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INFLUENZA VACCINE INDUCED ANTIBODY RESPONSES IN RELATION TO EVOLUTION OF INFLUENZA A VIRUSES

Anu Haveri

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To my parents

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ABSTRACT

Influenza A viruses (IAVs) cause yearly epidemics and occasionally appearing new pandemics. IAVs are subtyped based on their virion surface glycoproteins haemagglutinin and neuraminidase. Seasonal IA epidemics in humans are currently caused by A(H1N1) and A(H3N2) viruses with varying incidence. The natural reservoir of IAVs is birds, and the virus also infects swine and other mammalian species. Global monitoring of the genetic drift and antigenic characteristics of influenza viruses is needed for vaccine strain selection and pandemic preparedness.

Vaccine-induced humoral immunity can be analyzed by measuring serum antibodies. In this study, serum antibody levels in adults were assessed before and after vaccination with different seasonal influenza vaccines and pre-pandemic A(H5N1) vaccine candidate viruses. Traditional haemagglutination inhibition (HI) assay was used to measure antibody levels. In addition, a colorimetric microneutralization test (MNT) was optimized to measure functional neutralizing antibodies. The correlation between the methods was found to be high.

One of the most effective ways to prevent an infectious disease is vaccination, if circulating viruses are well-matched with vaccine viruses. The protective efficacy of influenza vaccines varies mainly due to virus evolution. In this study we reported reduced cross-protection against drifted A(H3N2) viruses.

The latest influenza pandemic was caused by a novel A(H1N1)pdm09 virus, since pre-existing immunity in the majority of human population was missing. After the pandemic, the A(H1N1)pdm09 viruses have continued to circulate as epidemic strains. In this study, seasonal influenza vaccines were found to induce high levels of cross-reactive antibody responses against different genetic group A(H1N1)pdm09 viruses for several years after the pandemic.

Avian influenza viruses usually do not easily transmit to humans. However, A(H5N1) viruses have caused serious disease and considerable mortality, which have led to the development of various avian influenza vaccine candidates. Prepandemic A(H5N1) stockpile vaccine was also purchased in Finland. In this study we demonstrated that two heterologous A(H5N1) vaccinations induced long-lasting cross-clade humoral immunity.

KEYWORDS: Influenza, vaccine, antibodies, microneutralization test, humoral immunity, viral evolution, pandemic

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
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TIIVISTELMÄ

Tarttuvuutensa ja muuntautumiskykynsä vuoksi influenssa A -virukset aiheuttavat vuosittaisia epidemioita ja ajoittain myös maailmanlaajuisia pandemioita. Influenssa A -virukset luokitellaan useisiin alatyyppeihin viruksen kahta eri pintaproteiinia määrittävien hemagglutiniini- ja neuraminidaasigeenien perusteella. A(H1N1)- ja A(H3N2)-alatyypin virukset aiheuttavat vuosittain epidemioita vaihtelevin valtasuhtein. Influenssa A -viruksia esiintyy erityisesti linnuissa, mutta myös sioissa ja joissain muissa nisäkäslajeissa. Influenssavirusten evoluution maailmanlaajuinen seuranta tukee rokotevirusten valintaa ja pandemioihin varautumista.

Rokotteen laukaiseman vasta-ainevälitteisen immuniteetin muodostumista voidaan arvioida mittaamalla virusvasta-aineita. Tässä tutkimuksessa tarkasteltiin eri kausi-influenssarokotteiden sekä prepandeemisten A(H5N1)- lintu-influenssarokotteiden synnyttämiä vasta-ainetasoja aikuisilla. Tutkimusmenetelmänä käytettiin perinteistä hemagglutinaation inhibitio -testiä, jonka rinnalle optimoitiin kolorimetrinen mikroneutralisaatiotesti. Menetelmien välinen korrelaatio oli erittäin hyvä.

Rokottaminen suojaa tehokkaimmin influenssaa vastaan silloin, kun kiertävät virukset ovat samankaltaisia ennakoidun rokoteviruksen kanssa. Kausi-influenssarokotteiden suojateho vaihtelee virusten evoluutiosta johtuen kaudesta toiseen. Tässä tutkimuksessa tunnistettiin alentunut immuunivaste muuntuneita A(H3N2)-viruksia kohtaan.

