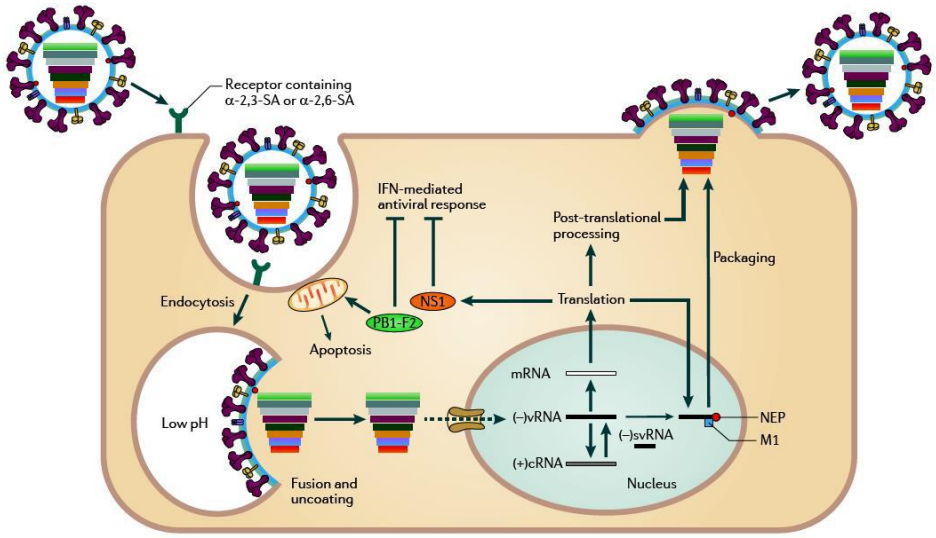


Figure 2: The life cycle of AI viruses [16]: 1. Attachment to host cell receptor (sialic acid), 2. Endocytosis, 3. Fusion with endosomal membrane and uncoating, 4. Replication of the genome, 5. Budding and release of viral progeny

2.1.4 Receptors and Host Spectrum

The HA molecule is thought to be the main mediator of host specificity, and binds, according to the subtype, to its suitable of receptor on the cell surface [17]. These receptors are oligosaccharides exhibiting terminal sialic acids, either linked by α2,6-linkages or α2,3-linkages to galactose. While human influenza viruses are prone to attach to Siaα2,6Gal constellations, AI viruses prefer Siaα2,3Gal linkages.

In the human upper respiratory tract, notably in the trachea, there are mostly α2,6-linked receptors and very few of α2,3 ones. However, in duck intestines where AI viruses replicate, a large amount of the latter is found.

Sporadically, AI viruses overcome the species barrier and infect people like in human cases of H5N1 (Egypt, Indonesia, Vietnam and others) and H9N2

(Hongkong). The pandemic strains of the 20th century (1918 H1N1; 1957 H2N2; 1968 H3N2) exhibited human-type receptor-binding specificity with HA molecules that originated from avian subtypes. This and the fact that HPAI H5N1 viruses that circulate in the Middle East, Europe and Africa have acquired a limited ability to attach to human α2,6-linked receptors suggest that the next pandemic might be set off by a H5N1 or H9N2 strain.

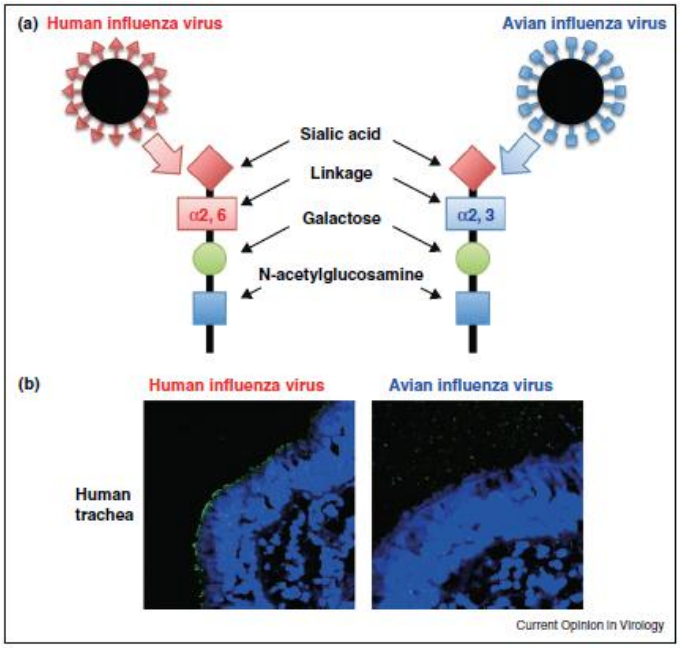


Figure 3: (a) Difference of linkage between the galactose and the sialic acid on cell surface receptors, determining the respective susceptibility either for human or avian influenza viruses. **(b)** Incubation of human trachea tissue sections with human or avian influenza virus and subsequent staining with antibodies and secondary fluorescent antibodies. Left image: The bright plating indicates virus binding [17].

2.1.5 Evolution of Avian Influenza Viruses – Antigenic Drift and Antigenic Shift

A characteristic of influenza viruses and one of the reasons why antiviral drug and vaccine development is very challenging is antigenic drift and shift. The viral polymerase complex has no proofreading activity and single point mutations in messenger RNA molecules occur frequently. This leads to slight alterations in the primary structure of one or more viral proteins and can cause substantial changes in the pathogenicity of a particle (antigenic drift). It is believed that LPAI viruses that have been introduced from wild birds into domestic poultry underwent

mutations in the hemagglutinin (HA) cleavage site. By doing so, HPAI viruses of the H5N1 subtype arose in domestic birds [16].
Antigenic shift denotes the exchange of whole gene segments between two viruses. This happens when an individual host cell gets infected by two identical or two different viruses at the same time and seems to happen preferentially in swine and ducks. Researchers assume that most human pandemic viruses arose this way. The first pandemic of the twenty-first century, a H1N1 from swine origin that killed more than 18,000 people and spread to 214 countries within a year [16], serves as an example of a virus that caught the world off-guard.
To what extend this novel pathogen affects humans (seasonal or pandemic characteristics, disease patterns, morbidity, and mortality) or if it doesn´t overcome pre-existing immunity is hard to predict. This demonstrates how dangerous and unpredictable newly-formed avian influenza viruses can be.

A way to respond viruses that circulate is annual vaccination of people. Researchers try to foresee which strain or strains might threaten the population during the upcoming flu season and adjust a vaccine correspondingly. There are even trivalent or quadrivalent influenza vaccines as a simultaneous protection against several strains seems to be appropriate. To what extend such yearly vaccines benefit public health is not always easy to assess since numerous studies are conducted in different conditions such as period of the year, geographical area, test persons and vaccine preparations. However, the conclusion has been drawn that annual flu vaccines can reduce the overall incidence of disease by about 60% [18].
If a newly designed vaccine protects from an infection by a field strain mainly depends on two aspects: 1) profile of the patient (age, state of health) and 2) how well the antibodies that are induced by the vaccine match the real pathogen. Therefore, the effectiveness of flu vaccines will continue to vary each year.

Besides, an easy way to lower the incidence of flu illness are strict hygiene habits such as covering your sneezes and coughs, the usage of paper instead of cotton handkerchiefs and regularly washing your hands with soap [18].

2.1.6 Clinical Signs in Birds

Clinical signs displayed by birds that are infected with avian influenza viruses can differ considerably. Factors influencing the course of the disease are the strain (Low/High Pathogenicity AI but also subtype), host family and subfamily (for example turkey vs. chicken), gender, age, presence of secondary pathogens (respiratory or digestive tract pathogens), management conditions and the route of inoculation.

In general, LPAI viruses create milder signs and cause less morbidity and mortality in infected flocks than HPAI strains. Physiological performances such as movement, vocalization, and egg production decrease only a little or not at all and postmortem examinations tend not to reveal prominent morphological changes. Non-specific symptoms like ruffled feathers and slight listlessness complicate the diagnostic in LPAI cases and require meticulous differential diagnostics. However, it has to be taken into consideration that LPAI viruses can cause severe symptoms and lead to death rates of 50% or higher, especially in young birds [19].

The clinical impact of LPAI in chickens will be discussed in more detail than HPAI infections as it is the object of this study.

Mainly three organ systems are affected: the respiratory, digestive, and reproductive systems. The pathophysiological changes in either one, several, or all three of these systems may result in clinical signs. The most frequent clinical signs seen are reduced activity, huddling, raised plumage, lethargy, and a drop in feed and water consumption [19]. Birds whose respiratory tract is affected can display coughing, rattles, rales, and heavy lacrimation in some cases. One of the most common lesions is the inflammation of mucosal tissues in the sinus, beak, and trachea and results in discharges of different qualities. In case the exudate blocks the airways, the chicken may die from asphyxiation. Bronchopneumonia is rarely seen and is normally the result of co-infections with secondary pathogens such as *Pasteurella multocida* or *Escherichia coli*. In severe cases, infected birds show purulent air sacculitis.

Within the reproductive tract, the production of follicles stagnates or regresses and congestion of the ovaries occurs. Prior to death, the last eggs laid by the dysfunctional uterus and an inflamed salpinx (oviduct) may be fragile and malformed, with a loss of shell pigment and reduced calcium levels [19].

HPAI viruses generally cause higher morbidity and mortality. By definition, these strains lead to very high mortality and can wipe out whole flocks within days without the appearance of clinical signs other than listlessness and agony. In order to estimate the pathogenicity of a field virus and for epidemic control decisions, the HA cleaveage site is sequenced. If this site within the HA gene consist of multiple basic amino acids, the virus is categorized as a HPAI virus. Clinical signs include nervous disorders like torticollis, opistotonus, and nystagmus; bile and urate stained droppings; cyanosis and hemorrhages causing discolorations on head, comb wattles, and shanks and precipitous decrease in egg production. Gross lesions include petechial stains in the epicardium, subcutaneous edema of head and neck, hemorrhagic pancreatitis, and splenic hyperplasia. Interestingly, respiratory disorders are less frequent and less prominent than in LPAI virus infections [19]. The clinical signs of AI can be very similar to other important diseases in poultry farming. Birds that are infected with the Newcastle Disease Virus (NDV) can show the same symptoms as AI. The disease is also named Pseudo Fowlpest. The Infectious Bronchitis Virus (IBV), a coronavirus, can cause respiratory signs, a decrease in egg production and is another differential diagnosis for AI. Another viral pathogen that leads to comparable clinical signs is the Gallid Herpesvirus 1 (GaHV-1), which causes Infectious Laryngotracheitis (ILT).

2.1.7 Approach to Epidemiological Processes using the example of HPAI H5N1

Influenza A viruses are circulating in humans and animal populations throughout the world, causing seasonal infections every year and therefore represent a serious health and economic burden. In addition, they were proven responsible for millions of deaths as in the 1918 Spanish Flu Pandemic [20, 21].

Influenza A viruses do not try to live in symbiosis with their human hosts since

they induce cellular death by necrosis and apoptosis [19]. This is followed by a quick elimination by the immune system or a fatal end. The reservoir from where viruses find their way into humans on a regular base must be found in a different species.

There is agreement that waterfowl are the natural source of all known subtypes [1-3, 19, 22], notably birds from to the orders Charadriiformes (gulls and shorebirds) and Anseriformes (waterfowl), although AI viruses have been found in more than 100 different bird and mammal species [1]. Areas where wild birds come into contact with domestic birds include farms situated in migratory flyways, or lakeshores where ducks are brought up feeding and resting with wild species. These areas allow viral transmission to poultry. In most cases, this transmission occurs by fecal-oral route via the water, sometimes termed "virus soup". Good examples of wetland areas located in a pathway of ducks are Poyang and Dongting lakes in eastern China [1]. Even if interspecies transmission of avian influenza viruses to mammals is not an uncommon event, the wild bird - domestic poultry barrier is passed much more frequently [1]. Clinical signs in domestic turkeys, ducks, geese and chicken – among others – can range from listlessness and production losses to heavy organ disorders and sudden death, whereas no overt disease is seen in waterfowl and virus is shed predominantly in the feces [23]. In early fall, in the Northern Hemisphere the virus prevalence peaks in birds at the moment or right before southbound migration, reaching values of up to 50% of infected individuals. In winter and spring time, these values drop almost zero with some birds that maintain a subclinical infection [24].

In highly pathogenic Avian Influenza (HPAI) H5N1 cases in humans, almost all confirmed 568 patients (as of 01/2015, WHO, 2003-15) had been in close contact to infected avian species before. Since 1997, the year of the first documented human HPAI case, ~59% of them succumbed to the H5N1 infection in 15 countries worldwide [16].

Another fact that supports the assumption of viral introduction from aquatic birds into humans via poultry kept in captivity is that all human pandemic viruses of the twentieth century contained gene segments of contemporary avian viruses in the moment of their emergence, as shown by phylogenetic analyses [1].

One of the key elements to stem the spread of AI infections in poultry and humans is a constant and nationwide surveillance of outbreaks. By quickly deploying reliable systems, like PCR analyses, lineage characterizations, and animal models, vaccines, biosafety measures, and culling, AI viruses are hindered from becoming endemic to terrestrial poultry in Europe and Northern America. According to Webster et al. [1], several subtypes such as H6N1 and H9N2 persist in Chinese domestic birds, where they have established multiple lineages since the 1990's. Subsequent to the HPAI H5N1 outbreak at Qinghai Lake in central China in 2005, antigenically and genetically distinct clades circulate constantly as an endemic pathogen in domestic poultry in large parts of Southeast Asia.

Fortunately, and maybe due to the strict disease control management, European and Northern American domestic bird populations do not perpetuate AI viruses although there have been outbreaks of both low and highly pathogenic AI in the past as well [1].

Two Worldwide Superfamilies

Krauss et al. [23] were the first to compare almost 250 different entire virus genomes from around the world and concluded that there are two 'superfamilies' of AI viruses with little exchange of genetic material: the Eurasian family, including Australia and Africa and, the Americas family. According to their findings, gene segments of a given virus can be classified as belonging to one of the two groups; and inter-hemispheric transfer is uncommon despite overlapping pathways in northeastern Russia and Alaska. In both superfamilies almost all combinations of HxNy could be detected, although the rarely isolated H14 and H15 antigens seem to exist in Eurasia only. Beside the separation into two geographical groups, AI viruses are classified according to the antigenic properties of their most prominent surface protein HA. H1,2,5,6,11,13, and H16 belong to Group 1, the viruses that express H3,4,14,7,10 and H15 to the second group. Concerns about the ability of migratory birds to scatter virus over tremendous distances were vocalized after the reemergence of HPAI H5N1 influenza virus in poultry in Japan, South Korea and Thailand in the winter of 2006-2007 [23] after the outbreak at Qinghai Lake (mentioned above). Only a few years prior to that, a

successful eradication campaign had been carried out.

In contrast, it should be noted that there is evidence that at least one of the HPAI H5N1 cases in Nigeria is due to importations of either frozen or live poultry. Furthermore, striking genetic similarity of a vaccine strain used in Central America and a japanese H5N2 isolate suggest that smuggled vaccine can cause long distances transmissions, in this case, since Japan bans the use of agricultural vaccines.

Hence, the introduction of H5N1 highly pathogenic AI viruses from Europe or Asia to the Americas through waterfowl or migratory birds is not very probable and could be caused by illegal or poorly controlled shipments of infected livestock [23].

Concerning humans, the mechanisms of human-to-human transmission of both LPAI and HPAI viruses are of particular interest to researchers and citizens. During infections of domestic poultry flocks, isolation methods can be applied quickly and epidemic control procedures such as culling are common, it is frightening to imagine what highly contagious people would mean for daily life. The exact underlying mechanisms of how sneeze droplets, aerosols, handshakes and suchlike work as a transmission vector were subjected to new studies [1] which revealed that influenza is less transmissible than previously thought. Transmission normally occurs, apart from annual fluctuations, from November to May in temperate regions and in less distinct patterns in tropical zones as there are no seasons like in Europe or Northern America. Young children make up the largest part of all infected patients although increasing rates are found among young adults who have a close contact to their children [1].

The suspicion that HPAI viruses can spread efficiently among humans has not been confirmed as there is no known case. Under laboratory condition in ferret models – the virus behaves similar in ferrets and humans – there was no aerosol transmission, except when a genetically modified HPAI H5N1 virus was used [25].

2.2 Low Pathogenic Avian Influenza H9N2 Virus

2.2.1 Geographical Distribution and Prevalence in Birds

H9N2 viruses are routinely found in wild birds and poultry, mostly in chicken, but also happen to infect mammals and humans. These LPAI viruses are widespread around the globe and were isolated in Germany, Italy, Portugal, Ireland, Finland, Sweden, Norway, France, Austria, Switzerland, Czech Republic, the Netherlands, South Africa, Russia, North and South Korea, Japan, Malaysia, Mongolia, Nepal, Vietnam, Canada, in the USA, Argentina, New Zealand, the Middle East (Kuwait, Saudi Arabia, United Arab Emirates, Iraq, Iran, Israel, Lebanon, Jordan, Egypt), in Libya, Tunisia, Pakistan, India, Bangladesh and China since the mid-1990s (as of 11/2014) [26-29].

Figure 4: Incidence of LPAI H9N2 viruses as of 11/2014. The grey circles indicate affected countries, not influenza epicenters. (Map provided [30].)

It has been shown that the spread of different lineages of AI viruses primarily depends on geographical influences, and less on host adaptions or temporal pressures [26]. In numerous countries where H9N2 infections in poultry have occurred, the disease has become endemic [29, 31, 32].

Examination studies showed that the prevalence of H9N2 in East and South East Asian countries reaches 7-8% in chickens in live bird markets in South Korea, followed by 16,5 % in retail markets in Bangladesh. Authors reported H9N2 prevalences of 13 % in chicken, 22% in minor poultry and 3% in ducks. Almost all samples (94%) that were tested AI positive in Bangladesh were of the H9N2 subtype [32].

2.2.2 Human Cases and Prevalence in People

Repeated zoonotic infections with H9N2 have been reported from 1998-2009 (as of 11/2010 [33, 34]). In 1999, for instance, in Hong Kong, China, two little children of one and four years of age had atypical AI viruses in their nasopharyngeal aspirates. Thorough analyses confirmed the presence of H9N2 [35]. The clinical signs including an inflamed oropharynx, vomiting, abdominal pain, fever below 40°C and poor appetite were self-limited and not life-threatening. The patients were treated in different hospitals and had lived geographically separated in Hong Kong, without having traveled to mainland China or abroad recently. In one of the patients, a contact with live chickens could not be excluded.

The same year, Peiris et al. investigated the titers of neutralizing antibodies against H9N2 in blood donor samples in Hong Kong. The fact that 2% of the people were tested positive suggests that they had had contact with the virus [35].

2.2.3 Evolution

In Southern China, a possible AI epicenter, H9N2 viruses divide into two lineages which are manly perpetuated by chicken and quail. Y280-like viruses, represented by a strain named A/Duck/Hong Kong/Y280/97 are predominately found in chicken, whereas quails rather tend to be susceptible for viruses subsumed under the term G1-like viruses, whose prototype is A/Quail/Hong Kong/G1/97 [36].

Li et al. showed that ducks that were infected with AI H9N2 had gene sequences for HA and NA surface proteins deriving from the Y280-like virus lineage. Their NS and M genes, however, originated from G1-like viruses. This suggests in both cases that gene material is transferred from terrestrial poultry, namely chicken and

quail, into aquatic birds, showing a movement of viral genetic information in the opposite direction than assumed previously. Until this moment, only a gene flux from aquatic birds to terrestrial poultry, pigs and men had been observed.
Beyond this, the ducks had H9N2 viruses with RNP genes that were closely related to a HPAI H5N1 strain that emerged in Hong Kong in 2001. Furthermore, they show parallels to HPAI H5N1 strains isolated in 1997 as the latter received 6 segments from H9N2 making them "most similiar in 6 of the 8 gene segments" [32]. Given this similarity of HPAI H5N1 viruses and LPAI H9N2 viruses, it is suggested that infections with the latter subtype may confer protection for HPAI H5N1 viruses and might have permitted them to circulate in poultry [32].
Li et al. provide evidence that ducks, situated on a point of bidirectional gene flow, may generate double or triple reassortants with amino acid signatures in their hemagglutinin that have a specificity for α2,6-Gal sialic acids residues, suggesting that the reassortants have the potential to infect people. As H9N2 viruses do not severely harm birds or people and spread without being noticed immediately but contribute to HPAI viruses, they are considered to be more, rather than less eligible to be at least partially responsible for a next pandemic and should be monitored continuously.

The current surveillance of newly emerged LPAI H7N9 in 2013 reveals similar findings in genetic analogy to H9N2: six of its internal genes are of avian origin and derived from H9N2. However, this upcoming strain poses new challenges as it does not cause clinical signs in poultry but leads to severe illness and death: at least 317 human cases have been reported with a mortality of 22% in humans (as of 01/2014 [37]).

Taken together, H9N2 viruses and their viral progeny threaten avian and human populations and highlight the urgent need of a better understanding of their diversity, the spread of emerging AI viruses and a reliable estimation of their pathogenic potential for the coming years.

2.2.4 Specific Clinical Signs

In comparison to other LPAI infections, H9N2 viruses do not cause strain specific clinical signs which could lead to a suspicion or confirmation of an infection with this subtype.

2.2.5 Pathogenesis in Different Species

Given that H9N2 viruses infected numerous species around the globe and have caused human infections, they are monitored continuously by the World Health Organization. They are considered a health threat for people due to repeated zoonotic infections and are therefore subjected to intensive research. A study conducted by Baranovich et al (SJCEIRS) [38] assessed the virulence and transmission of 12 different H9N2 viruses (see Table 1) in experimental conditions on normal Human Bronchial Epithelial Cells (NHBE) and in ferrets, mice, pigs and ducks. The study also dealt with the effectiveness of commercially available antivirals (all tested H9N2 viruses were sensitive to NA inhibitors, some were resistant to Adamantanes).

As expected, viruses from human origin grew to higher titers on human bronchial epithelial cells than viruses that originated from avian hosts. The swine/HK virus grew to the same titers as the human strains, but with a growth delay. In mice, most avian strains replicated at or below the limit of detection. Only the mice that had been inoculated with chicken/Beijing showed viral titers similar to those induced by mammalian strains. However, all virus strains caused seroconversion. In pigs, no clinical symptoms could be observed and none of the pigs seroconverted but a direct transmission could be seen in one case (with the human virus HK/33982), as well as moderate shedding titers in another single animal infected with HK/33982. The transmission experiments in ferrets revealed that only viruses from mammalian origin transmitted, and only by direct contact. Among the H9N2 viruses tested, aerosol transmission did indeed not occur. Only a single virus led to detectable shedding in ducks (shorebird/DE), suggesting that LPAI H9N2 viruses are almost fully adapted to gallinaceous poultry.

Table 1: Tabular overview of virus clades and strains, performed experiments, animal species and risk scores [38].