Viimeisimmän influenssapandemian aiheutti A(H1N1)pdm09-virus, jota vastaan valtaväestöllä ei ollut aiempaa immuniteettia. Pandemian aiheuttaneen viruksen jälkeläisvirukset jäivät kiertämään väestössä normaalina kausi-influenssana. Tässä tutkimuksessa osoitettiin, että kausi-influenssarokotteet saivat aikaan hyvän immuunisuojan eri jälkeläisviruskantoja vastaan useita vuosia pandemian jälkeen.

Lintuinfluenssavirukset tarttuvat ihmiseen yleensä huonosti. Vakavampia taudinkuvia ja huomattavaa kuolleisuutta aiheuttaneet A(H5N1)-tartunnat ovat aiheuttaneet huolta uudesta pandemiasta, mistä syystä on kehitetty erilaisia lintuinfluenssarokotekantoja. Prepandeeminen H5N1-mallirokote varattiin myös Suomeen. Tässä tutkimuksessa todettiin, että kahdella, eri viruskantoja sisältävällä H5N1-rokotteella saatiin aikaan laajakirjoinen vasta-ainevälitteinen immuniteetti.

AVAINSANAT: Influenssa, rokote, vasta-aineet, mikroneutralisaatiotesti, vasta-ainevälitteinen immuniteetti, virusevoluutio, pandemia

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Abbreviations

Ag	Antigen
AS03	Adjuvant system containing α-tocopherol and squalene in an oil-in-water emulsion
BSA	Bovine serum albumin
CC	Cell control
CD4	Marker on helper T lymphocytes
CI	Confidence interval
CPE	Cytopathic effect
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
Fc	Fragment, crystallizable. The tail region of an antibody.
FcR	Fc receptor
FDA	The United States Food and Drug Administration
FRNT	Focus reduction neutralization test
GMT	Geometric mean titre
HA	Haemagglutinin
HI	Haemagglutination inhibition
HPAI	Highly pathogenic avian influenza
HRP	Horseradish peroxidase
HUS	Helsinki University Hospital
IAV	Influenza A virus
Ig	Immunoglobulin
LAIV	Live attenuated influenza vaccine
M1	Matrix protein
M2	Protein on viral surface (ion channel)
M2e	M2 ectodomain
MDCK	Madin-Darby canine kidney cells
MF59	Oil-in-water emulsion used as adjuvant
MNT	Microneutralization test

mRNA	Messenger ribonucleic acid
NA	Neuraminidase
NP	Nucleoprotein
OAS	Original antigenic sin
OC	Oseltamivir carboxylate
OD	Optical density
OPD	O-phenylenediamine dihydrochloride
PBS	Phosphate-buffered saline
PeSt	Penicillin-streptomycin
PRNT	Plaque reduction neutralization test
RBC	Red blood cell
RDE	Receptor-destroying enzyme
RNA	Ribonucleic acid
RT	Room temperature
QIV	Quadrivalent influenza vaccine
SR	Seroprotection rate
SRH	Single radial haemolysis
$TCID_{50}$	50% tissue culture infectious dose
TIV	Trivalent influenza vaccine
THL	Finnish Institute for Health and Welfare
UD	Unpublished data
VC	Virus control
VE	Vaccine effectiveness
VLP	Virus-like particles
VN	Virus neutralization
WGS	Whole genome sequencing
WHO	World Health Organization

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals. Additionally, unpublished results linked to these publications are presented.

I Haveri A, Ikonen N, Julkunen I, Kantele A, Anttila VJ, Ruotsalainen E, Nohynek H, Lyytikäinen O, Savolainen-Kopra C. Reduced cross-protection against influenza A(H3N2) subgroup 3C.2a and 3C.3a viruses among Finnish healthcare workers vaccinated with 2013/14 seasonal influenza vaccine. Euro Surveill., 2015; 20(5):21028: 1–8. doi: 10.2807/1560-7917.es2015.20.5.21028.

II Haveri A, Ikonen N, Kantele A, Anttila VJ, Ruotsalainen E, Savolainen-Kopra C, Julkunen I. Seasonal influenza vaccines induced high levels of neutralizing cross-reactive antibody responses against different genetic group influenza A(H1N1)pdm09 viruses. Vaccine, 2019; 37(20): 2731–2740. doi: 10.1016/j.vaccine.2019.03.078.

III Haveri A, Ikonen N, Savolainen-Kopra C, Julkunen I. Long-lasting heterologous antibody responses after sequential vaccination with A/Indonesia/5/2005 and A/Vietnam/1203/2004 pre-pandemic influenza A(H5N1) virus vaccines. Vaccine, 2021; 39(2); 402–411. doi: 10.1016/j.vaccine.2020.11.041.