		Experimental setting and measures										
		NHBE cells	Ferrets			Mice		Ducks	Pigs			
Clade	Influenza virus	Score[a]	Score[b]	SC[c]	Trans[d]	Titer	SC[e]	Titer[f]	Trans	Risk score	Residues 226/228	Risk[g]
North Amer	Shorebird/DE	1.75	1	1	ND	0	1	2	0	5.75	L/G	Low
Chicken/Beijing	Chicken/Beijing	1	2	1	ND	2	1	0.5	0	7.5	Q/G	Low
	Chicken/HK/G9	2	2.5	1	0	1.5	1	1.5	0	9.5	L/G	Int
	Swine/HK	2.5	3	1	1	3.5	1	0.5	0	12.5	L/G	High
	Duck/Nanchang	1	4	1	ND	0.5	1	0.5	ND	8.0	L/G	Low–Int
Y280	Chicken/HK/TP38	2	2.5	1	ND	0.5	1	0.5	ND	7.5	L/G	Low
	Chicken/Nanchang	1	4	1	0	0	1	1	0	8.0	L/G	Low–Int
	Guinea fowl/HK	2.5	2.5	1	ND	1	1	0.5	ND	8.5	L/G	Low–Int
	HK/33982	4	4	1	1	3	1	0	1.5	15.5	Q/G	High
G1	HK/1073	4	4	1	1	4	1	0	ND	15	L/G	High
	Chicken/Dubai	1	2	1	ND	0.5	1	0	ND	5.5	L/G	Low
	Quail/Bangl	3	1	1	0	1	1	0	ND	7.0	L/G	Low

[a] Highest titers, the fastest kinetics indicated by score=4.
[b] Highest titers, the longest duration indicated by score=4.
[c] Seroconversion (SC) in ferrets was determined by HI assay.
[d] 1=direct contact (DC) transmission in one animal; 2=DC transmission in two animals; 3=aerosol-contact (AC) transmission in one animal; 4=AC transmission in two animals; 0, no transmission; ND, not done.
[e] Seroconversion (SC) in mice was determined by enzyme-linked immuno sorbent assay.
[f] 1=2 ducks shed for 1 day; 2=2 ducks shed for 2 days.
[g] Categories of risk were defined as follows: low=5–8; intermediate (Int) =8–10 or >10 with Q226; high ⩾10; very high >10 with L226.

2.2.6 Transmission

Negovetich et al. collected more than 5700 samples in Bangladesh, and found that there were more oropharyngeal than cloacal swabs positive for influenza [32]. They drew the conclusion that direct transmission and aerosol carryover are the predominant modes of transmission. Direct contact transmission could be observed in chickens and ferrets and is considered to be the route that lead to human infections [32, 39]. In comparison, aerosol transmission happens in chickens but has not been seen in ferrets, indicating that human to human transmission is limited at the moment [32].

2.3 MVA Vector Vaccine

The « Modified Vaccinia Virus Ankara », abbreviated MVA, serves as a safe and versatile viral vector system for vaccine development. MVA can be equipped with heterologous gene sequences in order to produce foreign proteins [40-45]. MVA was derived from the conventional Vaccina Virus Ankara through serial passages on chicken embryo fibroblasts (CEF). During this procedure, the virus had lost about 27 kilo bases of genetic information as demonstrated by six major genomic deletions. Resulting MVA became highly attenuated and replication-deficient in cells of mammalian origin.

By using a transfer plasmid, an artificially produced genetic sequence can be placed under the transcriptional control of an MVA-specific promoter and inserted into the virus' genome by homologous recombination [46].

The MVA vector has become replication-incompetent and avirulent due to the loss of many viral genes encoding for proteins interfering with host (cell) immune functions. Therefore, MVA was established as particularly safe viral vector without possibility to regain its pathogenic potential by further passaging [47].

In large field trials, over 120.000 humans have been successfully vaccinated with MVA [48]. It therefore serves as non-replicating, safe, and efficient expression vector and can be handled under biosafety level 1 in Europe and the USA.

To date only very few studies have used MVA vaccines in chicken or other birds. Yet, a first candidate recombinant MVA vaccine delivering AI H5 was proven to be immunogenic and protective in chicken vaccine experiments [40, 49].

2.3.1 Ancestral HA strategy

Due to the genetic but also antigenic diversity of many avian influenza viruses, it is challenging to find cross-reactive vaccines that would protect against several strains at a time within a given HxNy subtype. The most potent antibody response targets the most exposed but also the most variable viral protein: HA. At present, the World Health Organization seeks to keep pace with the continuous

diversification by producing new vaccine seed strains. Economic restrictions, but also the impossibility to stockpile a large number of different vaccination doses fuel the research on new approaches.

One of the possible strategies to obtain cross-reactive influenza vaccines is to use “ancestral” antigens. Ancestral sequences are artificial computationally derived amino acid (aa) sequences that would be located at the node of phylogenetic trees, for example the ancestral HA sequence used for the challenge (HAanc). Ducatez et al. generated ancestral H5N1 vaccines (whole virus inactivated vaccines) that conferred cross-clade protection for morbidity and mortality in the ferret model [50]. In the present study, we aimed at generating an ancestral cross-reactive MVA-H9 vaccine.

3. Material and Methods

Unless otherwise indicated, the laboratory procedures were carried out in strict accordance with the Manual on Animal Influenza Diagnosis and Surveillance of the World Health Organization [37].

The objectives of the strain selections for the phylogeny was to include a representative subset of all G1-like strains and to keep the number of sequences reasonable to be able to read the phylogeny and run marginal reconstruction algorithms.

3.1 Vaccine Production

3.1.1 Provision of Vaccine Components

The MVA vector platform was provided by the Institute for Infectious Diseases, LMU University of Munich, Germany. The ancestral sequence originates from the National Veterinary School in Toulouse, France. The cloning of the ancestral H9 (H9anc) sequence into the vector, the testing of its genetic purity and stability as well as the analysis for correct expression were done in Munich. The MVA-H9 vaccine was then ready to use and shipped to France.

3.1.2 Dataset selection and Phylogenetic Analyses

We downloaded the nucleotide sequences of HA genes of LPAI H9N2 viruses and aligned them with ClustalW [51]. We included in the phylogenetic tree reference H9N2 HA sequences as well as representative strains from the Middle East (Egypt, UAE, Israel, Iran), Tunisia and from the Indian subcontinent (G1-like strains). Estimates of statistical support for the observed phylogenies were calculated by performing 1,000 bootstrap replicates.

3.1.3 Generation of Ancestral Sequence

Nucleotide alignments are the base for the calculation of amino acid sequences. We used the ANCESCON computer program with a marginal reconstruction method to calculate ancestral sequences. The method was used with default settings except that a maximum likelihood rate factor was chosen. The ancestral protein sequence that is finally determined is located at a node point of several strain branches. To generate the HA nucleotide sequence that expresses the putative ancestral proteins, the amino acid (aa) sequences were compared with aa sequences available in GenBank using BLAST and served to convert the ancestral sequence into a nucleotide (nt) sequence at positions where several nt triplets were theoretically possible. The aa homology between the H9anc sequence and the HAtun sequence is 94%.

3.1.4 Generation of recombinant MVA vaccine

Generation, clonal isolation and in vitro characterization of the recombinant MVA (rMVA) vaccine and the purification and quality control of vaccine preparations were using established methods and protocols [46]. Briefly, plasmid vectors were used to insert the recombinant H9 gene sequence into the DNA MVA genome. They were transfected into MVA-infected cells where homologous recombination between plasmid DNA and MVA occurred. Plasmid vectors contained MVA flanks, authentic start and stop codons, the ancestral H9 sequence and a VACV-specific promoter, which arranges the right orientation of the recombinant gene. In order to harvest the highest possible amount of rMVA and obtain it as clonally pure as possible, well-separated viral foci from the wells infected with the highest dilutions were chosen for picking.

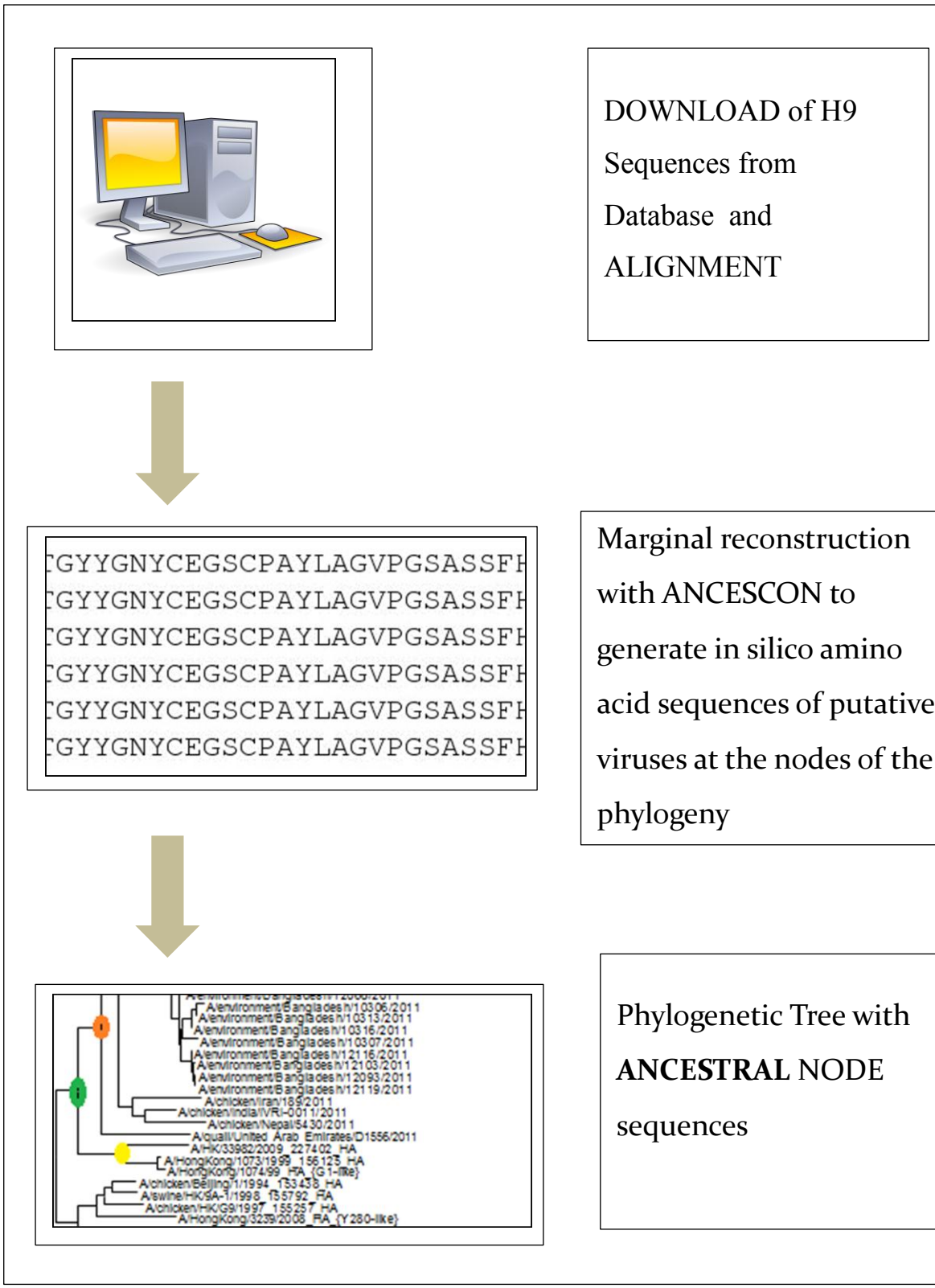


Figure 5: Schematic procedure to create an ancestral sequence by using National Center for Biotechnology Information Influenza Virus Resource Sequences and the ANCESCON Computer Programm.

3.1.5 Purification, Titration and Quality Control of the rMVA

To remove cellular debris and recombinant proteins the virus solution was frozen, thawed, and ultra-centrifuged through a sucrose cushion. The titration of infectivity of MVA stock preparation was done on CEF. Using a specific immune-peroxidase staining of cells that contained viral antigen, viral foci could be detected. The correct location of the inserted gene was checked by PCR and protein expression verified by Western Blot.

3.2 Birds and Challenge Virus Strain

3.2.1 Birds

44 one-day old specific pathogen free (SPF) White Leghorn chickens were purchased at the Institut National de la Recherche Agronomique (INRA) – Plateforme d'infectiologie expérimentale (PFIE), Nouzilly, France. As listed below, the breeder assures that the chickens are free from certain pathogens. For this study, it is necessary to work on animal flocks that have never been in contact with AIV before as antibodies that exist due to maternal immunity could interfere with the vaccine.

Table 2: Pathogens SPF chickens are screened for before being used for scientific purposes.

Microorganism	Detection method	Number and Frequency
Salmonella pullorum	Serum Agglutination Test	100% at 20-21 weeks of age + 6% every 8 weeks
Mycoplasma gallisepticum	Serum Agglutination Test	
Mycoplasma synoviae	Serum Agglutination Test	
EDS 76	HI	
Adenovirus	Gel Immunodiffusion	
Infectious Bursal Disease Virus/ Gumboro	Gel Immunodiffusion	

NDV	HI	32% (at 20-21 weeks) + 6% (every 8 weeks)
Influenza	Gel Immunodiffusion	32% (at 20-21 weeks) + 6% (every 8 weeks)
Infectious Bronchitis	Gel Immunodiffusion	100% (at 20-21 weeks) + 6% (every 8 weeks)
Infectious Anemia	ELISA	32% (at 20-21 weeks)
Avian Leucosis	ELISA (P27)	100% (cages at 24-26 weeks) + 2 eggs/ hen average
ILT	ELISA	32% (at 20-21 weeks)
Marek Disease	PCR	6% (at 8-10 and 20-22 weeks) + 6% of livestock (every 8 weeks)

3.2.2 Challenge Strain A/chicken/Tunisia/12/2010

A/chicken/Tunisia/12/2010 is an AI virus strain that expresses the hemagglutinin 9 and the neuraminidase 2 and belongs therefore to the H9N2 subtype. In 2010, it was isolated from a chicken in Tunisia, brought under the applicable safety measures to France and is currently stored at -80°C in the laboratory of virology of the ENV Toulouse. In order to make sure that the virus did not undergo genetic changes during the preparation of the experiment, we sequenced the hemagglutinin segment right before the challenge of the birds and could confirm genetic identity.

3.3 Immunization and Challenge

3.3.1 Setup and Protocol Timeline of the Animal Experiment

Figure 6 shows an overview of the course of the animal experiment. In total, it lasted 8 weeks and included the sampling of 183 blood tubes, 204 oropharyngeal swabs and the performance of 12 necropsies.

The birds were kept in within the animal facilities (biosafety level 2) of the ENV Toulouse and fed ad libitum with a commercially available mixture of poultry food according to their age. Each bird was individually tagged on the foot.

3.3.2 Vaccine Dose and Route of Inoculation

The birds from group A were vaccinated and boosted at day -21 and day 0 respectively with a dose of 10^8 PFU (100 µl) each by intramuscular route. The same route and dose was used for the birds from group B, which were vaccinated once at day 0. Each bird was infected with 10^9 EID_{50} A/chicken/Tunisia/2010 in 500µl total by ocular, nasal and intratracheal route.

3.3.3 Observation of Clinical Signs

Throughout the experiment the birds were monitored at least once a day to evaluate the clinical signs they may display. We paid attention to any kind of pathology but especially to disorders of the respiratory system. Clinical signs we expected ranged from none at all to rattles, light gasping and coughing as well as seromucoid airway exudates.

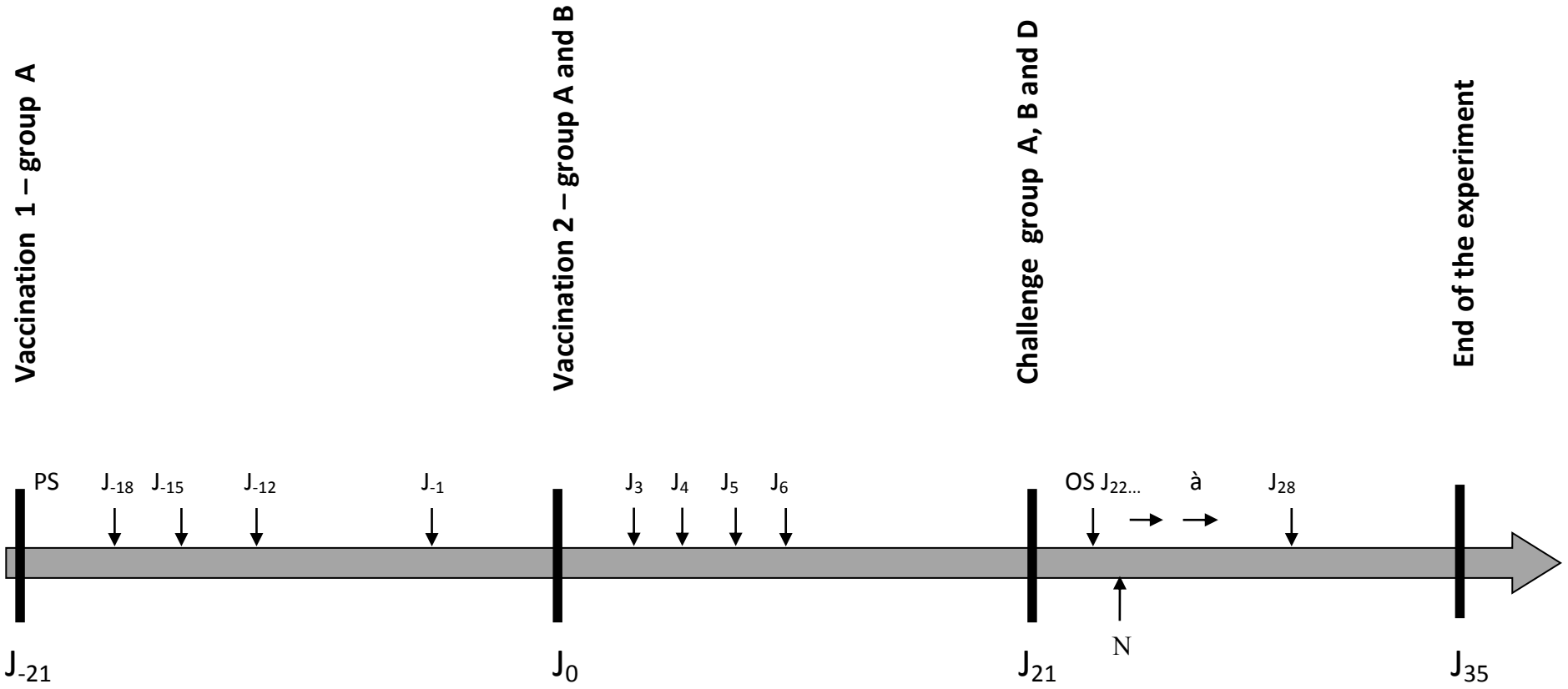
3.3.4 Swab Sampling

We used soft cotton swabs to get saliva and epithelial cells of the mucous membrane of the oral cavity. In order to avoid bleedings and suffering of the animals only oropharygeal swabs were preferred to tracheal swabs. Immediately after rubbing during approximately 5-8 sec, they were stored temporarily in 1 ml of refrigerated PBS supplemented with antibiotics (penicillin, streptomycin) and a 140 µl aliquot was collected for further RNA extraction before both tubes were frozen at -80°C. Thus, the following RNA extraction was performed on single thawed virus samples.

3.3.5 Necropsy

On day 25, i.e. four days post infection, we euthanized three birds per group to perform an autopsy. After a closer look on the general health status (plumage,

nutritional status, orifices...) we examined the inner surface of the abdominal cavity, the air sacs, the peritoneum of the organs and other tissues that are prone to suffer damage from an AI infection. For histological analyses, we preserved samples of the trachea, lungs with rip cage to avoid pulmonary collapse, small intestine and cloacal bursa in 10% formalin.



Group A : double vaccination, challenged
Group B : simple vaccination, challenged
Group C : not vaccinated, not challenged
Group D : not vaccinated, challenged

Necropsies at J_{24} of 3 chicken per group

TS from J_{22} to J_{28} for groups A, B, D

PS from J_{20} to J_{35} for all animals

Key: J= day, PS= blood sample, OS= oropharyngeal swab, N= Necropsie

Figure 6: Timeline of the animal experiment. Total duration: 8 weeks. Blood sampling (PS) at day (J) -22, -18, -15 etc. (red=group A, blue=group B). Oropharyngeal swabbing (OS) was performed from days 22-28. Necropsy (A) at day 25.

3.3.6 Histopathology and Immunohistochemistry

All animals were subjected to a complete post-mortem examination. Tissue samples of trachea (one transversal section in the proximal portion and another one in the terminal portion), lungs and caecum were taken and stored in 10 % neutral formalin. After fixation, tissues were routinely processed in paraffin blocks, sectioned at 4 µm and stained with hematoxylin and eosin (H&E) for microscopic examination. Lesions were assessed histologically and their intensity was graded as: no lesion, minimal, slight, moderate, marked or severe.

Immunostainings were performed on paraffin-embedded sections of trachea with a monoclonal mouse anti-nucleoprotein Influenza A virus antibody (Argene, 11-030, pronase 0,05 % retrieval solution, 10 minutes at 37°C: antibody dilution 1/50, incubation overnight, at 4°C). The immunohistochemical staining was revealed with a biotinylated polyclonal goat anti-mouse Immunoglobulins conjugated with horseradish peroxidise (HRP) (Dako, LSAB2 system-HRP, K0675) and the diaminobenzidine chromagen of the HRP (Thermo Scientific, TA-125-HDX).

3.4 Virus Shedding Titration

3.4.1 RNA extractions

The Qiagen QIAamp® Viral RNA Kit (third edition) was used for the RNA extraction on the QIAvac® 24 Plus vacuum system.

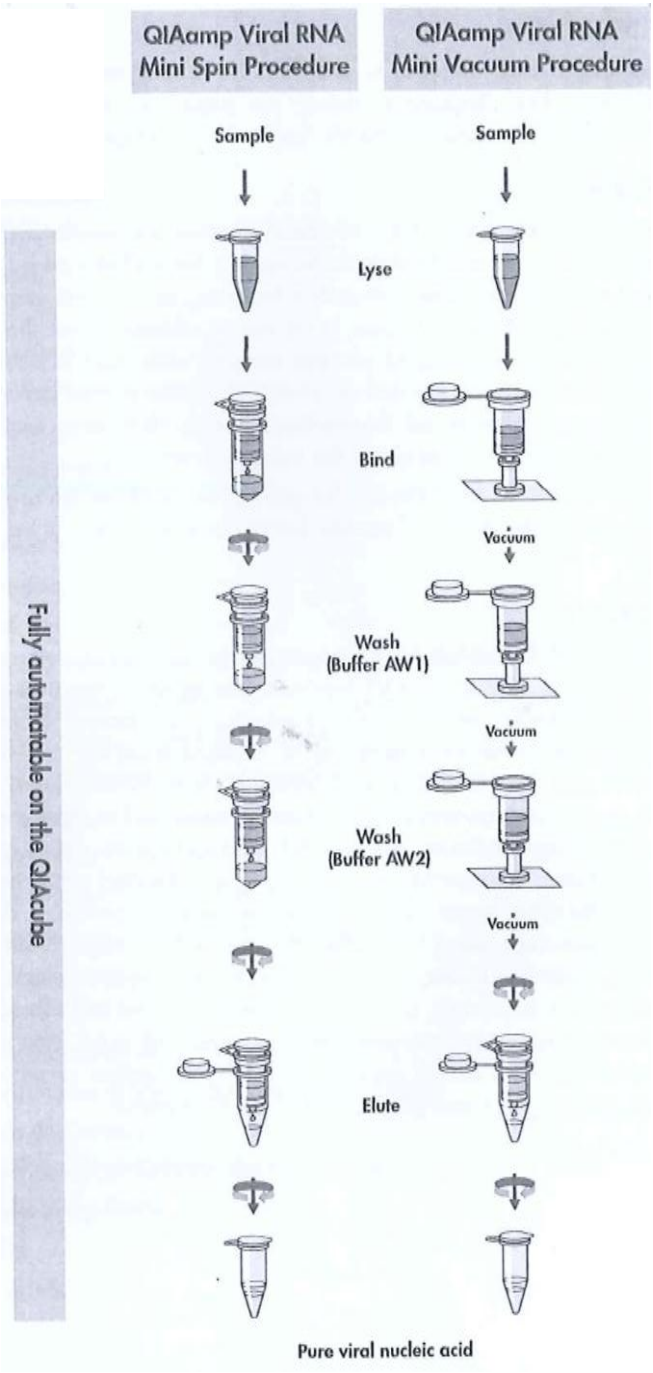


Figure 7: Process of viral RNA extraction with the Qiagen QIAamp® Viral RNA Kit (third edition) was done as shown on the right side.