1 Introduction

Influenza viruses are enveloped, negative-stranded ribonucleic acid (RNA) viruses. There are four types of influenza viruses, types A, B, C and D. Influenza A and B viruses circulate and cause seasonal epidemics in humans practically every year and novel influenza A reassortant viruses have caused occasional pandemics. Influenza C virus usually causes mild infections in humans with no public health importance. Influenza D viruses primarily affect cattle and have not yet been associated with clinical infection in humans.

Influenza A viruses (IAVs) are further classified into subtypes according to the combinations of two proteins on the surface of the virus: haemagglutinin (HA or H) and neuraminidase (NA or N). Subtypes A(H1N1) and A(H3N2) are currently circulating in humans. The A(H1N1)pdm09 virus caused the most recent influenza pandemic in 2009 and subsequently replaced the previous seasonal influenza A(H1N1) viruses that circulated prior to 2009. Emerging avian IAVs, especially those of the H5 and H7 subtypes, pose a constant pandemic threat, if they evolve the ability to transmit efficiently from person-to-person.

Vaccination remains one of the most effective strategies in controlling influenza disease burden. An inactivated influenza vaccine induces predominantly antibodies, while a live attenuated influenza vaccine (LAIV) and novel vaccine platforms also induce cell-mediated immune responses. However, circulating influenza viruses are constantly mutating to evade humoral immunity in the population. The HA and NA genes possess the highest evolutionary rates of all influenza virus proteins. Constant mutations in the antigenic sites of HA and NA molecules require annual adaptation of the influenza vaccine formulation.

Immunological techniques commonly used to quantify influenza-specific antibodies are the haemagglutination inhibition (HI), single radial haemolysis (SRH) and microneutralization tests (MNT). A fourfold increase or decrease in antibody titres in subsequent serum specimens is generally considered to be diagnostic.

In this study we measured HI and MNT titres as surrogate correlates of protective immunity before and after influenza vaccinations. The HI assay detects antibodies that interfere with influenza virus HA binding to sialic acid-linked residues on red blood cells (RBC). HI titre of 1:40 has been generally considered to indicate a 50%

reduction in the risk of IAV infection in adults. MNT quantifies functional neutralizing antibodies primarily directed towards the HA molecule that prevent infection of cells in tissue culture *in vitro*. There are various modifications of the MNTs, yet shorter protocols of two and three days are preferred for seroepidemiological studies. A global standardization and an agreement of a protective antibody level of influenza MNT techniques are still missing.

2 Review of the Literature

2.1 Influenza A virus evolution

IAV belongs to the genus *Alphainfluenzavirus* of the virus family *Orthomyxoviridae* [1]. IAVs are enveloped viruses with a rapidly evolving negative-stranded RNA genome comprising eight gene segments, each encoding one or more proteins [2]. IAVs have been suggested to have originated from wild waterfowl that remain generally asymptomatic during infection [3].

IAV strains are classified into types and subtypes based on their genetic and antigenic properties. The surface glycoproteins HA and NA are the major antigenic targets of the host immune response against IAV infection (Figure 1). So far 18 different HA subtypes and 11 different NA subtypes have been characterized [4].

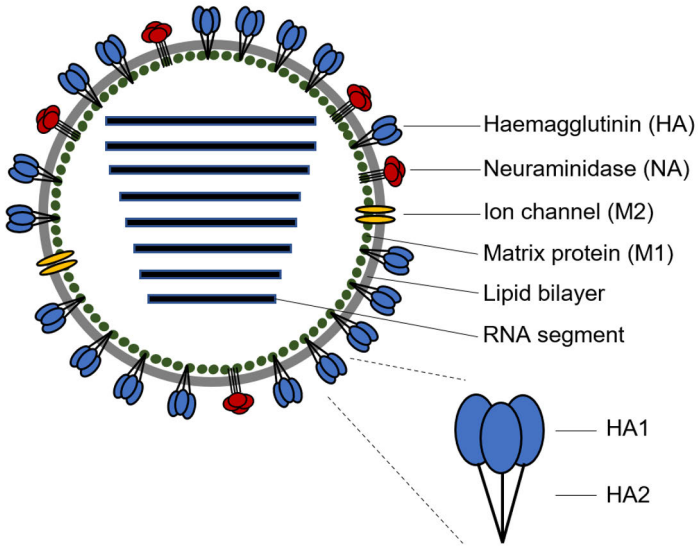

Figure 1. Simplified structure of influenza A virus. Modified from Webster et al. 2013 [2].