3.4.2 Reverse Transcription Real-Time Polymerase Chain Reaction (rt-RT-PCR)

We used the following one-step rt-RT-PCR amplification protocol and program to detect H9 AI viruses in the oropharyngeal swabs. The primers targeted conserved sequences of the matrix gene [52].

A known amount of plasmid (StrataClone Blunt PCR Cloning Vector pSC-B-amp/kan, Agilent Technologies) that contained the same matrix gene sequence was serially diluted. The resulting standard curves were compared to the curves obtained from the swab samples and allowed a calculation of the numbers of RNA copies with the following formula:

$$Number\ of\ RNA\ copies = \frac{6{,}022 * 10^{23} * template\ concentration\ (\frac{g}{\mu l})}{650 * (length\ of\ plasmid + length\ of\ insert\ (bp))}$$

Component	**Amount (for one reaction)**
Sybr G Quantitect Qiagen Mastermix (2x)	5 µl
RT-mix	0,1 µl
Forward primer: M52C (10 µM)	0,5 µl
Reverse primer: M253R (10µM)	0,5 µl
RNA free water	1,9 µL
RNA	2 µl
Total volume	**10 µl**

The RT-qPCR was run using the following program on a Roche LightCycler® 480.

Step Name	Temperature (°C)	Acquisition Mode	Number of Cycles	Duration (sec)
RT	50	none	1	1800
Activation	95	none	1	15
PCR	95	none	40	15
	60	single		40
Melting Curve	95	none	1	5
	65	none		60
	97	continuous		-
Total duration of the program: 1 hour 54 minutes				

3.5 Serology

3.5.1 Hemagglutination Inhibition (HI) Assay

In each HI Assay, the components added up to a final volume of 100 µl with a constant amount of virus solution (25 µl) and chicken red blood cells (RBC, 50 µl). The sera were serially diluted with PBS starting with a 1:10 receptor destroying enzyme (RDE) mix (25 µl) to remove unspecific inhibitors and gain better results. In case the antibodies match the antigen and the titer is high enough to link all viruses, the subsequently added RBC will sink to the bottom of the V-shaped well: the hemagglutination has been inhibited by the antibodies.

3.5.2 Seroneutralization

The seroneutralization assay is an assay to analyse virus-specific antibodies by visualizing the effect that a specific antibody-antigen mixture has on a cell monolayer.

AI H9N2 viruses A/chicken/Tunisia/2010 were mixed with serial dilutions of sera preparations and added after incubation to Madin-Darby Canine Kidney (MDCK) cells. During the following days, a cytopathic effect may be observed if 1) the serum antibodies do not match the antigen and do not inactivate the virus or 2) the serum antibody titer is too low to bind all viruses. This test allows for detection and titration of neutralizing antibodies.

In comparison to an in vivo study, the loss of cells can easily be observed, whereas in an animal model a cytopathic effect can be there but invisible because it does not cause any clinical signs.

4. Results

4.1 Vaccine design

Figure 8 shows a phylogenetic tree of LPAI H9 sequences. It includes 117 strains from 11 different countries that were isolated between 2011 and 2013. The majority belongs to G1-like H9N2 viruses, others are from the Y280-like group and few are wild bird strains (neither G1-like nor Y280-like viruses). The highlighted node #126 is the "G1-H9 ancestor", it subsumes 104 sequences and is a computationally derived, ancestral sequence that was chosen for insertion into the MVA genome to construct the recombinant MVA vaccine.

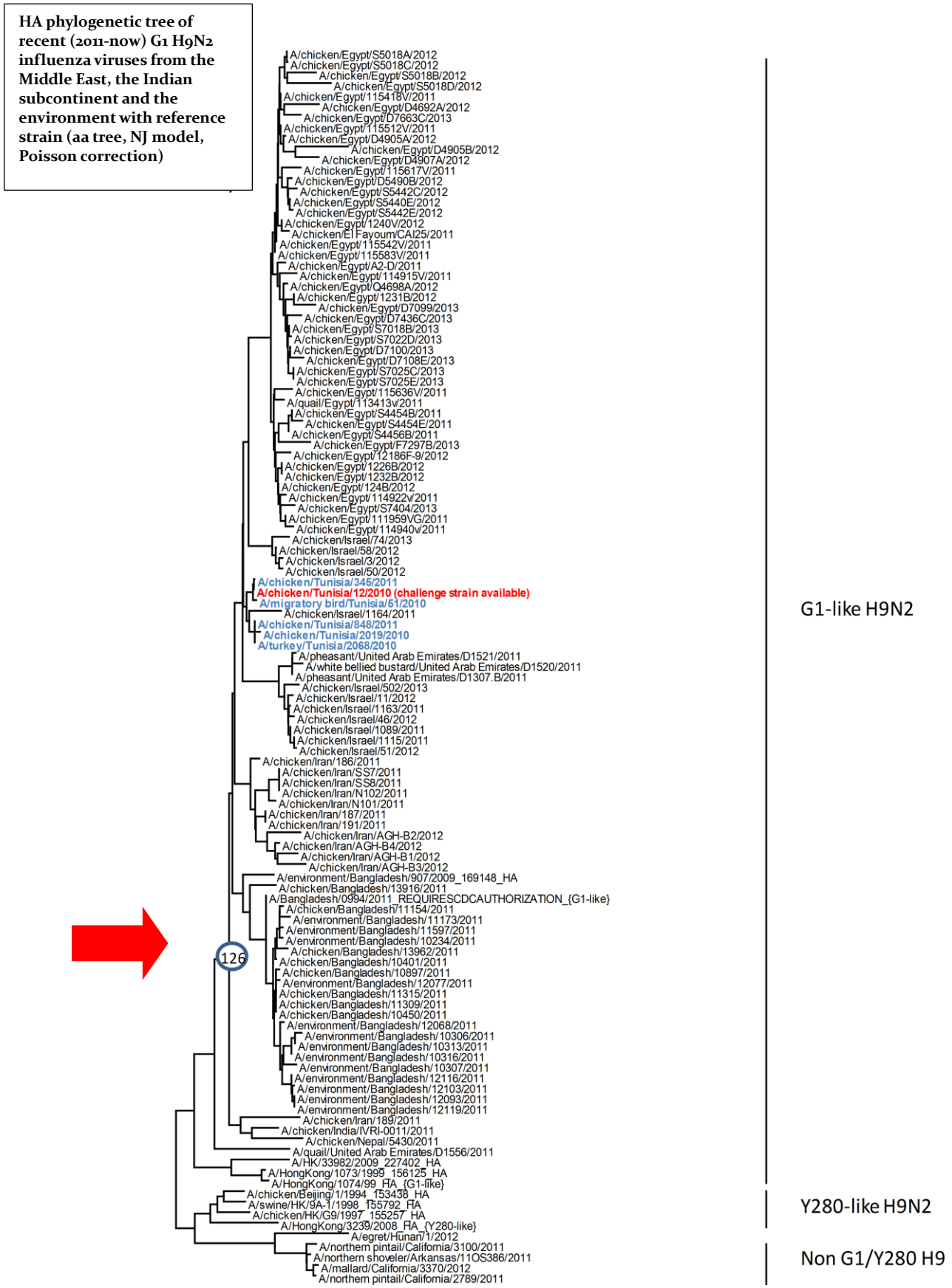


Figure 8: Phylogenetic tree of H9N2 viruses. The highlighted node (#126) corresponds to the ancestral sequence that formed part of the vaccine.

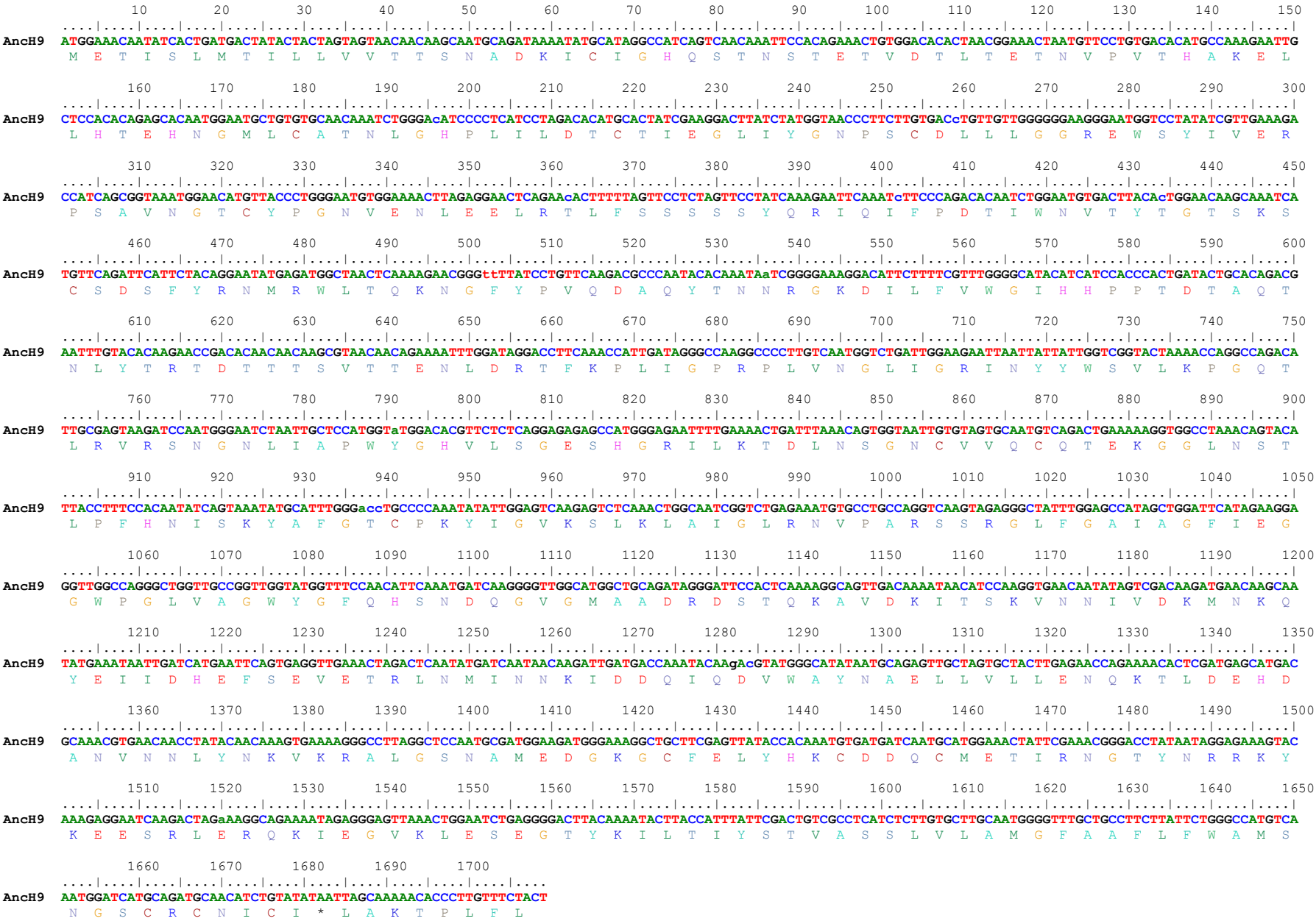


Figure 9: Full ancestral sequence with according one-letter-code nucleotides and correlative numbering.

4.2 Clinical Appearance

4.2.1 Clinical Signs

Throughout the eight weeks of the animal experiment, not a single chicken of group A (double vaccinated), group B (single vaccinated) and group C (control group) displayed any clinical signs. However, in group D chickens number 3 and 10 showed light gasping on day 3. Bird number 5 showed comparable signs on days 4 and 5.

4.2.2 Macroscopic Lesions

During the necropsy, none of the chicken showed macroscopic lesions, regardless of the group.

4.2.3 Histopathology

The main histopathological lesions were observed in the trachea and the bronchi. These lesions consisted of diffuse to multifocal mild tracheitis and multifocal to focal mild bronchitis (all lesions are summarized in figures 11-12).

The main elementary lesions of subacute tracheitis were:

- Loss of ciliature,
- Focal necrosis and exfoliation of the superficial mucosal epithelium,
- Regenerative epithelial hyperplasia,
- Squamous epithelial metaplasia,
- Inflammatory cellular infiltrates in the lamina propria with mononuclear cells (lymphocytes, plasmocytes and macrophages) and a few heterophils.

In the lesions of subacute bronchitis, regenerative epithelial hyperplasia and inflammatory cellular infiltrates in the lamina propria with mononuclear cells (lymphocytes, plasmocytes and macrophages) and a few heterophils were also seen.

Double vaccinated group A birds show only a partial loss of ciliature, whereas group C control birds were entirely healthy. The strongest lesions were seen in the unvaccinated group D with all animals displaying tracheitis and bronchitis (multifocal moderate to diffuse marked inflammations). Intermediate respiratory lesions and lesions of the digestive tract were observed in group B chickens only.

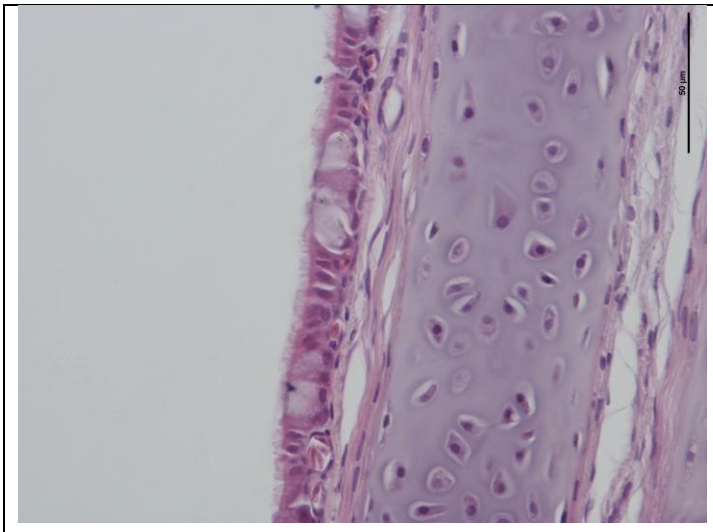


Figure 10: Healthy control bird C8. Trachea, hematoxylin & eosin X 50): tracheal epithelium with ciliature.

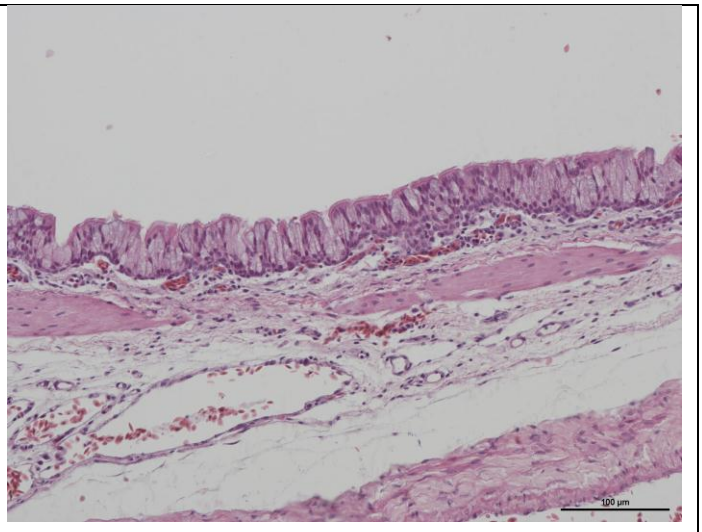


Figure 11: Healthy control bird C8. Bronchus, hematoxylin & eosin, X50): bronchial epithelium with ciliature.

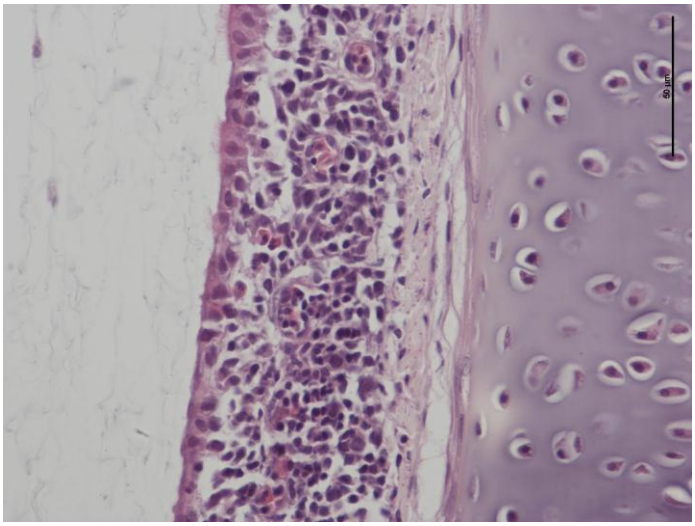


Figure 12: A10, trachea, hematoxylin & eosin X 50): focal slight subacute tracheitis with loss of ciliature of some epithelial cells and small inflammatory cellular infiltrates in the lamina propria with mononuclear cells (lymphocytes, plasmocytes and macrophages) and a few heterophils.

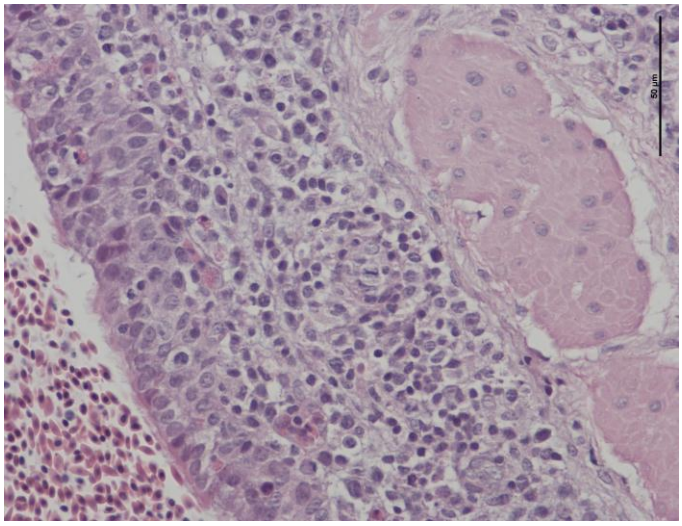


Figure 13: A10, bronchus, hematoxylin & eosin, X50): focal moderate subacute bronchitis with regenerative epithelial hyperplasia and inflammatory cellular infiltrates in the lamina propria (mononuclear cells (lymphocytes, plasmocytes and macrophages) and a few heterophils).

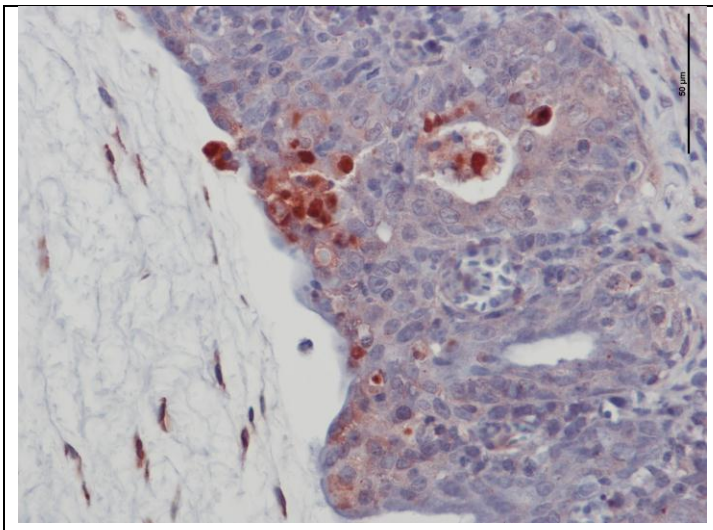


Figure 14: D11, trachea, immunoperoxydase with a monoclonal mouse anti-nucleoprotein Influenza A virus antibody, x50): labelling of the nucleus of epithelial cells (chromogen: aminoethylcarbazole).

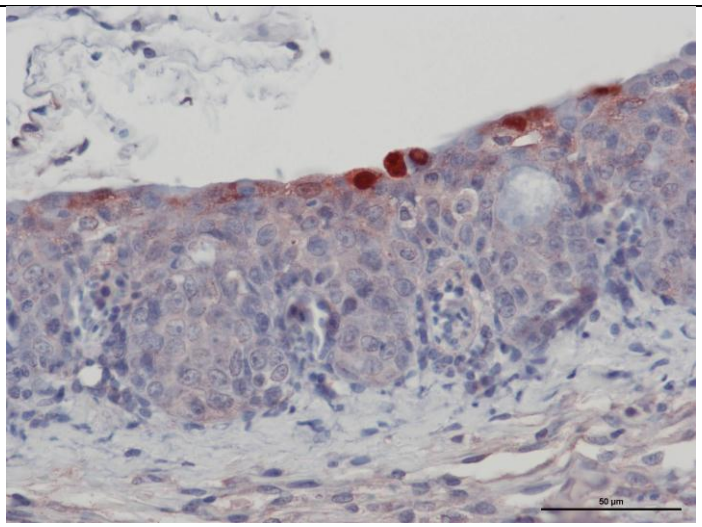


Figure 15: D11, trachea, immunoperoxydase with a monoclonal mouse anti-nucleoprotein Influenza A virus antibody, x50): labelling of the nucleus of epithelial cells (chromogen: aminoethylcarbazole).

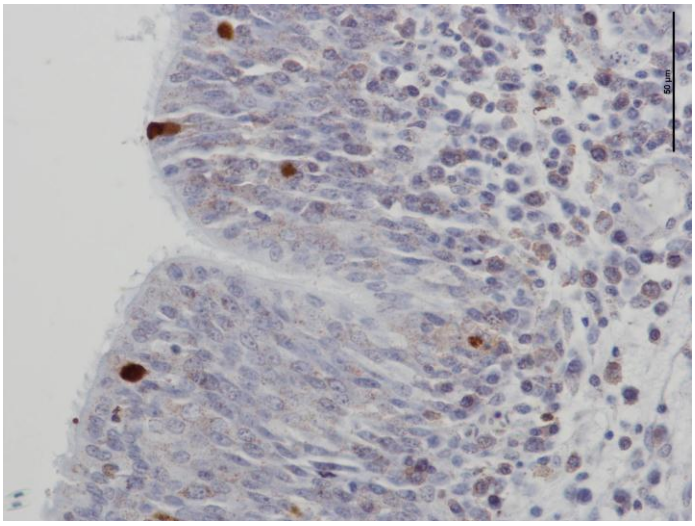


Figure 16: D11, bronchus, immunoperoxydase with a monoclonal mouse anti-nucleoprotein Influenza A virus antibody, x50): labelling of the nucleus of epithelial cells (chromogen: diaminobenzidine).

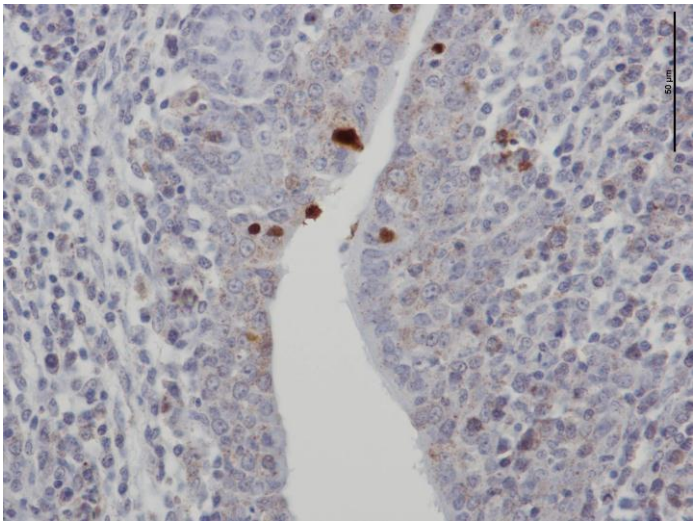


Figure 17: D11, bronchus, immunoperoxydase with a monoclonal mouse anti-nucleoprotein Influenza A virus antibody, x50): labelling of the nucleus of epithelial cells (chromogen: diaminobenzidine).

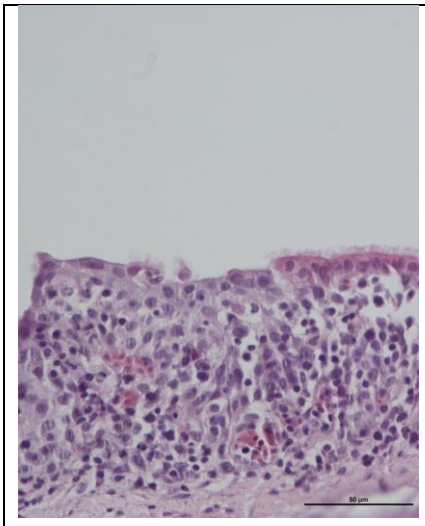


Figure 18: D9, trachea, hematoxylin & eosin X 50): diffuse marked subacute tracheitis with loss of ciliature of many epithelial cells and large inflammatory cellular infiltrates in the lamina propria with mononuclear cells (lymphocytes, plasmocytes and macrophages) and a few heterophils.

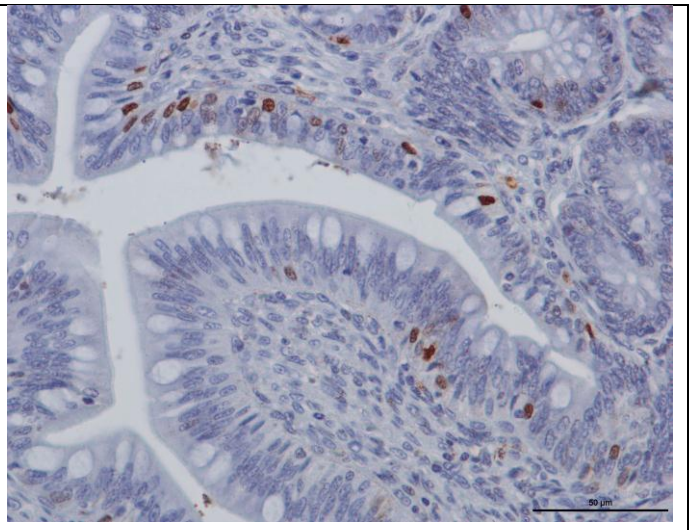


Figure 19: B11, caecum, immunoperoxydase with a monoclonal mouse anti-nucleoprotein Influenza A virus antibody, x50): labelling of the nucleus of some epithelial cells (chromogen: diaminobenzidine).

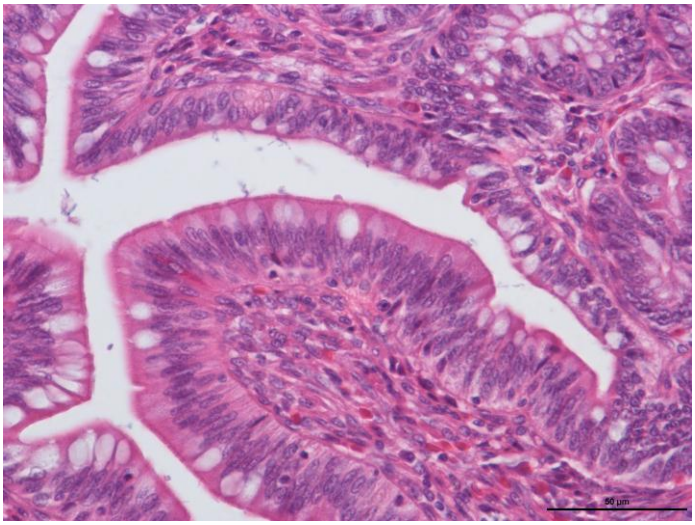


Figure 20: (B11, caecum, hematoxylin & eosin, X50): no histopathological lesion.

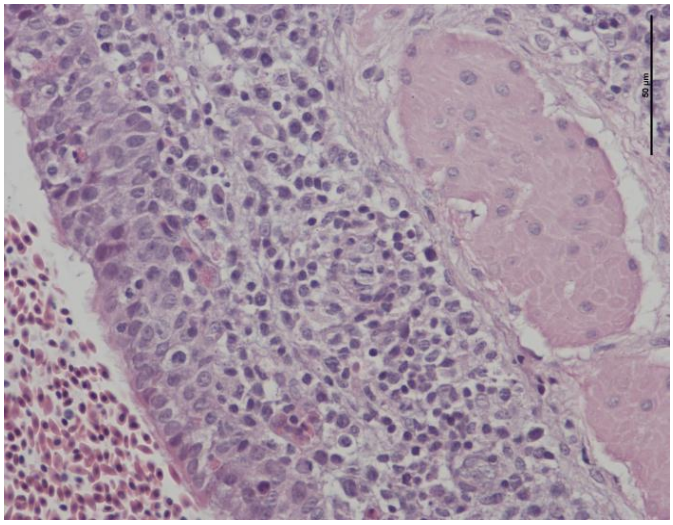


Figure 21: (A10, bronchus, hematoxylin & eosin, X50): focal moderate subacute bronchitis with regenerative epithelial hyperplasia and inflammatory cellular infiltrates in the lamina propria (mononuclear cells (lymphocytes, plasmocytes and macrophages) and a few heterophils).

4.3 Virus Shedding

4.3.1 Group Mean Virus Titres

Figure 22 and 23 show the group average virus titers form day 1 to 7 post infection (pi). In both diagrams the same data was used. Three chickens per group were euthanized on day 4 pi.

The viral shedding peaked on day 4 pi with $9.35x10^3$-$11.46x10^3$ RNA copies. The curves then dropped similarly to values of ~30-100 copies on day 6 pi, whereas on day 7 the vaccinated groups had virus titers at or below the limit of detection. Group D birds (not vaccinated, challenged) showed slightly higher titers.

Figure 23 accentuates the longer clearance of the D group. The last two columns are significantly higher than in group A or B. In addition to that,

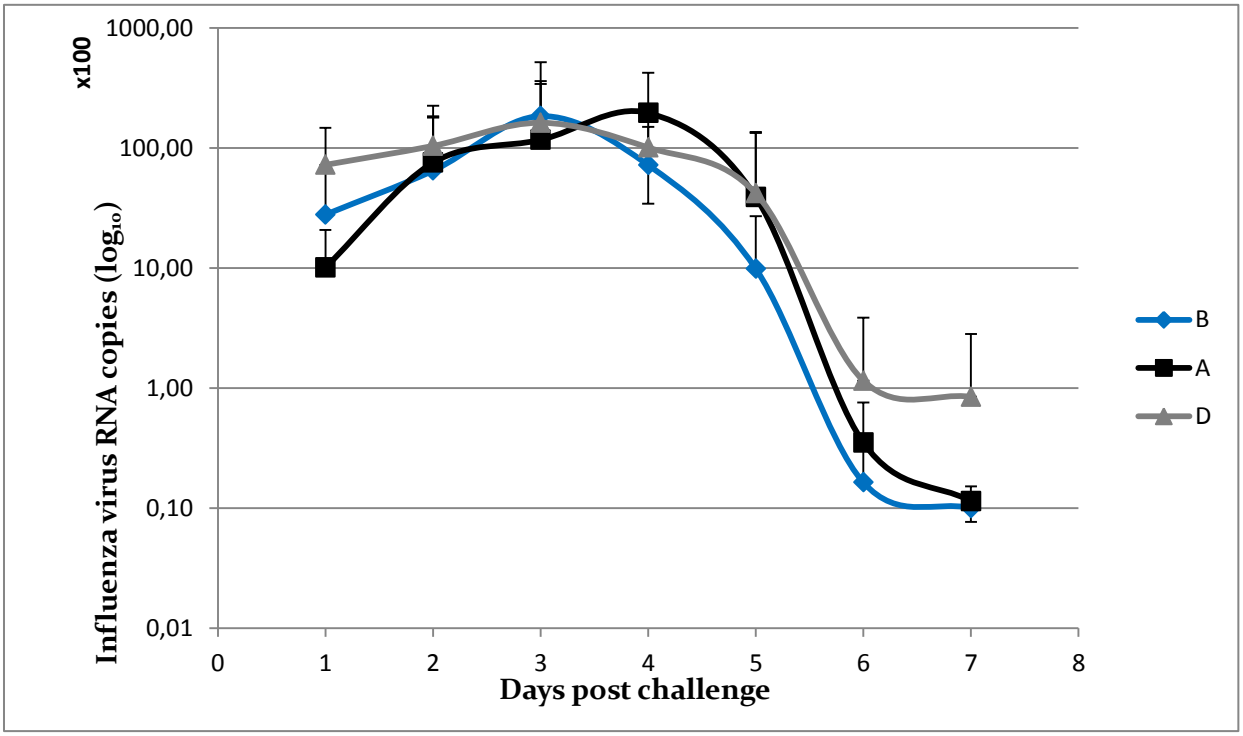


Figure 22: Group average virus titers for all challenged birds from day 1 to 7 post infection. Each line represents one group: A (double vaccinated in black), B (single vaccinated in blue), D (not vaccinated in grey). Virus titers at day 7 are slightly higher (1 log) in group D than in the others.

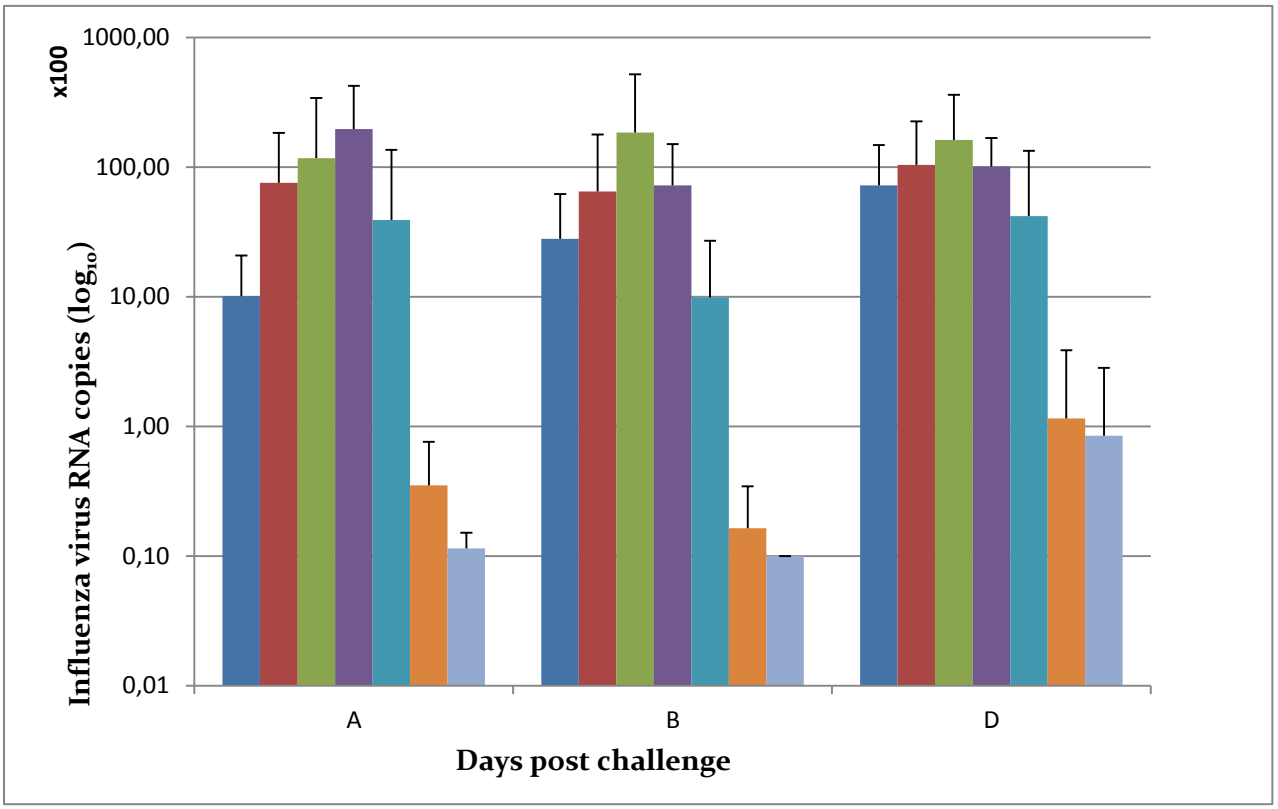


Figure 23: Group average virus titers for all challenged groups from day 1 to 7 post infection.

the curves are overlapping but the columns are not, presenting the results separated from each other.

4.3.2 Individual virus titers

Figures 24, 25 and 26 show individual virus titers of all chickens of a group from day 1 to 7 post infection. Each line represents a single animal. Three birds per group were euthanized on day 4 pi.

Regardless the group, the virus titers vary strongly from one individual to another. In general, the birds excrete significant higher amounts of virus from day 1 to 4 pi than the following days. The diversity of excretion curves makes it difficult to find a description that is valuable for all of them. Their mean virus titers are summarized in Figure 22 and 23.

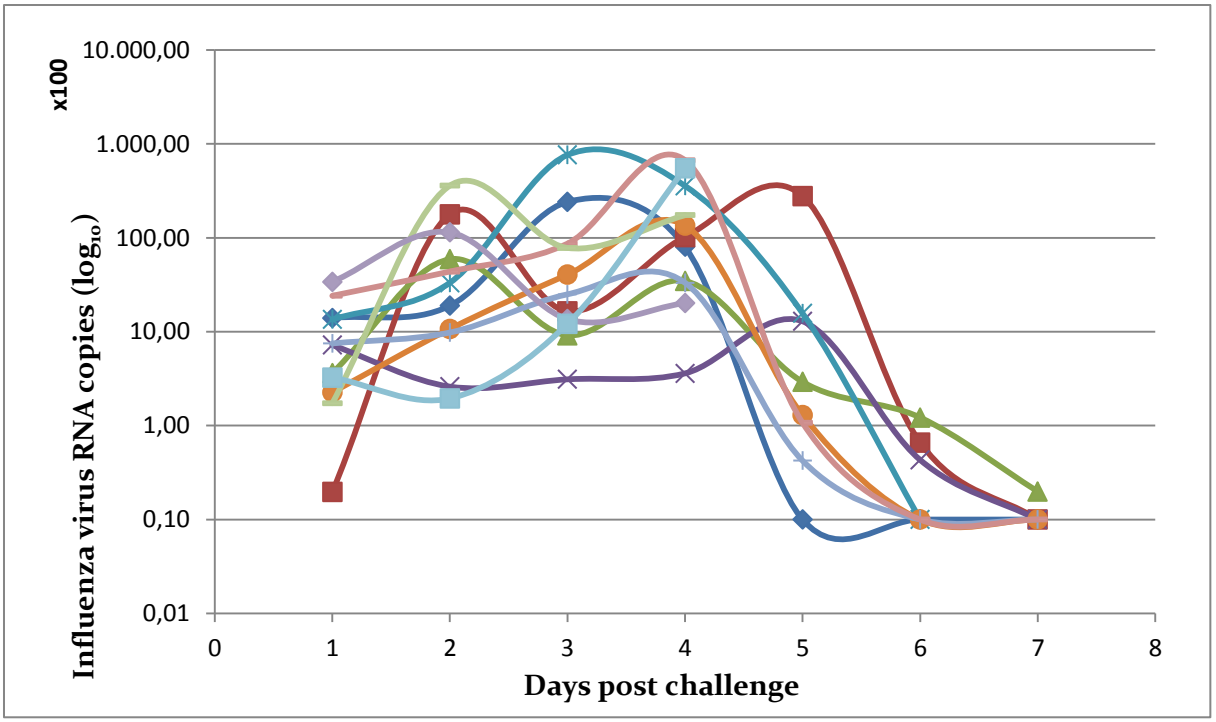


Figure 24: Individual virus titers of group A (double vaccinated) from day 1 to 7 post infection. Each line represents a single chicken.

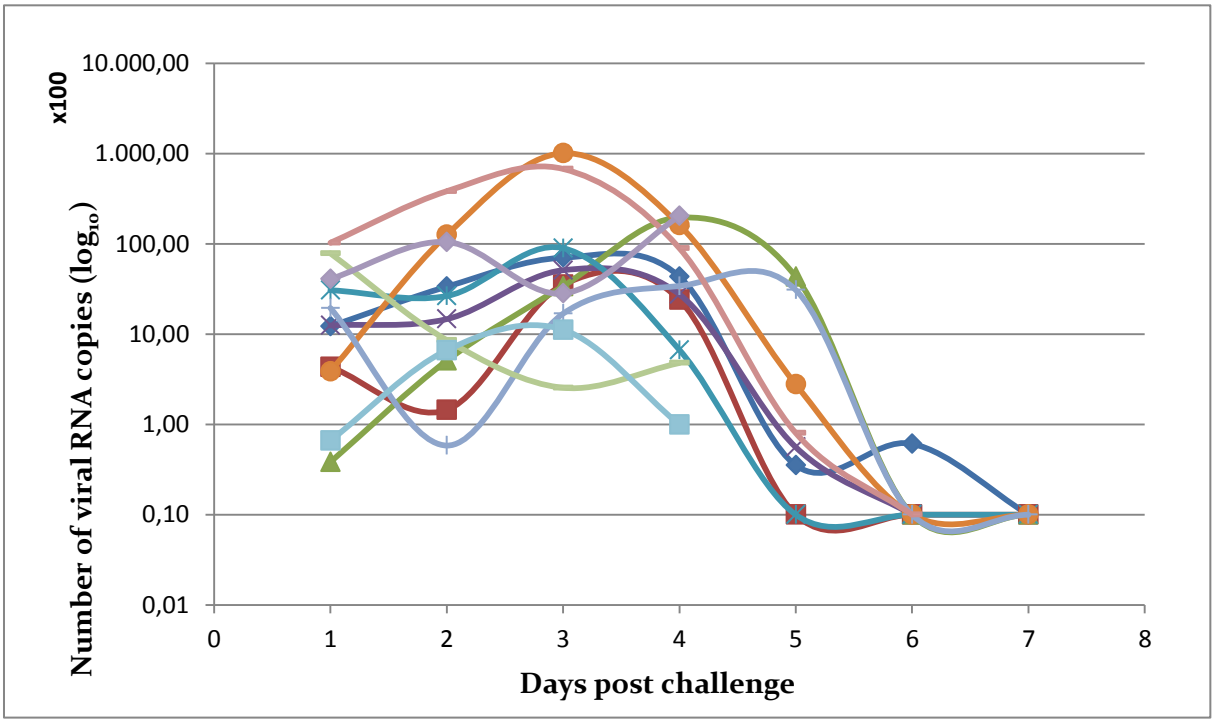


Figure 25: Individual virus titers of group B (single vaccinated) from day 1 to 7 post infection. Each line represents a single chicken.

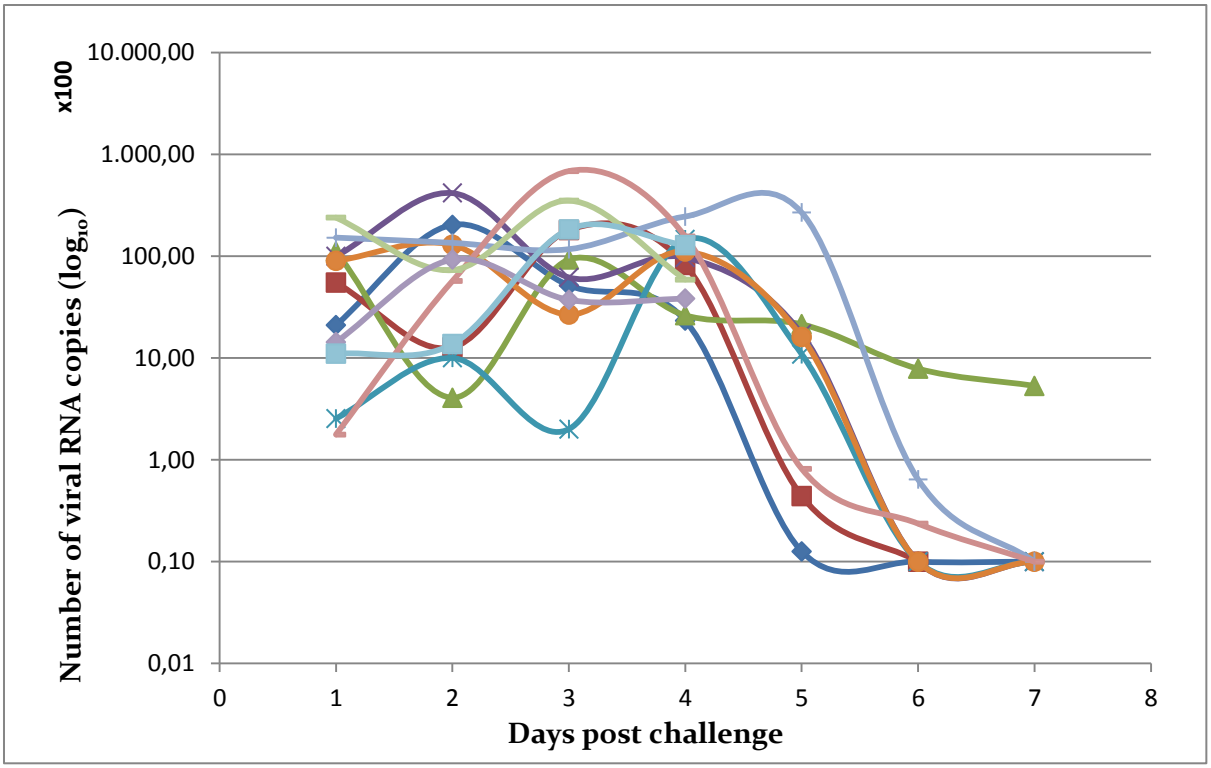


Figure 26: Individual virus titers of group D (not vaccinated, challenged) from day 1 to 7 post infection. Each line represents a single chicken. One animal shows a significantly higher titer on day 7.