Continuous evolution of influenza viruses enables the escape of prior infection- or vaccination-induced immunity. Five basic mechanisms determine the genetic

structure and evolution: mutation, natural selection, genetic drift, recombination, and migration [5]. Compared to the high mutation rate of IAVs, the effective spreading of a newly emerged antigenic variant is, however, quite rare and subject to the biology of the virus, the role of hosts' innate and adaptive immunity and epidemiological processes. To cause an epidemic, antigenic change of the mutated virus should not disrupt the receptor binding ability, as well as a sufficient viral density within-host should be reached. After exiting the cell, the virus should survive mucociliary clearance and finally be able to replicate well in individuals who are infected. Moreover, the wide viral diversity in an ongoing epidemic combined with the short duration and seasonal nature of influenza epidemics cause competition with existing variants [6].

2.1.1 Mechanisms of evolution

Significant evolution occurs in each of the eight gene segments of influenza viruses but is most prominent in the surface glycoproteins. Mutations are one of the most important mechanisms for producing variations by substitutions, deletions, and insertions [3]. The IAV RNA polymerase lacks the 3' to 5' exonuclease proofreading capability, which results in a high spontaneous mutation rate [7–9].

Antigenic drift

Antigenic drift happens in both influenza A and B viruses and causes periodic epidemics. Antigenic drift is caused by relatively continuous, gradual modification by point mutations. Mutations in the surface glycoproteins HA and NA may lead to the inability of antibodies produced against previous strains to neutralize the altered virus [6].

Rates of adaptation vary significantly among influenza genes. HA and NA genes have the highest rates of mutations, HA evolving at the highest rate [10]. The adaptation in the NA gene is concentrated on sites at the surface of a tetrameric NA molecule [11]. Similarly, the globular head domain of HA1 is distal from the virus surface and readily accessible for immune recognition [12]. The mutation rate for the antigenic HA1 domain is much higher for both subtypes H1N1 and H3N2, whereas the mutation rate has shown to be slow for the conserved HA2 stem domain [11].

Five major antigenic sites in the HA1 globular head domain for both H1 and H3 influenza viruses have been identified. Substitutions of amino acids at these antigenic sites are associated with changes in the antigenic properties of a trimeric HA molecule [13]. H1 HA major antigenic sites are Sa, Sb, Ca1, Ca2, and Cb [14, 15] (Figure 2) and those of H3 HA sites A–E [16, 17]. The antigenic sites of Sa, Sb

and Ca2 of H1 HA, and locations of A and B of H3 HA partially overlap with the receptor binding site, while Ca1 and Cb of H1 HA, and antigenic sites C-E of H3 HA are more distant from the receptor binding site [12]. The antigenic sites of H5 HA have similarities with the antigenic sites of H1 and H3 HA. Site 1 in H5 HA corresponds to site A in H3 HA and site Ca in H1 HA. H5 HA site 2 corresponds to site B in H3 HA and site Sa in H1 HA [18, 19].

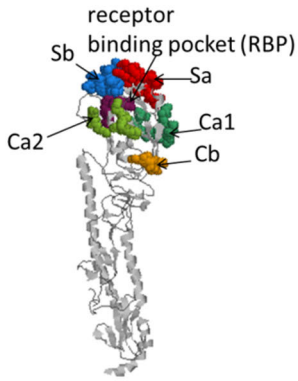

Figure 2. A side view of the monomeric structure of haemagglutinin molecule of influenza A(H1N1)pdm09 (A/California/04/2009; RCSB Protein Bank accession number 3LZG) with previously identified H1 protein-specific antigenic sites of influenza A(H1N1) viruses: Sa in red, Sb in blue, Ca1 in dark green, Ca2 in light green, and Cb in orange. The receptor binding pocket (RBP) in purple. Reprinted with permission from Original publication II.

Antigenic shift

Antigenic shift is less frequent than antigenic drift. The phenomenon occurs in IAVs only and may lead to the development of viruses that cause worldwide pandemics. In a shift event, influenza genes between two or more strains are reassorted during a coinfection in the same host. The new subtype virus contains a novel HA protein with or without a novel NA protein that is immunologically different from those viruses that have been circulating previously [2].