4.4 Serology

In order to exclude an environmental contamination with H9 viruses, a HI was performed on the control chickens at three weeks of age. None of the chickens had antibodies anti-H9 would lead to a positive hemagglutination inhibition assay (HI).

All chicken were SPF chickens and free of any flu antibodies or other cross reactive antibodies that could alter our results.

Table 3: HI titers anti-A/chicken/Tunisia/12/2010 (Group C at three weeks of age)

Chicken # / Titer	1	2	3	4	5	6	7	8	9	10	11	12
Anti-H9	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

Three weeks after the first vaccination of Group A, thus pre-boost, HI titers revealed detectable antibodies titers ranging from 10 to 160, with an average value of 35 (Table 3).

Table 4: HI titers of Group A before boost (upper line: chicken number, lower line: antibody titers)

bird #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
titers	60	20	20	15	20	15	160	40	10	15	20	30

Figures 28 and 29 show the results from seroneutralization assays using sera of birds from all groups. Whereas the controls chickens and the group D chickens do not show neutralizing antibodies, we found them in the vaccinated animal with titers ranging from 160 to ≥1280 for group A and 80 to 640 for group B. We tested the sera at the day before challenge.

Table 5: HI (antiH9) titers of five consecutive days after vaccination (Group B), *doubtful HI titer

bird # / day p. vacc.	1	2	3	4	5	6	7	8	9	10	11
d3	10					10					
d4	20	10	10	10	10	10	10	10	10	20	10
d5	20	20	20	40	20	40	20	20	20	10	10
d6	40	40	10	40	30	40	40	20	20	20	20
d7	80	80	20	*10**	40	80	40	20	30	40	40

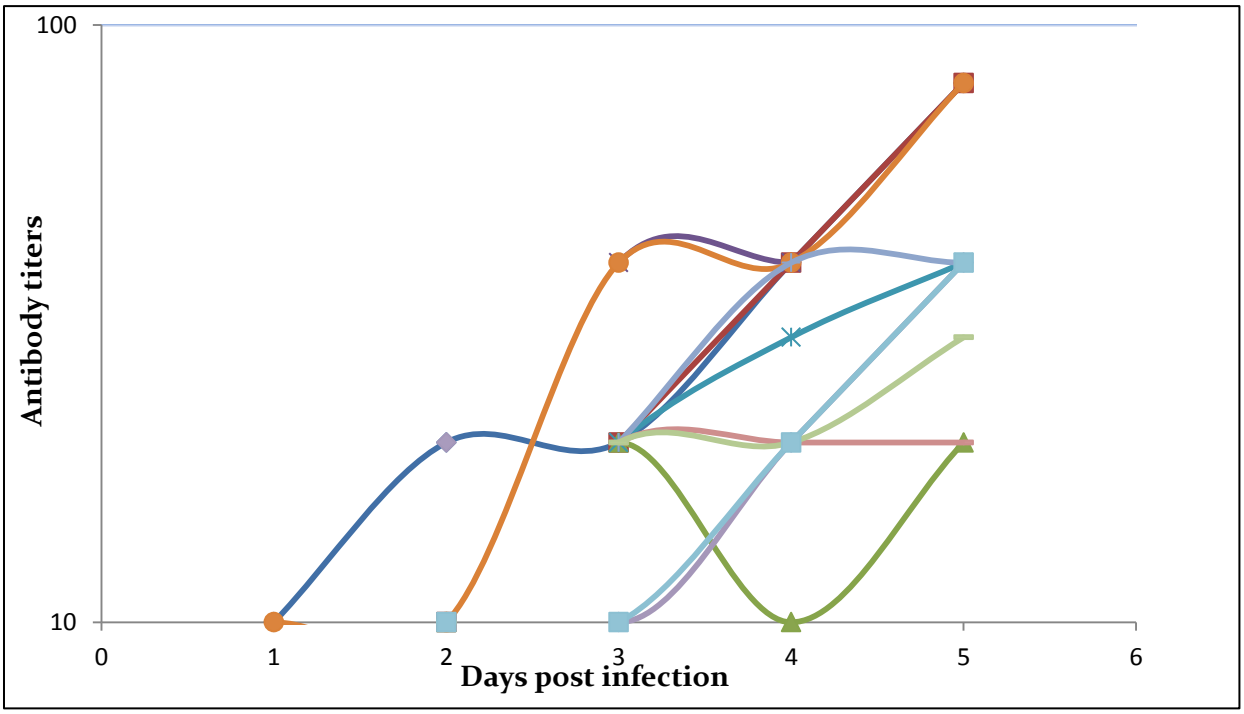


Figure 27: HI (antiH9) titers of Group B during five consecutive days after vaccination. The same data as presented in Table 4 was used.

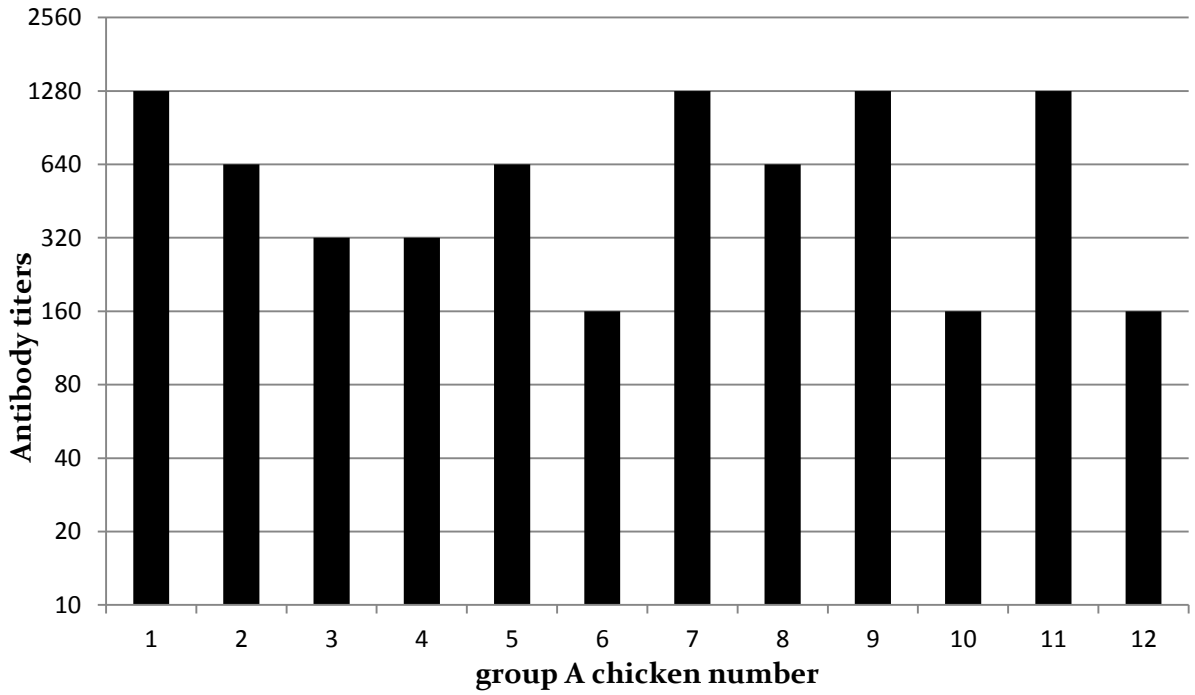


Figure 28: Group A seroneutralization assay results one day before challenge.

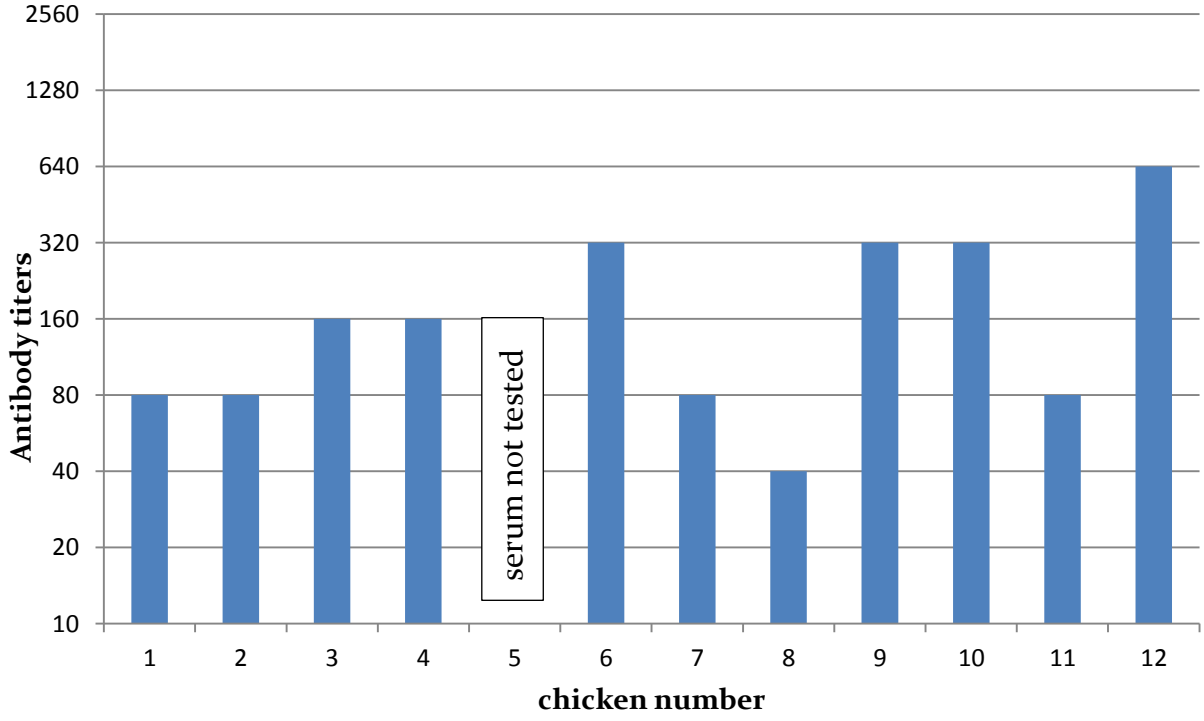
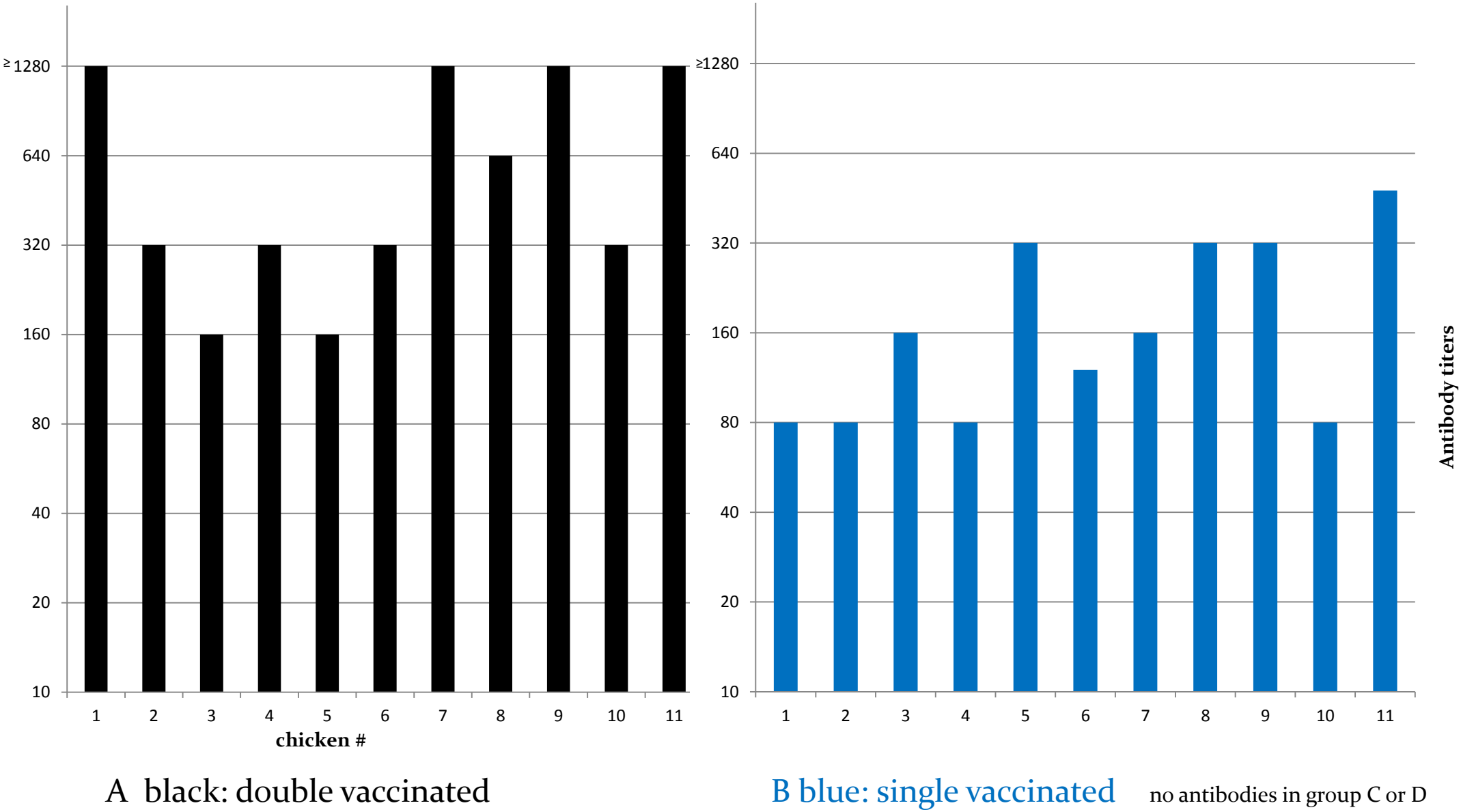


Figure 29: Group B seroneutralization assay results one day before challenge.

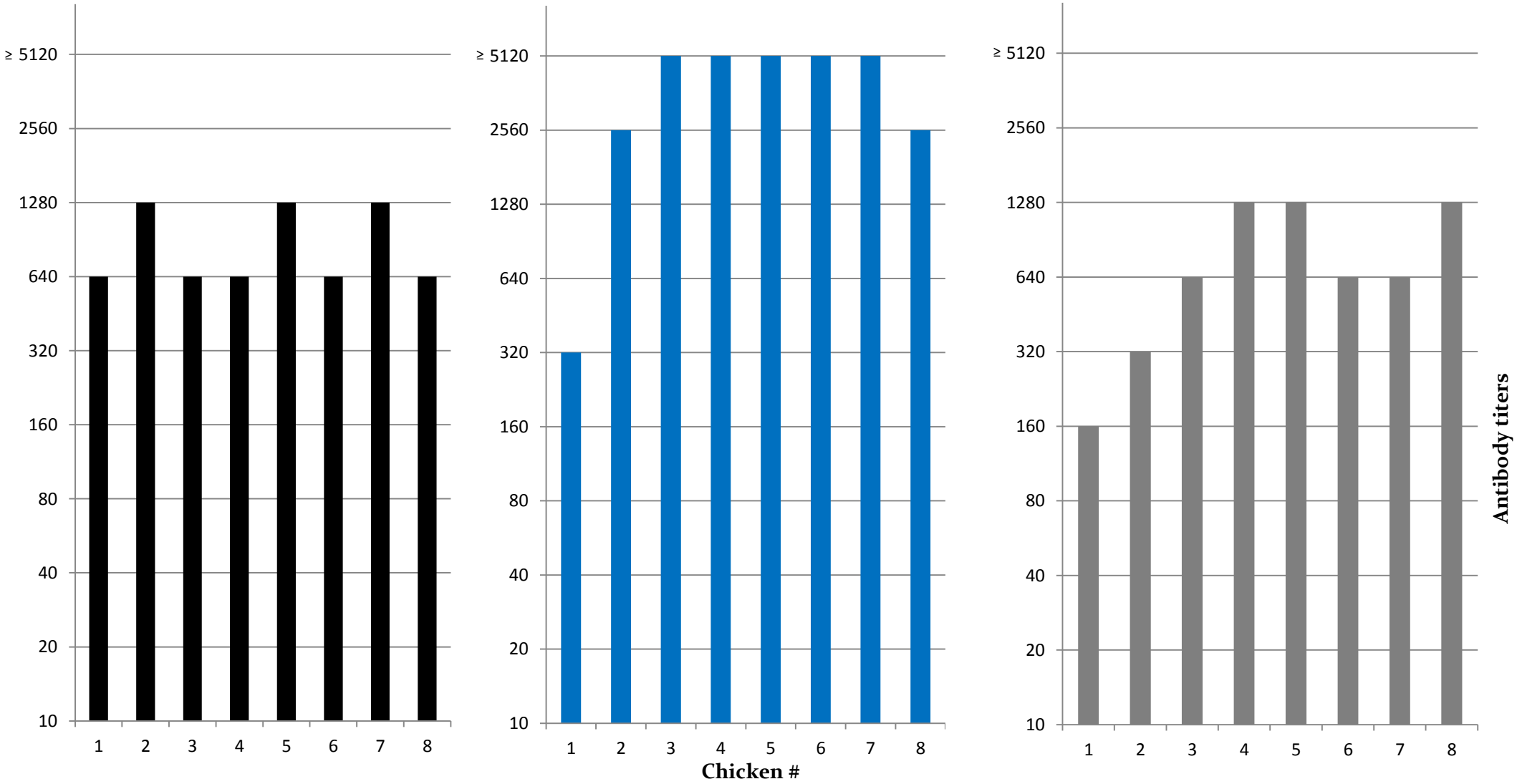


A black: double vaccinated

B blue: single vaccinated

no antibodies in group C or D

Figure 30: HI antibody titers of Group A and B before challenge. The highest anti-H9 titers are found in Group A, however, several chickens of Group B (3/2) reached the same titers (320/160) as Group A chicken. Group D's chicken 11 is the single one showing a higher titer than most Group A animals.



black: A, double vaccinated

blue: B, single vaccinated

grey: D, not vaccinated

no antibodies in control group C (unvaccinated, not challenged)

Figure 31: HI antibody titers of Group A, B and D two weeks after challenge. While Group A and D show comparable results, Group B chickens had significantly higher anti-H9 antibody titers.

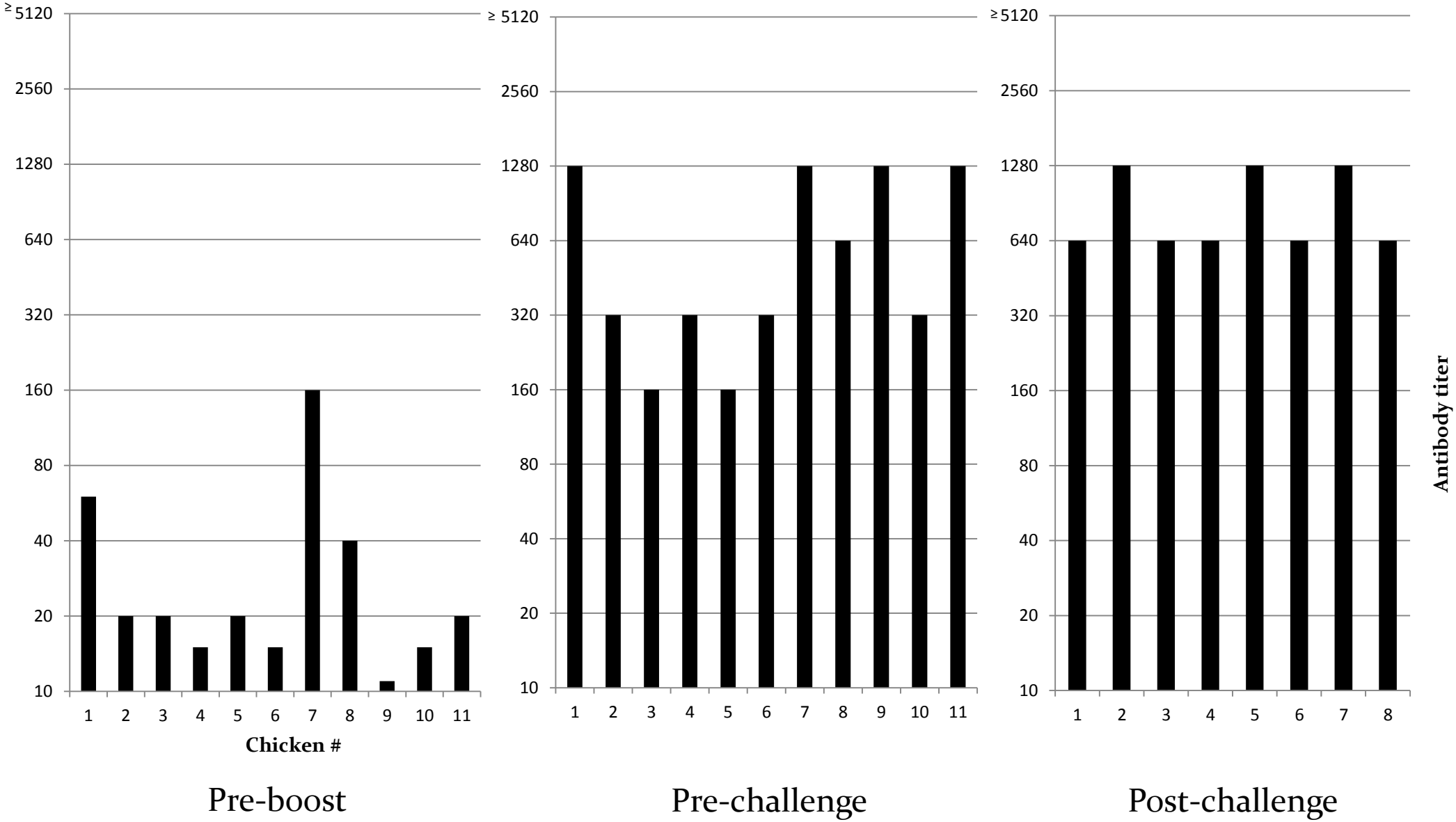


Figure 32: Kinetics of anti H9 antibodies for group A. Evolution of HI antibody titers of Group A after two 3-week intervals and one 2-week interval. Three birds were euthanized on day four post-challenge.

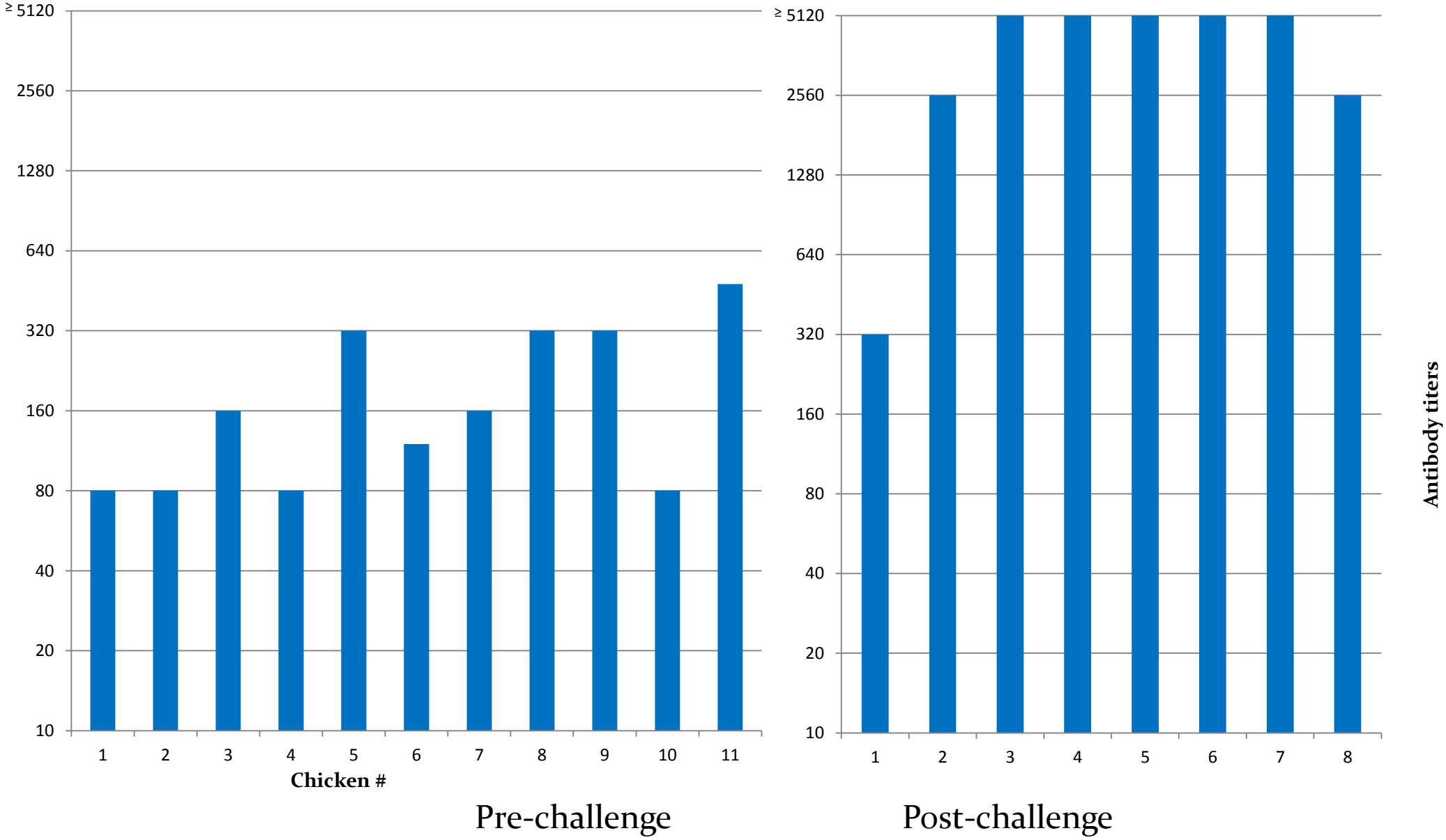


Figure 33: Kinetics of HI antibody titers of Group B. Unlike Group A birds, these were vaccinated at three weeks of age and challenged at week 5.

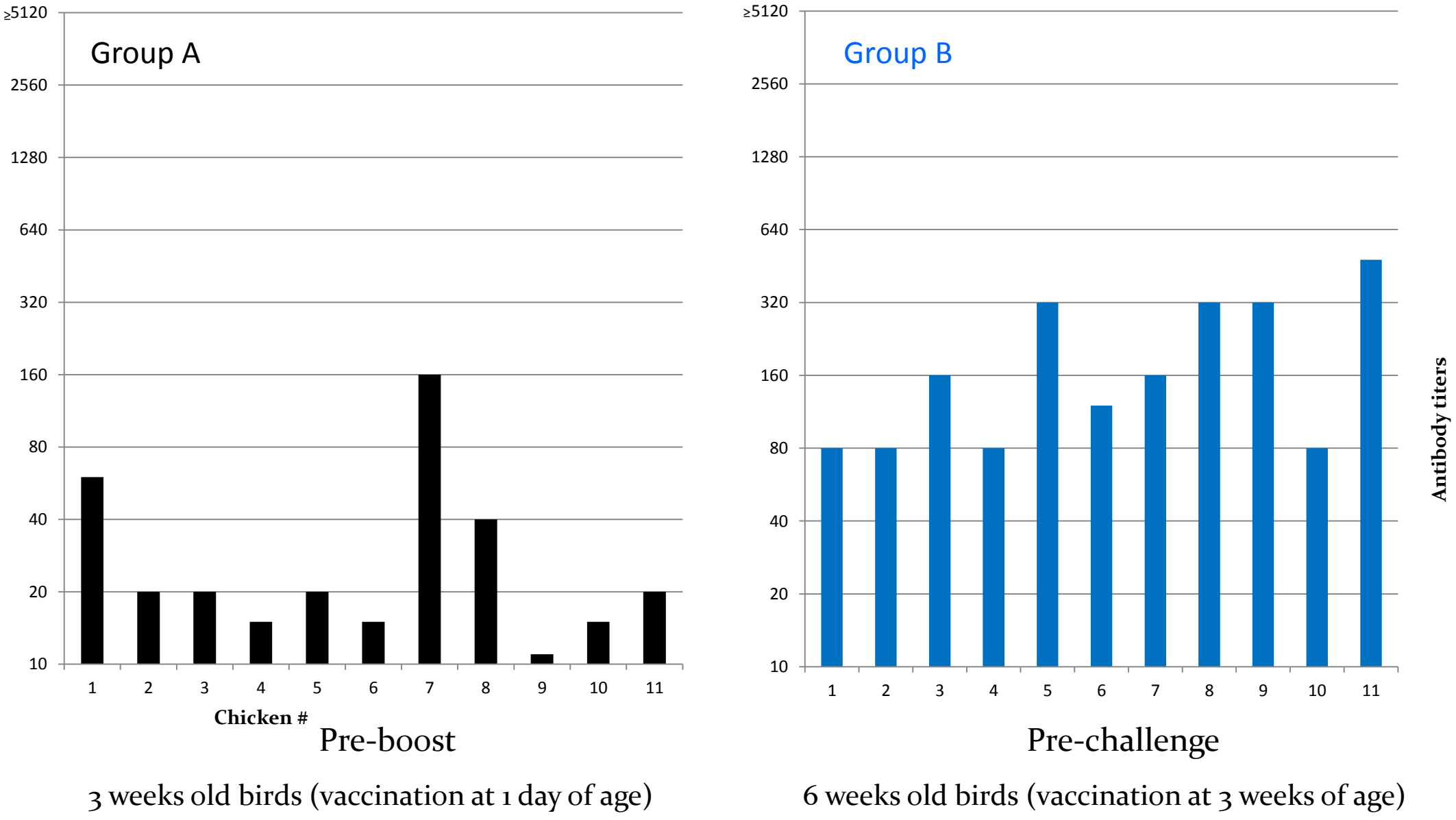


Figure 34: Age-dependent anti-H9 HI antibody response after 1 vaccination with the MVA-H9 vaccine. Comparison of Group A and B anti-H9 titers three weeks after vaccination. Note that Group A chickens were vaccinated at one day of age, while Group B chickens were three weeks older.

Figures 30 to 34 present HI titers results. The antibodies (anti-challenge strain A/chicken/Tunisia/12/2010) were extracted from blood samples taken before the boost, before the challenge and two weeks post challenge.

Pre-challenge antibody titers were higher in double vaccinated group A chickens, although single vaccinated group B bird titers' resulted higher than group A titers before their boost. It has to be taken into consideration that group B animals were vaccinated at three weeks of age, and not at day 1 like group A.

Post-challenge titers, however, generally reached higher levels in single vaccinated group B birds, measured two weeks after the infection. Chickens that had not been vaccinated (group D) showed two-fold to five-fold lower titers compared two double-vaccinated group B, or the same to three-fold lower titers than group A.

4.5 Cross-reactivity of ancestral MVA H9 sera with different H9N2 viruses

We tested the pooled sera of the double vaccinated group A one day before challenge for cross-reactivity against the following H9N2 strains from Europe, Asia and the Middle East.

The H9N2 viruses tested were:

A/PHEASANT/UAE/D1521/2011 (G1)
A/QUAIL/UAE/D1550/2011(G1)
A/ENVIRONMENT/BANGLADESH/10307/2011(G1)
A/HONG KONG/33982/2009 (G1)
A/ENVIRONMENT/BANGLADESH/907/2009 (G1)

A/CHICKEN/HONG KONG/TP38/2003 (Y280)
A/CHICKEN/HONG KONG/G9/97 (Y280)
A/CHICKEN/BEIJING/1/94 (Y280)

A/SHOREBIRD/DE/249/2006 (wild bird H9N2)

Although the pooled sera antibody titer amounted to 320 in a HI assay with the Tunisian challenge strain, no cross-reactivity was observed with any other tested H9N2 virus.

5. Discussion and Conclusion

The object of this study was to evaluate the efficiency of the recombinant MVA-H9 vaccine in the chicken model. We vaccinated naive birds and used an H9N2 field strain for challenge in order to measure the protection it confers in two different vaccination regimens.

According to the results of previous publications, we also observed a fast and substantial onset of antibody production in vaccinated chickens [40, 53]. However, while the immunoglobulins were highly reactive in HI assays and seroneutralization tests, we found no evidence for substantial protective efficacy in vivo. Given the fact that little is known about experimental vaccination with MVA in chickens [49], we presume that there is a low degree of correlation between the reduction of virus shedding, clinical protection of the birds and HI titers of circulating antibodies as suggested by Terregino et al. [54]. In a comparative study of several commercially available inactivated whole virus H5 vaccines and two recombinant vector platforms (NDV-H5/MVA-H5) in a HPAI H5 challenge, one of the former vaccines that induced reasonable HI titers ($6.1\log_2$) could not protect 20% of the chicken which succumbed to the infection. In contrast, MVA-H5 induced lesser HI titers but protected all birds from fatal disease [49]. Therefore, the outcome of challenge studies in chickens is somewhat difficult to predict by serological tests evaluating circulating antibodies before the challenge infection.
In contrast, in other animal models, the levels of antibody responses measured by HI or seroneutralization appear to correlate well with protection obtained upon challenge, as recently shown with MVA-H7 immunization and LPAI H7N9 challenge in the ferret model [53].

The amino acid homology between the vaccine antigen and the field virus has been shown to influence strongly the capacity to confer in vivo protection, notably in HA expressing recombinant vaccines, such as infectious laryngotracheitis and

fowlpox virus vectors [55, 56]. A Newcastle Disease Virus platform expressing a H5 (NDV-H5) with less than ~94% aa homology could not stimulate immune responses comparable to those obtained with a NDV vector vaccine delivering an HA antigen nearly identical to the one of the challenge virus used for infection [49].

In the present study, an amino acid homology of 94% between the HA of the Tunisian strain and the ancestral HA sequence could be one reason for insufficient protection in vivo. Ancestral whole-influenza virus vaccines have, however, provided robust protection in vitro and in vivo and against several HPAI H5N1 strains in the ferret model [50].

Due to the fact that ancestral sequences are computationally derived nucleotide alignments, there is no naturally occurring live virus expressing the ancestral H9 (H9anc). If this AI H9ancN2 virus had existed, it could have been used for the challenge, representing a 100% HA homology with the challenge strain. Besides, there are more factors that influence the generation of the immune response, for example the addition of adjuvants like aluminum hydroxide, paraffin oil or amine polymers [57].

By using rt-RT PCR, we detected viral RNA until day 6 post-challenge, with a precipitous decline after the fourth day. Unexpectedly, 100% of all vaccinated and infected chicken were shedding virus at least during four days whereas in a former study values of only 50% are mentioned [49]. Evidently, the birds were not able to avoid viral replication efficiently, irrespective of their age and the vaccine regimen. To evaluate whether the virus that was obtained by oropharyngeal swabbing once a day during 7 days post-challenge is dead or still replication-competent, in-ovo amplification on embryonated chicken eggs can be done and, subsequently, the Egg Infectious Dosage (EID_{50}) is calculated. As we did not perform such experiments, we do not know, if the birds shed live LPAI H9N2 viruses. Veits et al. found positive swab samples on day four during their MVA-H5 study in chicken [49].

Interestingly, the histopathological results indicated that the vaccination reduced

microscopic lesions, suggesting a positive effect of the MVA-H9 vaccine on disease outcome.
Virus replication and microscopic lesions after intranasal inoculation are normally confined to epithelial cells of the upper respiratory tract. Additionally, we inoculated the birds intratracheally, causing moderate subacute bronchitis with regenerative epithelial hyperplasia and inflammatory cellular infiltrates in the lamina propria. Alterations of tissues of the digestive tract occurred. No histological slide preparations of nervous tissues were done as we did not observe CNS signs during the animal experiment. Ascending infections from the olfactory nerves leading to neurologic manifestations in the brain subsequent to intraocular and intranasal infection are not to be expected in LPAI studies. If the bird is infected through an intravenous route it is primarily the kidneys that exhibit tubular damage, likely because of virus strains with renal tropism.

Audible and visual signs of illness were hardly observed and reveal an ambivalent fact: LPAI viruses like the Tunisian strain we inoculated the chicken with do not lead to clinical signs and, as they do so, there are no signs that can be reduced or prevented by any vaccine. Such strains are considered to be less dangerous for humans and animals and can be handled under less strict biosafety measures (biosafety level 2). This means that studies using LPAI AI viruses are conducted in favor of animal welfare politics and permissions from ethical committees are granted more easily. Moreover, in comparison to studies using HPAI viruses, they are less expensive.
By using a different species, such as mice, it could be possible to evaluate the vaccine's effect on the reduction of clinical signs, since mice are more susceptible to LPAI infections. Among others, they may display a loss of weight, respiratory symptoms, and a lower daily food intake.

On the other hand, we cannot evaluate whether this MVA-H9 vector vaccine is able to reduce any sign of sickness, which is not due to the vector but to the low pathogenicity of the influenza virus. This important information about the direct effect of MVA-H9 vaccines on disease outcome could not be obtained.

It has to be mentioned that the reduction of clinical signs by a vaccine is a theoretical, scientific but not economically exploitable capacity. A spread of AI viruses in poultry populations due to covered signs of illness would fuel the distribution of the pathogens. For biosafety reasons, an ideal vaccine therefore should lower the clinical signs and, as an inevitable addition, be able to stop viral replication and the shedding of live virus.

The antibodies we obtained during the study did not show any cross-reactivity with other H9N2 viruses. Concerning non-reactive G1 strains one could hypothesize that single amino acid mutations on the HA sequence prevent the immunoglobulins from binding the antigens.

For the Y280 and shorebird strains this could be due to genetic and antigenic variation, given that the inserted H9 sequence was only an ancestor of G1 viruses. Seo et al. and Khalenkov et al. showed that concerning cross clade protection the cellular immunity plays an important role [58, 59]: seroneutralization tests and HI assays using antibodies of chicken that were infected with H9N2 viruses on H5N1viruses came out negative. H9N2 immunoglobulins are, at least in vitro, not mediating a cross-clade protection. In 1997, during a period of co-circulation of both AI virus clades in retail markets in Hong Kong, lethal HPAI H5N1 viruses caused hardly any signs of disease, although 18 confirmed human infections were noted the same year. These pathogens normally cause mortality rates of up to 100% within 48 hours in chicken.

Naive chicken that had never been in contact with either H9N2 or H5N1AI viruses were then transferred T lymphocytes or CD8+ T cells from H9N2 infected chicken. Seo et al. showed that by doing so, the birds survived a subsequent H5N1 infection. Virus shedding in the feces continued.

According to these findings, T cells are crucial for cross-clade protection, whereas antibodies are not able to neutralize the virus in vitro neither in Seo's study nor in ours.

The results of the control group came out as we expected. All animals of this group were free of antibodies against the Tunisian challenge strain we had used for infection. This means that the control birds fulfilled their role as no contamination

occurred by the staff, the material or the handling. The existence of maternal immunity can be excluded as all birds originated from specific pathogen free flocks.

In our setting, the vaccination was done by intramuscular route in contrast to the challenge virus, which was inoculated intraocularly, intranasally and intratracheally. The lack of mucosal immunoglobulin A antibodies could be responsible for a non-adequate immune response of the birds. A nasal application of MVA-H9 might induce a better level of protection and has to be evaluated in further studies.

We found histological damages mostly in the upper and lower respiratory tract. Chen et al. attained a sterile immunity against homologous and heterologous pathogens in ferrets using a nasally applied live-attenuated H7N9 vaccine in the ferret model [60]. The challenge was done by using the same route of inoculation. This supports the theory of virus neutralization of airborne transmitted AI viruses by mucosal antibodies at the orifice of the body.

Vectored MVA influenza vaccines have provided evidence to be safe and immunogenic in numerous preclinical studies in chicken, mice, macaques and ferrets [42, 44, 45, 49, 53, 61, 62]. In comparison to whole AI vaccines which have to be administered in inactivated form due to safety reasons, recombinant carrier delivering vaccine antigens are advantageous as they require only a little amount of antigen, making the production of such live vaccines cost-effective [49]. Moreover, they can be produced on large scale in a short period of time in order to provide sufficient doses for each individual [53].

As AI viruses, notably HPAI H5N1 viruses, which have caused hundreds of deaths among humans, have become endemic in several countries like Egypt and China [63], it is crucial to be able to differentiate vaccinated from infected animals (DIVA). Although AI vaccines are banned from public use in many countries, undetectable spread under the vaccination coverage has to be avoided for any AI virus.

Inactivated whole virus vaccines with the same HA but a different NA than the

circulating strain work according to the DIVA strategy. However, it is expensive and labour intensive to differentiate NA antibodies, making whole virus vaccines not suitable for mass screening tests. In addition to that, several subtypes can circulate in the field at the same time and lead to false-negative results.

Common ELISA tests allow the simple detection of serum anti-NP or anti-MP immunoglobulins. Given the fact that vectored vaccines do not induce the generation of antibodies against conserved AI virus proteins as they typically express only the most prominent surface protein HA, they fulfill the requirements of the DIVA strategy and thousands of samples could be screened within reasonable expenditures.

To investigate further advantages and inconveniences of ancestral MVA-H9 vaccines in the chicken model, we propose a similar experimental setting, in which challenge virus and vaccine is applied intranasally. This could lead to a protection through mucosal antibodies. Besides, poultry farming needs vaccine strategies like nebulizing sprays that work without individual handling of each bird. Ancestral HA sequences with a higher degree of amino acid homology than the one used in our study may lead to cross-reactive antibodies. Therefore, it would be of interest to create phylogenetic trees of AI virus strains which are more closely related than G1 and Y280 strains, providing new ancestral sequences.

In order to obtain comparable results regarding signs of illness, another species such as mice should serve as animal model in a future study.

Safe and efficient vaccines are indispensable for the decrease of the virus load in the animal organism, thus lowering the infection pressure towards their fellow species and the environment, as well as to humans.

6. Summary

This work evaluates the efficiency of a recombinant vector vaccine (Modified Vaccinia Ankara, MVA) which has been equipped with a computationally derived, artificial genetic H9 influenza virus sequence named “ancestral sequence” in the chicken model. The aim of the study was to show that this vaccine induces protection from an experimental infection with low pathogenic Avian Influenza (AI) H9N2 viruses. As the high genetic and antigenic diversity of H9N2 viruses increases vaccine design complexity, one strategy to widen the range of vaccine coverage is to use an ancestor sequence.

This work was done in cooperation between the National Veterinary School of Toulouse, France and the Ludwig-Maximilians-University of Munich, Germany. The genetically altered MVA vector vaccine was engineered in Munich. The inserted sequence was provided by the French laboratory where the animal facilities are located and the study was conducted.

Due to their large poxvirus genomes and extensive manufacturing processes during the past decades, MVA vectors that are capable of carrying an inserted sequence and express the according protein on their surface without being replication competent in human and most mammalian cells. The immune system of the vaccinated organism produces antibodies that target this protein and are supposed to confer protection in our chickens. The inserted genetic material is an artificial hemagglutinin sequence that combines characteristics of multiple AI H9N2 viruses. Hemagglutinins are the most important surface protein of influenza viruses as they are the main target of the immune response, among others.

This clinical trial revealed that after an experimental infection, vaccinated animals show less histological damage, less influenza-positive cells and can eliminate the virus faster than unvaccinated individuals. All infected birds were shedding virus and a large majority stayed clinically healthy during the whole study. Although *in vitro* seroneutralization assays showed that the induced antibodies are neutralizing and protective on MDCK cell layers, they do not confer protection from virus replication in the chickens.

Further studies are warranted to explain this outcome of the study.

7. Résumé

Ce travail évalue l'efficacité d'un vaccin ancestral recombinant MVA (Modified Vaccinia Ankara) muni d'une séquence artificielle chez le poulet. Cette séquence porte le nom d'« ancestrale » car elle a été générée *in silico* afin de regrouper certaines caractéristiques des virus influenza aviaires H9N2. La diversité génétique et antigénique importante complexifie la production d'un vaccin efficace. Une stratégie pour élargir son spectre d'action est l'usage d'une séquence ancestrale.

Ce travail a été réalisé en coopération entre l'Ecole Nationale Vétérinaire de Toulouse, France et la Ludwig-Maximilians-Universität de Munich, Allemagne. Le virus recombinant provenait des laboratoires de Munich alors que la séquence à insérer a été conçue en France. L'infection expérimentale a été menée à Toulouse car les locaux étaient adaptés.

Grâce à sa taille et aux procédures des modifications moléculaires des dernières décennies, le MVA est capable de porter cette séquence artificielle et d'exprimer la protéine correspondante à sa surface. C´est elle qui induit la production d'anticorps protecteurs chez les poulets. La séquence insérée code pour une hémagglutinine qui est caractéristique de nombreux virus H9N2. Cette hémagglutinine est importante chez les virus influenza aviaires car c'est elle qui est ciblée par la réponse immunitaire de l'hôte.

Cette épreuve vaccinale a révélé que suite à une infection expérimentale les animaux vaccinés présentent moins de lésions microscopiques, moins de cellules influenza-positives et arrivent à éliminer le virus plus rapidement que les animaux non-vaccinés. Bien que les tests de séroneutralisation *in vitro* montrent la présence d'anticorps neutralisants et protecteurs sur des cellules MDCK, ils n'empêchent pas la réplication virale. Tous les oiseaux excrétaient du virus tout au long de l'expérience tandis qu'ils étaient cliniquement sains. Des études supplémentaires doivent être envisagées pour expliquer pourquoi les anticorps neutralisants ne protégeaient pas les animaux.