Adaptive mutation

A pandemic virus may arise also by a process called adaptive mutation. For example, an avian influenza A(H5N1) virus crosses the species barrier when infecting human beings. In humans, the adaptive mutation of the virus may increase the capacity of the virus to bind to human cells during subsequent rounds of infection, thus increasing its transmissibility among human beings [20].

RNA recombination

Although relatively rare among IAVs, also recombination contributes to the generation of genetic diversity. Recombination can only occur among viruses that replicate within the same cells [21]. Recombination in IAVs can occur through two mechanisms: a non-homologous recombination that occurs between two different RNA fragments [22] or through a homologous recombination during IAV replication [23], yet homologous recombination is controversial [24] and thought to be rare in influenza viruses [25].

2.1.2 Modes of transmission of influenza A viruses in humans

Influenza transmission is a dynamic and opportunistic process with factors affected by the host, the virus, and the environment [26]. Natural influenza transmission in humans is suggested to occur over short rather than long distances, likely through multiple ways via the aerosol [26], droplet and contact routes [26, 27].

Other species as reservoirs for zoonotic viruses and barriers of transmission

Birds are the natural hosts of influenza viruses. Migratory waterfowl are the predominant animal reservoirs of IAVs [3]. Domestic poultry can be infected with highly pathogenic (HPAI), low pathogenic, and non-pathogenic avian IAVs [28]. Human infection with avian IAVs seems to be quite rare despite of the proximity of people and poultry. However, pigs are like blenders where influenza viruses from birds and people can converge with adaptations and reassortments [2]. Swine influenza viruses can be transmitted also to humans, causing even pandemic outbreaks [28]. IAVs can infect a variety of other animals, including horses, dogs, cats, minks, sea mammals [2, 3] and bats [29].

Differences of host cell surface receptors constitute a barrier to cross-species transmission of influenza virus. The influenza virus binds to sialic acid residues on the host cell glycoproteins or glycolipids through the head of the HA molecule. Receptor-binding specificity differs between avian and human influenza viruses. Human-adapted influenza viruses preferentially bind to sialic acid with α-2,6-linkage, which are found on bronchial epithelial cells of the human upper respiratory tract [30]. The avian IAV preferentially recognizes sialic acid with α-2,3-linkage, which are found on epithelial cells of the birds' intestine and on the lower respiratory tract of humans [30]. Expression of both α-2,3- and α-2,6-linked sialic acids in swine tissue enables infection by both avian and human viruses, thus creating a favourable environment for reassortment of influenza gene segments [2].

Geographic and environmental differences

Seasonal influenza virus epidemics show global variation. In temperate regions of the Northern and Southern hemisphere, most infections occur in wintertime and the influenza activity peaks during cold and dry conditions [31]. However, in some tropical countries, influenza activity coincides with the rainy season, yet timing is variable, and all tropical countries do not have a clear influenza season [6, 32]. Environmental conditions may also be linked to changes in human behaviour favouring confined spaces, which has a role with more efficient IAV transmission [6].

2.1.3 Influenza A pandemics

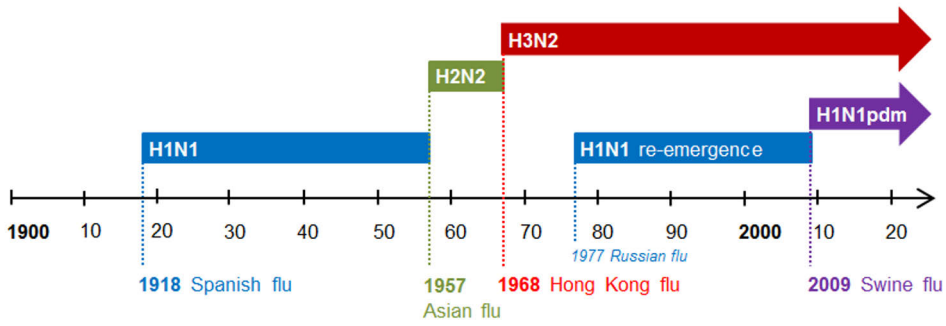

Figure 3. The emergence of pandemic influenza during the 20th and 21th century and the timespan of their subsequent circulation as epidemic strains. The re-emergence of H1N1 virus in 1977 "Russian flu" is not classified as a new pandemic virus. Modified from Webster et al. 2013 [2].

Influenza pandemic is a worldwide outbreak of influenza caused by IAV that has undergone antigenic shift for which the human immune system is relatively naïve [33]. IAVs expressing H1, H2, or H3 and N1 or N2 subtype proteins are known to have caused human pandemics. Global spread of pandemic IAV follows over a rather short period of time. After emergence of a novel pandemic IAV, the previous subtype usually disappears (Figure 3), with the exception of an ongoing circulation of A(H3N2) virus and the reintroduction of the A(H1N1) virus in 1977 [2]. An antigenically novel virus of an existing subtype may also be capable of pandemic-like spread.

During the 20th century and 21st century so far (Figure 3), three and one influenza pandemics have occurred, respectively [34].

- 1918–1919 the Spanish flu pandemic has been the most severe pandemic of influenza, which led globally to an estimated 50 million deaths [2]. Spanish flu was caused by A(H1N1) virus [35]. The origin of the genes in Spanish flu has remained unresolved [34].
- 1957–1958 the Asian flu caused by A(H2N2) virus was the second most severe influenza pandemic [2]. A(H2N2) virus was derived from genes from the circulating human A(H1N1) virus reassorted with avian virus-like HA, NA and polymerase protein PB1 segments [36, 37].
- 1968–1969 A(H3N2) virus was responsible for the Hong Kong flu, which caused lower morbidity and mortality during the pandemic presumably due to pre-existing antibodies against the N2 of the A(H2N2) pandemic/seasonal virus [38]. However, seasonal A(H3N2) strains have caused significantly more morbidity and mortality than co-circulating A(H1N1) strains [39]. The A(H3N2) virus comprises six segments from the seasonal A(H2N2) virus and two novel avian virus-like segments, HA and PB1 [37]. The HA of A(H3N2) virus derived from an avian virus with altered receptor binding properties changed from a preference for avian receptors to a preference for human receptors [40].
- 2009–2010 the Swine flu pandemic was caused by a new strain of A(H1N1)pdm09 virus. It was derived as a reassortant of avian, human, and swine origin viruses from North America and Eurasia [41–43]. While most cases were mild, A(H1N1)pdm09 virus caused still extensive morbidity and mortality [44]. Children and young adults were significantly more affected by A(H1N1)pdm09 virus compared to previous periods of epidemic influenza [45]. Moreover, in the elderly the pre-existing humoral immunity [46] and antibody response following infection suggested the existence of cross-reactive antibodies induced by the 1918 influenza virus [47].

2.1.4 Phylogenetic analysis

The evolutionary history and relationship between genes are routinely represented by phylogenetic trees. During tree building, homologous regions such as the coding region of the HA gene are compared in a multiple sequence alignment [2]. Phylogenetic analyses of the sequences enable among other things the estimation of the geographical spread and the rates of growth of influenza lineages, estimation of the timing of lineage divergence, the determination of the rates of evolution, and detection of reassortment events [2].

Until recently, Sanger sequencing of the HA gene has been the most used method to genetically characterize influenza viruses, although a Sanger-based sequencing method only partially covers one of the eight RNA segments of the influenza genome [48]. Nowadays, a whole genome sequencing (WGS) of all segments of the viral genome in one single reaction is also used, and it offers better resolution and substantially improved phylogenetic classification for genetic characterization compared to Sanger sequencing [49]. WGS with a high-throughput method such as next-generation sequencing has enabled the detection of mutations in all segments linked to potential virulence, drug resistance or other viral characteristics [50–52], and combined with clinical data also the possibility to predict the severity of an influenza infection [48, 51].

Based on HA phylogenetic relatedness, IAVs can be classified into two groups. H1 and H5 belong to group 1, and H3 to group 2. In addition, NA genetically forms two groups without connection to the two groups of HA. N1 and N2 belong to groups 1 and 2, respectively [2].

2.1.5 Antigenic characterization

Antigenic characterization of influenza viruses is an important methodological tool for understanding the virus evolution and infection mechanisms, the prevention of and response to outbreaks, and the selection of strains for influenza vaccine development [28]. While the sequence-based antigenic analysis has been focusing on amino acid substitutions and drug resistance markers of HA and NA, the antigenic impact of genetic changes varies [53]. Several attempts have been made to integrate phylogenetic analyses with antigenic analyses [54–59].