8. Zusammenfassung

Die vorliegende Arbeit beschreibt eine Impfstudie am Huhn bei der die Immunogenität und Wirksamkeit eines rekombinanten Vektorimpfstoffes (Modified Vaccinia Ankara, MVA) gegen Aviäre Influenza evaluiert wird. Dieser Vektorimpfstoff wurde mit einer künstlichen H9 Gensequenz ausgestattet, die am Computer errechnet worden war und sich „ancestral sequence/Vorläufersequenz“ nennt.
Ziel der Immunisierung war, die Tiere damit gegen die Infektion mit einem Influenza A Virus des Subtyps H9N2 zu schützen. Die genetische und antigenetische Vielfalt von Vogelgrippeviren ist eine Herausforderung für die Impfstoffherstellung und die Nutzung künstlicher Antigene basierend auf Vorläufersequenzen daher eine neue experimentelle Möglichkeit das Wirkspektrum von Impfstoffen zu erweitern.

Die Arbeit wurde in Zusammenarbeit der Ecole Nationale Vétérinaire de Toulouse, Frankreich und der Ludwig-Maximilians-Universität München, Deutschland erstellt. Der genetisch veränderte Impfstoff MVA kommt aus den Laboratorien der Fakultät für Tiermedizin in München. Die eingefügte Sequenz stammt aus Toulouse, wo darüber hinaus auch geeignete Stallungen für den Impfversuch am Huhn vorhanden sind und diese Arbeit angefertigt wurde.

Bei dem MVA Vektorimpfstoff handelt es sich um ein Pockenvirus, das dank seines großen Genoms und intensiver Bearbeitung über Jahrzehnte hinweg in der Lage ist, eine künstlich eingefügte Genesequenz abzulesen und in Form eines Oberflächenproteins zu präsentieren. Gegen dieses Protein bildet der Organismus dann Antikörper, die den Schutz gegen das Antigen vermitteln sollen. Bei der künstlichen Sequenz handelt es sich um eine artifizielle Hämagglutininabfolge, die die Charakteristika mehrerer Grippeviren in sich vereint. Hämagglutinine sind die wichtigsten Oberflächenproteine von Influenzaviren, unter anderem weil sich gegen sie die Immunantwort des Wirtes richtet.

Die klinische Prüfung des Impfstoffes hat gezeigt, dass geimpfte Tiere im Vergleich zu nicht geimpften nach einer experimentellen Infektion weniger histologische Veränderungen zeigen, weniger Influenza infizierte Zellen aufweisen und das Virus schneller eliminieren können. Vor einer Infektion schützen die induzierten Antikörper nicht. Im Labortest hingegen sind die gleichen Antikörper neutralisierend und vermitteln einen effektiven Schutz vor in vitro Infektion mit Titern bis zu 1280 auf MDCK Zellenkulturen. Alle infizierten Hühner schieden Viren aus, wobei die große Mehrheit der Tiere während des gesamten Experiments klinisch gesund blieb.

Es sind weitere Untersuchungen nötig, um erklären zu können, warum neutralisierende Antikörper nicht in der Lage waren, die in vivo Virusreplikation zu verhindern.

9. Danksagung

Am Ende dieser Arbeit richtet sich mein Dank im Besonderen an

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für die Konstruktion und Charakterisierung des Impfvirus, auf dessen Basis die Studie am Huhn durchgeführt werden konnte. Für die sorgfältige Herstellung nach den Prinzipien der ‘ancestral sequences’ und die Übersendung nach Toulouse, die den eigenlichen Startschuss für die Impfstudie symbolisiert hat.

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Allen Labormitarbeitern

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Für gezielte Power, als sie nötig war.

Meinen Eltern und Geschwistern

dafür, dass sie wirklich immer für mich da sind.

10. List of Figures

11. List of Tables

12. Abbreviations

AI	Avian Influenza
CEF	Chicken Embryo Fibroblast
CNS	central nervous system
cRNA	complementary RNA
Day p. vacc.	Day post vaccination
DIVA	differentiating infected from vaccinated animals
DNA	deoxyribonucleic acid
et al	et alii/et aliae
H9	hemagglutinin 9
H9N2	(Avian Influenza) virus with hemagglutinin 9 and neuraminidase 2
HA-/+	hemagglutinin assay positive/negative
HAanc	ancestral hemagglutinin sequence
HAtun	Tunisian challenge strain hemagglutinin sequence
HI	hemagglutinin inhibition assay
HPAI	highly pathogenic avian influenza virus
LMU	Ludwig Maximilians Universiät
LPAI	low pathogenic avian influenza virus
MEM	Minimal Essential Medium
MP	matrix protein
MVA	Modified Vaccinia Virus
N2	neuraminidase 2
NDV	Newcastle Disease Virus
NP	nucleoprotein
nt	nucleotide
RDE	receptor destroying enzyme
RNA	ribonucleic acid
rMVA	recombinant Modified Vaccinia Virus
rt-RT PCR	reverse transcription real time polymerase chain reaction
p.i.	post infection
vRNA	viral RNA

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14. Appendix

14.1 Poster « Low Pathogenic Influenza (H9N2) in chicken: Evaluation of ancestral MVA vaccine »

14.2 Application Document for Animal Experiment to the Ethical Committee

Low Pathogenic Influenza (H9N2) in chicken: Evaluation of an ancestral MVA-H9 vaccine

Jens Becker[1], Mariette Ducatez[2], Jean-Luc Guérin[2], Lisa Marr[1], Anna Lülf[1], Gerd Sutter[1], Asisa Volz[1]

[1] Institute for Infectious Diseases and Zoonoses, Department of Veterinary Sciences, LMU University of Munich, Germany

[2] UMR 1225 IHAP, INRA-ENVT, National Veterinary School, Toulouse, France

Email: jens@beckerkarlsruhe.de

Introduction and objectives

- Influenza A viruses are segmented RNA viruses that show substantial genetic variability.

  Avian influenza viruses (AIVs) can be transmitted from the waterfowl reservoir to domestic poultry or to mammals including humans. In 2013, low-pathogenic AIVs of the subtype H9N7 have been identified as zoonotic pathogens and continue to cause severe respiratory disease in humans (China). Therefore, AIVs of H9 subtypes are considered a threat to human health.
- Here we portray the three fundamental steps of a new vaccine development study. A Modified Vaccina virus Ankara (MVA) poxvirus vector[1,2] that expresses an ancestral hemagglutinin (HA) sequence is tested for immunogenicity and capacity to induce protection against a AIV H9 field virus in chicken.
- Prevention of flu in domestic birds lowers the risk of infection for humans as low pathogenic AIV H9N2 transmission among people is probably very limited[6].

Structure

1. Creation of ancestral Hemagglutinin Sequence

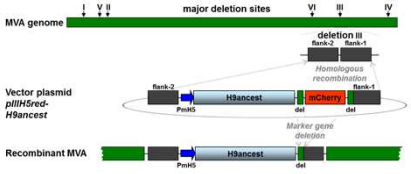
DOWNLOAD of H9 Sequence from Database and ALIGNMENT[5]

MARGINAL RECONSTRUCTION with *Ancescon* to generate in silico amino acid sequences of putative viruses at the nodes of the phylogeny

Phylogenetic tree with **ANCESTRAL** NODE[4]

2. Construction of MVA vector as experimental vaccine

Selection of the ancestral RNA sequence located at the node section of phylogenetic tree and insertion of synthetic cDNA into vector plasmid. Recombinant MVA viruses were generated by homologous recombination and cloned in plaque passages.



Quality control for clonal purity, genetic stability, and expression of the inserted synthetic H9 ancest sequence by PCR analysis of viral DNA and Western blot. High titer viruses purified by ultracentrifugation and reconstituted in physiological saline served as vaccine preparations[3].

3. In-Vivo Evaluation: Challenge of Vaccinated Chicken

- 44 specific pathogen free (SPF) chickens were divided into four groups
  - Double Vaccinated Group A
  - Single Vaccinated Group B
  - Not Vaccinated Group D
  - Control Group C

  Infected with 500µl of 10^9 EID_{50}
- Oropharyngeal swab samples were used to obtain RNA concentrations by reverse transcription real time PCR

  Serum antibodies were used to perform hemagglutination inhibition assays and microneutralization tests

Results

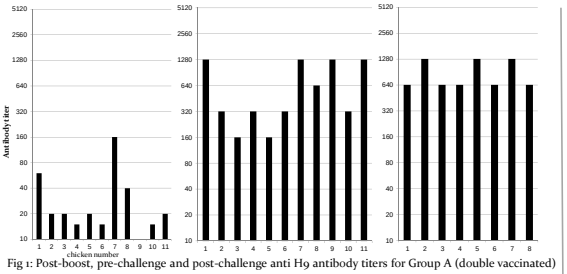


Fig 1: Post-boost, pre-challenge and post-challenge anti H9 antibody titers for Group A (double vaccinated)

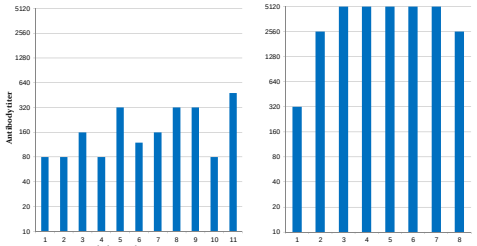


Fig 2: Pre-challenge and post-challenge anti H9 antibody titers for Group B (Single vaccinated)

- None of the birds of Group D (not vaccinated) seroconverted pre-challenge
- None of the birds of Group C (not vaccinated, not challenged) shed virus nor seroconverted.
- Birds that were vaccinated twice seroconverted to higher titers pre-challenge than single vaccinated chickens
- Virus shedding mean values are simliar for birds of all infected groups for 5-6 days post challenge
- Group A birds show higher neutralizing anti H9 antibody titers (in vitro, serum pre-challenge)

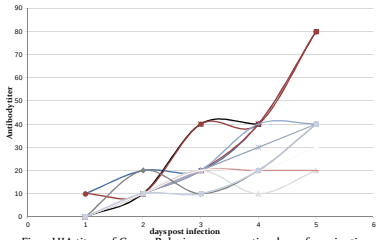


Fig 3: HIA titers of Group B during 5 consecutive days of vaccination

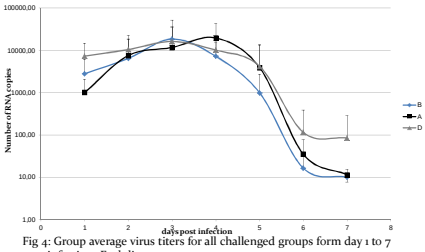


Fig 4: Group average virus titers for all challenged groups form day 1 to 7 post infection. Each line represents one group.

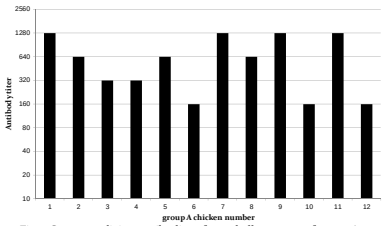


Fig 5: Seroneutralizing antibodies of pre-challenge sera of group A (double vaccinated) on MDCK cells

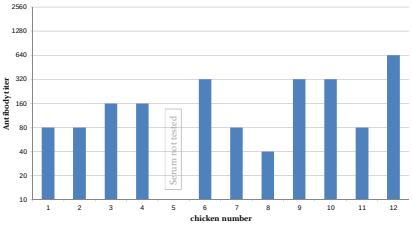


Fig 6: Seroneutralizing antibodies of pre-challenge sera of group B (single vaccinated) on MDCK cells

Conclusion (Intermediate Step Results)

The MVA vector vaccine reliably expressed the ancestral hemagglutinin H9 sequence and lead to a production of serum antibodies in chicken that neutralized H9N2 Avian Influenza with titers up to ≥ 1280 (in vitro). We observed delayed clearance of non vaccinated Group D chickens in comparison to the vaccinated animals. Given that the H9N2 Avian Influenza viruses used in this study caused hardly any clinical signs we can not provide evidence on whether the vaccine is able to lower any clinical symptoms but H9N2 virus shedding was not prevented in the vaccinated chicken. Further studies are ongoing to understand why pre-challenge antibodies are only neutralizing in vitro and are not able to reduce viral shedding in the chicken.

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Demande d'Autorisation de Projet utilisant des Animaux à des Fins Scientifiques

Ce formulaire a pour objectif de rassembler les informations permettant au comité d'éthique dont relève l'établissement utilisateur où sera réalisé le projet utilisant des animaux à des fins scientifiques, d'évaluer éthiquement le projet et au Ministère de l'Enseignement Supérieur et de la Recherche d'autoriser le projet suite à l'évaluation éthique.

Le fichier de ce formulaire ainsi que la notice d'aide pour le renseigner peuvent être demandés à :
autorisation-projet@recherche.gouv.fr

Le projet est défini comme un programme de travail répondant à un objectif scientifique défini, utilisant un ou plusieurs modèles animaux et impliquant une ou plusieurs procédures expérimentales (article R-214-89 du Code rural et de la pêche maritime).

Le formulaire renseigné sera envoyé par lettre recommandée avec accusé de réception à l'adresse suivante :
Ministère de l'Enseignement Supérieur et de la Recherche – Direction Générale pour la Recherche et l'Innovation – Secrétariat « Autorisation de projet » - 1, rue Descartes, 75231 PARIS cedex 5

N.B. A l'exception des duplications des blocs permises et repérées par …, aucune modification des items de ce formulaire ne doit être effectuée.

1. INFORMATIONS GÉNÉRALES

1.1. TITRE DU PROJET :

Epreuve vaccinale : efficacité du Vecteur Virus MVA pour vacciner contre un virus influenza aviaire faiblement pathogène de sous-type H9N2.

1.2. Durée du projet :

1 an

1.3. Date prévue de début du projet :

1/6/2014

2. RÉSUMÉ NON TECHNIQUE

Les virus Influenza se trouvent dans le monde entier, infectant certains animaux et l'homme, avec un impact sur les populations qui diffère fortement selon le sous-type, la région et l'hôte. Chez la volaille, certains virus Influenza ne causent pas de signes cliniques alors que d'autres sont responsables de mortalité très élevée en l'espace de 48 heures. Dans certains élevages, on observe des pertes économiques importantes.

Les virus influenza sont des virus à ARN de la famille des Orthomyxoviridae qui comprend trois Genres : A, B, et C. Nous allons travailler sur des virus influenza A aviaires faiblement pathogènes de sous-type H9N2. Les virus H9N2 faiblement pathogènes sont enzootiques en Tunisie, en Egypte, et dans de nombreux pays d'Asie. Ce sous-type est déjà passé à l'homme à plusieurs reprises (toujours sous une forme peu sévère). Le meilleur moyen à l'heure actuelle de réduire la charge virale dans les élevages est la vaccination. La difficulté est de vacciner avec une souche suffisamment proche de la souche du terrain pour s'assurer d'une réactivité croisée, sans être obligé de générer une nouvelle souche vaccinale pour chaque souche du terrain.

Nous allons vacciner des poulets et des dindes avec un nouveau vaccin généré en collaboration avec l'Université de Munich (vaccin « MVA-H9 ancestral » décrit dans ce document) puis faire une épreuve de virulence pour vérifier l'efficacité du vaccin. Le virus influenza H9N2 que nous utiliserons pour l'épreuve virulente ne causant pas de signe clinique en conditions expérimentales, nous suivrons la charge virale post-infection pour déterminer l'efficacité vaccinale. Des prélèvements sanguins seront également effectués pour suivre la production d'anticorps au cours de l'expérience. Le nombre d'animaux sera au plus bas, suivant la règle des 3R, tout en s'assurant que nous aurons suffisamment de réplicas pour que nos données soient valables statistiquement et éviter de devoir répéter inutilement une expérience : 32 poulets et 32 dindes au total seront nécessaires (8 oiseaux vaccinés 2 fois avec MVA-H9 ancestral puis infectés avec un virus H9N2 ; 8 oiseaux vaccinés 1 fois avec MVA-H9 ancestral puis infectés avec un virus H9N2 ; 8 oiseaux non vaccinés infectés avec un virus H9N2 (contrôles non vaccinés) ; 8 oiseaux non vaccinés et non infectés (contrôles). Les oiseaux seront hébergés sur copeaux puis en isolateurs pour volailles une fois infectés pour éviter toute contamination de l'environnement.

3. INFORMATIONS ADMINISTRATIVES ET RÉGLEMENTAIRES

3.1. L'établissement utilisateur (EU)

3.1.1. Agrément de l'EU où seront utilisés les animaux :

0. **Nom : UMR INRA/ENVT 1225 « Interactions Hôtes- Agents Pathogènes »**
   - **Numéro d'agrément : C 31 555 27**
   - **Date de délivrance de l'agrément : 19 août 2010**
   - **Nom et prénom du responsable : MILON Alain**
   - **Adresse électronique du responsable : a.milon@envt.fr**
   - **Nom et prénom de la personne délégataire du responsable présente dans l'EU : SCHELCHER François**
   - **Adresse électronique de ce délégataire : f.schelcher@envt.fr**

3.1.2. Comité d'éthique agréé par le MESR dont relève l'EU :

Sciences et santé animale (n°115)

3.1.3. Responsable(s) de la mise en œuvre générale du projet dans l'EU et de sa conformité à l'autorisation de projet :

Nombre de responsables :

1

Responsable :

- Nom et prénom : Mariette Ducatez
- Adresse postale (avec le nom du laboratoire) :
  UMR INRA/ENVT 1225
  23 chemin des Capelles
  31076 Toulouse Cedex 3
- Adresse électronique : m.ducatez@envt.fr
- Téléphone : 05 61 19 32 49

3.1.4. Responsable(s) du bien-être des animaux :

Nombre de responsables :

1

Responsable :

- Nom et prénom : Jean-Marc Delmas
- Adresse postale (avec le nom du laboratoire) :
  UMR INRA/ENVT 1225
  23 chemin des Capelles
  31076 TOULOUSE Cedex 3
- Adresse électronique : jm.delmas@envt.fr
- Téléphone : 05 61 19 38 89

3.2. Le personnel

- **Compétences des personnes participant au projet :**

- **la conception des procédures expérimentales et des projets** **oui**
- **l'application de procédures expérimentales aux animaux** **oui**
- **les soins aux animaux** **oui**
- **la mise à mort des animaux** **oui**

3.3. Le projet

3.3.1. L'objectif du projet :

Est-il :

- ○ **Justifié du point de vue éducatif ?**
- ○ **Requis par la loi ?**
- ☑ **Justifié du point de vue scientifique ?**

Informations sur cette justification :

La grippe aviaire est causée par un virus qui évolue très rapidement. Les virus H9N2 faiblement pathogènes sont enzootiques en Tunisie, en Egypte, et dans de nombreux pays d'Asie. Ce sous-type est déjà passé à l'homme à plusieurs reprises (toujours sous une forme peu sévère). Le meilleur moyen à l'heure actuelle de réduire la charge virale dans les élevages est la vaccination. La difficulté est de vacciner avec une souche suffisamment proche de la souche du terrain pour s'assurer d'une réactivité croisée, sans être obligé de générer une nouvelle souche vaccinale pour chaque souche du terrain.

Les virus influenza faiblement pathogènes de sous-type H9N2 sont classées en de nombreux groupes de virus distincts sur la base de leurs séquences génétiques et de leur antigénicité : les souches « G1-like », « Y280-like » regroupant la plupart des virus H9N2 circulant dans les élevages aviaires dans le monde (SJCEIRS H9 Working Group, Emerging Microbes and Infection, 2014). En Tunisie, en Egypte, et dans une bonne partie de l'Asie, les souches « G1-like » dominent.

Le vecteur MVA a déjà été utilisé avec succès dans des études de vaccination contre plusieurs virus dont le virus influenza et dans de nombreux modèles animaux (souris, furet, singe, poulet, etc). Chez le poulet, il a été testé une seule fois, contre un virus influenza hautement pathogène de sous-type H5N1 et a protégé les oiseaux lors de l'épreuve virulente (Veits et al, Vaccine, 2008).
La stratégie de « vaccin ancestral » a été fructueuse pour protéger des furets contre 3 clades de virus influenza hautement pathogènes de sous-type H5N1 (Ducatez et al, *Proceedings of the National Academy of Sciences*, 2011).

Nous voulons à présent tester l'efficacité du vecteur MVA combiné à la stratégie de vaccin « ancestral » pour le sous-type H9 (« G1-like ») du virus influenza. Cette approche innovante est prometteuse pour réduire la charge virale des souches de virus influenza H9N2.

3.3.2. Description du projet :

Le vaccin MVA-H9 ancestral est produit par nos collaborateurs à l'Institute for Infectious Diseases and Zoonoses Ludwig-Maximilians-Universität München, en Allemagne.

La souche du terrain (wild type) faiblement pathogène qui va être utilisée pour infecter les animaux a été isolée en Tunisie, il s'agit de A/chicken/Tunisia/12/2010(H9N2). Nous allons travailler sur poulets et dindes.

Quatre groupes d'oiseaux vont être utilisés. Les groupes seront constitués comme suit (vue d'ensemble sur le schéma de procédure expérimentale page 7) :

Groupe A : vaccination double (vaccination et rappel), challenge
Groupe B : vaccination simple (1 injection unique), challenge
Groupe C : non vacciné, challenge
Groupe D : non vacciné, pas de challenge

La durée totale de l'expérience est de 54 jours ; elle se divise en trois phases (vue d'ensemble à la fin de cette section) :

Phase 1 : Vaccination (à J_{-21}) du premier groupe (A) (avec prises de sang pour tous les oiseaux avant la vaccination pour s'assurer de leur séronégativité)

Phase 2 : Vaccination (à J_0) du premier groupe (A, rappel) et du deuxième groupe (B), (avec prises de sang à J_{-1} pour suivi des anticorps post-première vaccination pour les oiseaux du groupe A et pour s'assurer de la séronégativité des poulets du groupe B)

Phase 3: Inoculation du pathogène (J_{21}), test de l'excrétion virale (écouvillons oropharyngés quotidiens de J_{22} à J_{28} et autopsies de 3 oiseaux par groupe à J_{24}) et de la réponse immunitaire (prise de sang pour suivi d'anticorps à J_{35})

Un suivi de cinétique de développement d'anticorps post-vaccination sera effectué en 2 étapes : d'abord avec 5 oiseaux du groupe A à $J_{-18/-15/-12}$ (pour savoir si les anticorps anti-H9 apparaissent entre 3, 6, et 9 jours post vaccination) ; puis avec 5 oiseaux du groupe B (vaccinés 1 seule fois) pour affiner la cinétique avec des prélèvements quotidiens en ciblant les 3-4 jours lors desquels les anticorps apparaissent sur la base des prélèvements sanguins du groupe A (J3 à J6 sont indiqués sur le schéma expérimental ci-dessous, nous ajusterons le timing en fonction des résultats sérologiques du groupe A).

Tous les animaux auront 1 jour au début de la procédure expérimentale (primo-vaccination du groupe A sur poussins d'1 jour) pour que le challenge ait lieu à 6 semaines conformément aux expériences du même type dont les résultats sont déjà publiés (Veits *et al*, Vaccine, 2008).

Le vaccin sera utilisé en intra-musculaire (IM), et une dose comprendra 10^8 plaque forming units (PFU) de vaccin puisque l'efficacité d'un vaccin MVA-H5 a déjà été démontrée en IM à cette dose

dans le modèle poulet par Veits *et al* en 2008.

Tous les animaux seront hébergés en isolateurs pour volailles pour l'épreuve de virulence (groupes de 6-8 animaux par isolateur), sauf ceux du groupe D (groupe de contrôle, pas de vaccination, pas d'infection) qui seront hébergés sur copeaux dans un box (dimensions de la zone avec copeaux dans le box : 2,5m x 3m, conformes à la directive 2010/63/EU).

Photo d'un isolateur volaille Allentown

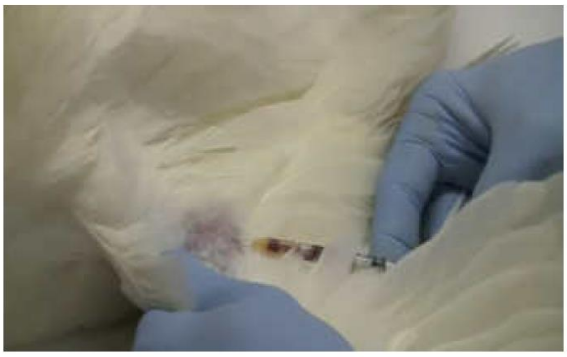
- Référence de nos isolateurs Allentown : SH12isol

- Superficie dans un isolateur : 1,2 m^2

- Surface minimale pour des poulets de moins de 600g d'après l'AM du 1er février 2013 (AGRG1238753A) : 1 m^2 (0.05 m^2/poulet) ; pour les dindes et pour les poulets de plus de 600g, ces surfaces passent à 2 m^2 minimum.

En accord avec le personnel vétérinaire, et de par la courte durée de l'hébergement en isolateurs (2 semaines, durée minimale pour une épreuve de virulence influenza dans le modèle aviaire), nous serons donc amenés à héberger les oiseaux dans des compartiments plus petits que ce que leur poids nécessiterait normalement.

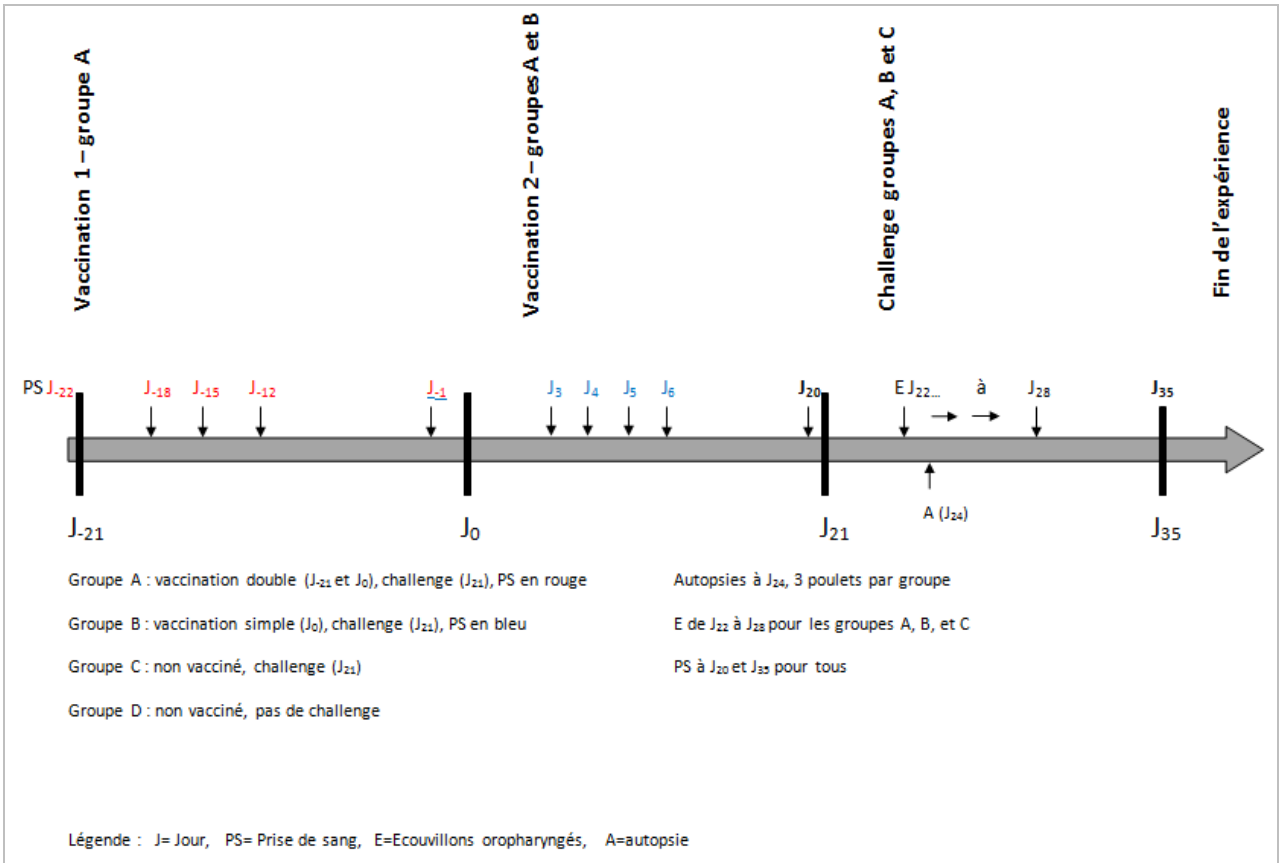
Les prises de sang seront effectuées à la veine alaire (cf illustration ci-dessous). Les écouvillons oropharyngés seront effectués comme illustré ci-dessous :



Prise de sang à la veine alaire

Ecouvillonnage oropharyngé

La procédure expérimentale est schématisée ci-dessous :



Nous allons travailler en animalerie de niveau 2 (A2) en respectant les mesures de biosécurité obligatoires en A2 afin d'éviter la contamination de l'environnement par le pathogène utilisé. Les manipulateurs porteront l'équipement de protection individuelle suivant : combinaison, bottes en caoutchouc, gants et sur-gants, masque, charlotte. Les salles dans lesquelles sont installés les isolateurs sont équipées de pédiluve à l'entrée pour éviter toute contamination de l'environnement. Enfin, les isolateurs dans lesquels seront hébergés les oiseaux infectés ont une pression négative, qui permet d'éviter la sortie de pathogène.

3.3.3. Précisez, le cas échéant, la ou les méthodes de mise à mort prévue(s) :

Anesthésie/euthanasie fixe au barbiturique (0.5 ml de Pentobarbital sodique® en intraveineuse)

3.3.4. Précisez, le cas échéant, les éléments scientifiques justifiant la demande de dérogation concernant la méthode de mise à mort envisagée :

NA

3.3.5. Stratégie d'expérimentation ou d'observation et approche statistique utilisée afin de réduire au minimum le nombre d'animaux, la douleur, la souffrance et l'angoisse, infligées et l'impact environnemental, le cas échéant – si une étude statistique est prévue, indiquez et justifiez les tests choisis :

Des données de la littérature (Tang et al, Vet Microbiol, 2014 (étude vaccinale avec des groupes de

10 poulets) et Tsunekuni et al, Veterinary Immunology and Immunopathology, 2014 (étude vaccinale avec des groupes de 4 poulets)) ainsi qu'un test statistique (critère de jugement binaire, essai de différence, avec un risque alpha de 5% et une puissance de 80%) indiquent que l'on peut travailler sur 8 animaux vaccinés pour une étude d'efficacité de vaccin pour avoir des données nécessaires et suffisantes pour être valables statistiquement.
Nous allons suivre les oiseaux quotidiennement pour repérer au plus vite tout animal qui souffrirait (très peu probable) et procéder à une anesthésie suivie d'une euthanasie pour limiter au minimum douleur, souffrance et stress.

3.4. Les animaux

3.4.1. Justifiez la nécessité d'avoir recours à des animaux pour atteindre les objectifs du projet :

De par sa nature, et l'impact de la réponse immunitaire de l'hôte, l'étude de l'efficacité de vaccins MVA ne peut se faire in vitro : nous sommes obligés d'avoir recours à des animaux pour évaluer la protection engendrée par un vaccin contre un virus.

3.4.2. Espèces animales ou types d'animaux utilisés :

- Souris (*Mus musculus*) ☐
- Rats (*Rattus norvegicus*) ☐
- Cobayes (*Cavia porcellus*) ☐
- Hamsters (syriens) (*Mesocricetus auratus*) ☐
- Hamsters (chinois) (*Cricetulus griseus*) ☐
- Gerbilles de Mongolie (*Meriones unguiculatus*) ☐
- Autres rongeurs (*Rodentia*) ☐
- Lapins (*Oryctolagus cuniculus*) ☐
- Chats (*Felis catus*) ☐
- Chiens (*Canis familiaris*) ☐
- Furets (*Mustela putorius furo*) ☐
- Autres carnivores (*carnivora*) ☐
- Chevaux, ânes et croisements (*Equidae*) ☐
- Porcs (*Sus scrofa domesticus*) ☐
- Caprins (*Capra aegagrus hircus*) ☐

Ovins (*Ovis aries*)	☐
Bovins (*Bos primigenius*)	☐
Prosimiens (*prosimia*)	☐
Ouistitis et tamarins (*par exemple, Callithrix jacchus*)	☐
Singe cynomolgus (*Macaca fascicularis*)	☐
Singe rhésus (*Macaca mulatta*)	☐
Vervets *chlorocebus* spp. (généralement soit *pygerythrus*, soit *sabaeus*)	☐
Babouins (*Papio* spp.)	☐
Saïmiris (par exemple, *Saimiri sciureus*)	☐
Autres espèces de primates non humains (autres espèces de *Ceboidea* et *Cercopithecoidea*)	☐
Singes anthropoïdes (*Hominoidea*)	☐
Autres mammifères (autres *Mammalia*)	☐
Poules domestiques (*Gallus gallus domesticus*)	☑
Autres oiseaux (autres *Aves*)	☑
Reptiles (*Reptilia*)	☐
Grenouilles Rana (*Rana temporaria* et *Rana pipiens*)	☐
Grenouilles Xenopus (*Xenopus laevis* et *Xenopus tropicalis*)	☐
Autres amphibiens (autres *Amphibia*)	☐
Poissons zèbres (*Danio rerio*)	☐
Autres poissons (autres *Pisces*)	☐
Céphalopodes (*Cephalopoda*)	☐

Espèces : poulet et dinde

3.4.3. Justifiez la pertinence de l'(des) espèce(s) animale(s) choisie(s) :

Le projet nécessite l'utilisation de l'hôte naturel du pathogène étudié : le poulet et la dinde

3.4.4. S'agit-il de spécimens d'espèces menacées énumérées à l'annexe A du règlement (CE) n° 338/97 du Conseil du 9 décembre 1996 relatif à la protection des espèces de faune et de flore sauvages par le contrôle et leur commerce ? **non**

Si oui, éléments scientifiques démontrant que la finalité de la procédure expérimentale ne peut être atteinte en utilisant d'autres espèces que celles énumérées dans cette annexe :

3.4.5. S'agit-il de spécimens de primates non humains ? **non**

Si oui, éléments scientifiques démontrant que la finalité de la procédure expérimentale ne peut être atteinte en utilisant d'autres espèces de primates non humains

3.4.6. S'agit-il d'animaux capturés dans la nature ? **non**

Si oui, éléments scientifiques démontrant que la finalité de la procédure expérimentale ne peut être atteinte en utilisant d'autres animaux que ceux capturés dans la nature :

3.4.7. S'agit-il d'animaux d'espèces domestiques, errantes ou vivantes à l'état sauvage ? **non**

Si oui, éléments scientifiques démontrant que la finalité de la procédure expérimentale ne peut être atteinte qu'en utilisant ces animaux.

3.4.8. Catégorie des animaux utilisés dans le projet :

Animaux tenus en captivité (domestiques ou non domestiques) ☑

Animaux non domestiques non tenus en captivité ☐

Animaux génétiquement altérés ☐

- **Animaux non domestiques non tenus en captivité**
  - **Si les animaux utilisés sont des spécimens d'espèces protégées en application de l'article L. 411-1 du Code de l'environnement, indiquez les références de la dérogation accordée pour effectuer la capture des animaux dans le milieu naturel (4° de l'article L. 411-2 du Code de l'environnement) :**
  - **Si les animaux utilisés sont des spécimens d'espèces dont la chasse**

est autorisée, indiquez ici les références de l'autorisation de prélèvement accordée pour effectuer la capture des animaux dans le milieu naturel (article L. 424-11 du Code de l'environnement) :

- **Justification scientifique montrant que l'objectif de la procédure expérimentale ne peut être atteint en utilisant un animal élevé en vue d'une utilisation dans des procédures expérimentales :**

- **Animaux génétiquement altérés**

Animaux génétiquement modifiés non

Si oui :

Création d'une lignée oui non
Maintien d'une lignée établie et/ou utilisation oui non
Phénotype non dommageable oui non
Phénotype dommageable oui non
Souche mutante autre oui non

Si oui :

Création d'un mutant oui non
Maintien d'une lignée établie et/ou utilisation oui non
Phénotype non dommageable oui non
Phénotype dommageable oui non

3.4.9. Origine des animaux tenus en captivité :

- **Les animaux destinés à être utilisés dans les procédures expérimentales appartenant aux espèces dont la liste est fixée réglementairement sont-ils élevés à cette fin et proviennent-ils d'éleveurs ou de fournisseurs agréés ?**

oui et en cours

- **Si oui, nombre d'établissements éleveur ou fournisseur agréés fournissant tout ou partie des animaux du projet :**

2

Etablissement :

- **Nom :**

PFIE - Centre Val de Loire - INRA

- **Adresse postale :**

Secteur 2 - Bâtiment 249
37380 NOUZILLY
France

- **Animaux fournis :**

Poulets EOPS

- **Si non, justifier scientifiquement l'utilisation d'animaux qui ne proviennent pas d'éleveurs ou de fournisseurs agréés :**

Il n'existe pas d'éleveur ou fournisseur agréé de dindes. Nous travaillons en collaboration avec le groupe Grelier en Vendée qui nous fournira les dindes nécessaires aux expériences. Nous avons lancé la procédure de certification du groupe Grelier.

- **Votre propre établissement utilisateur fournit-il tout ou partie des animaux du projet ? non**

- **Un autre établissement utilisateur fournit-il tout ou partie des animaux du projet ? non**

- **Nombre d'établissements éleveur occasionnel non agréés fournissant tout ou partie des animaux du projet ?**

Etablissement :

- **Nom :**

Grelier

- **Adresse postale :**

La Bohardière - BP1
49290 St-Laurent de la Plaine

- **Animaux fournis :**

dindes de statut sanitaire sain

- **Nombre d'établissements éleveur ou fournisseur localisés dans des Etats membres autres que la France fournissant tout ou partie des animaux du projet ?**

aucun

Etablissement :

Nom de cet éleveur ou fournisseur et pays :

- **Nombre d'établissements éleveur ou fournisseur localisés dans des pays tiers fournissant tout ou partie des animaux du projet ?**

Aucun

Etablissement :
Nom de cet éleveur ou fournisseur et pays :

- **Les animaux sont-ils des animaux réutilisés d'un projet précédent ?** **non**

  Si oui, veuillez compléter le chapitre 4.3 de ce formulaire.

3.4.10. Nombre estimé d'animaux utilisés dans le projet :

Nous utiliserons au total 32 poulets et 32 dindes

- **Justification de ce nombre pour chacune des espèces animales utilisées :**

Les données de la littérature ainsi qu'un test statistique (critère de jugement binaire, essai de différence, avec un risque alpha de 5% et une puissance de 80%) indiquent que on peut travailler sur 8 animaux par expérience/groupe pour avoir des données nécessaires et suffisantes pour être valables statistiquement. Pour réduire le nombre d'animaux utilisés, nous avons décidé de n'utiliser que six animaux pour les groupes non-vaccinés (C et D) puisque nous avons des données préliminaires (infections avec le même virus influenza H9N2 dans le cadre du projet MODELAFLU).

3.4.11. Indiquez à quel(s) stade(s) de développement les animaux seront utilisés et le justifier :

Sur la base de publications décrivant des essais vaccinaux chez le modèle aviaire, nous allons utiliser des poulets et des dindes de 1 jour (à J_{-21}).

3.4.12. Indiquez le sexe des animaux utilisés et le justifier :

Mâles et femelles (le sex ratio est subi (nous sommes dépendants de la disponibilité des oiseaux en élevages sains) mais à l'heure actuelle aucune différence entre mâles et femelles n'ayant été observée, ceci nous importe peu).

3.4.13. Indiquez pour chaque espèce les points limites adaptés, suffisamment prédictifs et précoces pour permettre de limiter la douleur à son minimum, sans remettre en cause les résultats du projet :

Les données de terrain et nos expériences préliminaires montrent que les poulets vaccinés ne devraient pas avoir de signes cliniques. Même si improbables, tout signe clinique d'apathie, d'arrêt de prise d'aliment, ou de détresse respiratoire, constitueraient des points limites chez le poulet et la dinde et seraient suivis d'anesthésie/euthanasie.

4. LES PROCÉDURES EXPÉRIMENTALES

4.1 Objet(s) visés par les procédures expérimentales

A - La recherche fondamentale. ☑

B - Les recherches transactionnelles ou appliquées menées pour :

- la prévention, la prophylaxie, le diagnostic ou le traitement de maladies, de mauvais états de santé ou d'autres anomalies ou de leurs effets chez l'homme, les animaux ou les plantes ;

- l'évaluation, la détection, le contrôle ou les modifications des conditions physiologiques chez l'homme, les animaux ou les plantes ;

- le bien-être des animaux et l'amélioration des conditions de production des animaux élevés à des fins agronomiques. ☐

C - L'une des finalités visées au point précédent (B) lors de la mise au point, de la production ou des essais de qualité, d'efficacité et d'innocuité de médicaments à usage humain ou vétérinaire, de denrées alimentaires, d'aliments pour animaux et d'autres substances ou produits. ☐

D - La protection de l'environnement naturel dans l'intérêt de la santé ou du bien-être de l'homme ou de l'animal. ☐

E- La recherche en vue de la conservation des espèces. ☐

F- L'enseignement supérieur, ou la formation professionnelle ou technique conduisant à des métiers qui comportent la réalisation de procédures expérimentales sur des animaux ou les soins et l'entretien de ces animaux, ainsi que la formation professionnelle continue dans ce domaine. ☐

G - Les enquêtes médico-légales. ☐

4.2 Nombre de procédures expérimentales :

1

4.2.1 NOM DE LA PROCÉDURE EXPÉRIMENTALE N° 1 :

épreuve virulente du vaccin recombinant MVA-H9 ancestral contre une souche tunisienne de virus influenza faiblement pathogène H9N2.

- PROPOSITION DE CLASSIFICATION DE LA PROCÉDURE SELON LE DEGRÉ DE SÉVÉRITE (conformément à l'annexe de l'arrêté relatif à l'autorisation de projet) **:**

○ classe légère

☑ classe modérée

○ classe sévère

○ classe sans réveil

- Description détaillée de la procédure expérimentale :

- Pertinence et justification de la procédure expérimentale :

L'expérience porte sur l'efficacité d'un vaccin MVA-H9 ancestral chez le poulet et la dinde. Bien que le vecteur exprime les gènes recombinants in vitro, il est absolument nécessaire d'avoir un modèle in vivo pour s'assurer que le vecteur n'interfère pas avec d'autres composants de l'organisme. A la fin de la procédure on saura si on est capable d'éviter une infection avec la souche A/chicken/Tunisia/10/2012(H9N2), que l'on trouve réellement sur le terrain.

- Indiquez le nombre de lots et le nombre d'animaux par lots, et les justifier :

32 oiseaux au total par expérience, quatre lots (quatre lots de 8 oiseaux) (32 poulets et 32 dindes au total)

- Indiquez le cas échéant le prélèvement, ainsi que la fréquence et le(s) volume(s) prélevés :

Prélèvement	*Fréquence et volume prélevé*	*Animaux et Date (jours post infection)*
Prise de sang pour suivi sérologique	1 ml, 2 à 7 fois (suivi d'anticorps pour comprendre la cinétique de développement des anticorps post vaccination et post challenge)	Groupe A : J_{-22}/J_{-18} /J_{-15} /J_{-12} /J_{-1}/J_{20} et J_{35} Groupe B : J_{-1}, J_3 /J_4 /J_5 /$J_{6,}$ J_{20} et J_{35} Groupes C et D : J_{-20}, J_{35}
Ecouvillon oropharyngé	une fois par jour et par animal	Groupes A, B et C : J_{22} -J_{28}
Autopsie	Une fois sur 3 animaux par groupe, fragment de trachée et de poumon prélevés sur chaque animal	Groupes A, B et C : J_{24}

- Indiquez le cas échéant les méthodes pour réduire ou supprimer la douleur, la souffrance et l'angoisse (liste des médications - anesthésiques,

analgésiques, anti-inflammatoires…en précisant les doses, voies, durées et fréquences d'administration), y compris le raffinement des conditions d'hébergement, d'élevage et de soins :

Les oiseaux seront hébergés en isolateurs pour volailles, respectivement sur des copeaux, et anesthésiés/euthanasiés pour limiter la douleur à son minimum en cas de signes cliniques importants (décrits en 3.4.13) avant la fin de l'expérience. Nous allons accrocher des CDs dans les isolateurs (sur des fils accrochés au plafond de l'isolateur, référence travaux ITAVI) pour enrichir l'environnement.

- Indiquez le cas échéant les dispositions prises en vue de réduire, d'éviter et d'atténuer toute forme de souffrance des animaux de la naissance à la mort :

Les oiseaux seront hébergés en isolateurs pour volailles, respectivement sur des copeaux, et anesthésiés/euthanasiés pour limiter la douleur à son minimum en cas de signes cliniques importants (décrits en 3.4.13) avant la fin de l'expérience.

- Indiquez le cas échéant les raisons scientifiques justifiant une dérogation à l'anesthésie des animaux :

Sans objet

- Indiquez le cas échéant les raisons scientifiques justifiant une dérogation aux conditions d'hébergement des animaux :

NA

- Dispositions prises pour éviter tout double emploi injustifié des procédures expérimentales, le cas échéant :

NA

- Devenir des animaux à la fin de cette procédure expérimentale :

- mise à mort ?

Précisez les animaux concernés :

tous

- animal gardé en vie ?

précisez les animaux concernés et si la décision a été prise par le vétérinaire ou toute autre personne compétente désignée par le responsable du projet :

aucun

- placement ou mise en liberté des animaux ?

précisez les animaux concernés :

non

4.3. Si le projet utilise des animaux réutilisés d'un projet antérieur : NON

- GRAVITÉ RÉELLE DES PROCÉDURES EXPÉRIMENTALES ANTÉRIEURES :
légère
modérée
sévère

Précisez les éléments scientifiques justifiant la demande de dérogation pour autant que les animaux n'aient pas été utilisés plus d'une fois dans une procédure expérimentale entrainant une douleur intense, de l'angoisse ou une souffrance équivalente :

- Effet cumulatif de cette réutilisation sur les animaux :

- L'avis vétérinaire est-il favorable en prenant en considération le sort de l'animal concerné sur toute sa durée de vie ? **oui** **non**

- L'animal réutilisé a-t-il pleinement recouvré son état de santé et de bien-être général ? **oui** **non**

4.4. Cas particulier des projets contenant une procédure expérimentale impliquant une douleur, une angoisse ou une souffrance sévère et susceptible de se prolonger sans qu'il soit possible de les soulager

- De quelle(s) procédure(s) expérimentale(s) du projet s'agit-il ?

- Justifiez scientifiquement les raisons à l'origine d'une demande de dérogation :