Serological methods used in antigenic characterization include the HI test, virus neutralization (VN) assays and the SRH assay described later in more detail. Other methods involve measuring NA-inhibiting antibody titers using a traditional method [60] or an enzyme-linked lectin assay [61]. The binding strengths of antibodies can be compared and quantified using Enzyme-linked immunosorbent assay (ELISA) [62]. The primary structure of the viral antigen as well as antigenicity of a specific epitope of the virus can be determined using mass spectrometry [63]. Moreover, a computational approach called antigenic cartography can be used to visualize the antigenic diversity based on antigenic distances [64–66].

Traditional serological methods comprise several challenges and potential bias with virus propagation, such as culture-adapted mutations during viral propagation [67, 68], limited growth ability in Madin-Darby canine kidney cells (MDCK) [69] and embryonated chicken eggs without culture adaptions [70]. Moreover, altered viral receptor binding properties [71] have complicated HI assays. A clinical sample-based antigenic characterization, which requires a low amount of influenza viruses

could overcome virus isolation and propagation challenges. The sequence-based analyses combined with a novel quantitative polyclonal antibody-based proximity ligation assay [72], which can detect antigenic variations in both HA and NA proteins, may serve as a next-generation platform for antigenic characterization of influenza viruses [28].

2.1.6 Nomenclature

The nomenclature for influenza virus strains is based on type of influenza, geographic location of isolated virus, strain number and year of isolation. If the virus is isolated from a different species than human, this is defined between the type of influenza and the geographic location. Examples are A/Finland/385/2013 and A/duck/Hunan/795/2002 for human influenza A(H3N2) and avian A(H5N1), respectively.

2.2 Influenza vaccines for humans

Immunization against influenza has been the main strategy for prevention and control of seasonal and pandemic influenza. The goal of the vaccination is the generation of immunological memory, which means the ability of the immune system to specifically and quickly recognize and respond to a foreign, previously encountered antigen [73, 74]. The first inactivated influenza vaccines were licensed for human use in 1945 [2]. Moderate protection against laboratory-confirmed influenza can be achieved by vaccination, yet protection differs between seasons and antigenic match between vaccine viruses and epidemic strains [75].

Most licensed seasonal influenza vaccines have been produced in embryonated chicken eggs [76]. However, egg adaptation can introduce amino acid mutations in the HA of particularly the A(H3N2) viruses, potentially altering the antigenicity of the vaccine virus [77, 78]. In 1995, World Health Organization (WHO) recommended to investigate cell-grown vaccine production in MDCK cells, Vero cells, and other cell lines [79]. The first MDCK cell culture-derived seasonal influenza vaccine Optaflu and the first Vero cell-derived pandemic vaccine were approved by the European Medicines Agency (EMA) in 2007 and 2009, respectively [76]. The most recent technology in next generation influenza vaccines utilizes e.g. recombinant proteins, virus vector approaches, peptides, nucleid acids and nanoparticles [76, 80].

The dose of the vaccine antigen has an effect on the vaccine response [81]. A high-dose inactivated influenza vaccine Fluzone® was licensed by the United States Food and Drug Administration (FDA) in 2009. It contains four times more HA antigen compared to the standard dose [82]. A high dose of HA has been shown to

improve the protection against influenza by reducing influenza disease and associated clinical complications in elderly individuals compared to the standard dose [81–83]. Furthermore, the use of adjuvants induces a better response also with a lower dose of antigen [84].

Administration of inactivated influenza vaccines is usually intramuscular, which is considered better than the subcutaneous route because of the milder local adverse reaction and a greater increase in the influenza antibody titre compared to subcutaneous delivery [85]. The LAIV is given as an intranasal spray to induce better mucosal immunity [86]. Intradermal inoculation utilizes dermal immune-stimulatory antigen-presenting cells to increase vaccine immunogenicity [87, 88]. Intradermal administration frequently cause stronger local adverse reactions, yet has shown to be as effective as intramuscular injection, even providing a dose-sparing strategy [86, 87, 89], and the EMA approved intradermal seasonal influenza vaccine Intanza® for adults in 2009 [90]. In addition, influenza vaccine delivery via e.g. oral capsule [91] and tonsillar route has been investigated [92].

Influenza vaccines can be separated into different categories and types, such as live and inactivated vaccines, or according to the purpose, production process, dose, route of delivery or use of adjuvant.

2.2.1 Influenza vaccine categories

2.2.1.1 Seasonal influenza vaccines

Trivalent vaccine (TIV)

The most widely used seasonal influenza vaccine has been the trivalent influenza vaccine (TIV). TIV has been used since 1977 [2]. It contains a representative of the corresponding epidemic A(H1N1), A(H3N2) and B viruses as a standard adult dose ranging from 15 µg up to 60 µg of HA antigen per strain [93].

Quadrivalent vaccine (QIV)

In addition to a representative of A(H1N1) and A(H3N2) viruses, quadrivalent seasonal influenza vaccines (QIV) comprise both B virus lineages Victoria and Yamagata. These antigenically distinct B virus lineages have existed and co-circulated since 1983 [94]. Using QIV the chance for a vaccine mismatch is reduced [95, 96]. QIV was licensed for use since the 2013–2014 influenza season [2].

Annual selection of vaccine virus strains

Annual adaptation of the seasonal influenza vaccine formulation is needed because circulating influenza viruses evolve antigenically to escape antibody-mediated immunity. The goal is to predict and achieve the closest possible match between the influenza vaccine strains and the circulating influenza strains [97].

The WHO issued the first formal recommendations on the composition of the influenza vaccine in 1971. Since 1998, separate recommendations have been issued twice a year: in February and in September for Northern hemisphere and Southern hemisphere, respectively [98]. Lists of prototype viruses for egg-propagated, cell culture-propagated and recombinant-based vaccines are available on the WHO website [99]. The decisions on vaccine composition have an impact on the following influenza season. The influenza vaccine production and licensing process takes about six to eight months [100].

WHO collaborative centers generate the seed viruses of each vaccine component and provide them to vaccine manufacturers. Reverse genetics is used to improve vaccine virus growth in eggs. For inactivated recombinant based influenza vaccines, high-growth A/Puerto Rico/8/34 or a derivative strain with HA and NA genes from the candidate vaccine IAVs are generated [100]. For LAIVs, reassortment between the HA and NA genes of influenza A and B candidate vaccine viruses and a cold-adapted master donor virus is needed [28].

Since the onset of the pandemic of coronavirus disease 2019 (COVID-19) the global influenza circulation was widely suppressed by travel restrictions and public health measures. Sporadic case detection and waning population immunity challenged influenza epidemic control and vaccine strain selection [101].

2.2.1.2 Pre-pandemic influenza vaccines

Pre-pandemic avian IAV vaccines have been developed to protect humans against future epidemics and pandemics [102]. WHO coordinates the development of zoonotic influenza candidate vaccine viruses intended for human vaccine production. Lists of prototype viruses are available on the WHO website [103]. National authorities may consider applying these candidate vaccine viruses for example in clinical trials and other pandemic preparedness purposes based on their assessment of public health risk and need [104].

2.2.1.3 Pandemic influenza vaccines

Rapid vaccine production in response to a novel pandemic virus strain is essential to mitigate global morbidity and mortality [105]. When an influenza virus with pandemic potential has acquired the ability to maintain human-to-human

transmission, WHO accelerates the selection, development, and distribution of vaccine viruses for pandemic vaccine production. Access to the pandemic vaccine is influenced by the sharing of viruses and clinical specimens with WHO to produce an appropriate vaccine, and the global production capability [106]. Furthermore, a fair, effective and timely allocation of vaccines with sufficient doses is needed to protect the global population [107].

Vaccines were created as updated inactivated influenza vaccines in response to pandemics of Asian flu (A/Japan/1957, H2N2) and Hong Kong flu (A/Hong Kong/1968, H3N2), and also for Russian flu (A/Russia/1977, H1N1) [2]. A pandemic influenza vaccine against the Swine flu (A/California/07/2009, H1N1pdm09) was developed, produced, and deployed for the first time during the first year of the influenza pandemic [108].

2.2.1.4 Universal and broadly protective influenza vaccines

A desirable goal for influenza vaccines would be the development of a universal influenza vaccine. The universal vaccine would ideally protect against all influenza A and B viruses independent of HA and NA subtypes or antigenic drift. An effective defense against viruses with a pandemic potential could be provided and the need for seasonal reformulation of vaccines avoided [109]. Compared to universal vaccines, the broadly protective supraseasonal vaccines would cover a large subset of influenza viruses such as all human seasonal influenza virus subtypes [110, 111].

Both types of vaccines are based on the conserved domains or proteins of the influenza virus. The mechanism of protection depends on the conserved target of the vaccine as well as the vaccination method and strategy [110]. The main target for universal influenza virus vaccines has been the conserved stalk domain of the HA [109, 112]. Other targets include the ectodomain of the matrix protein (M2) ion channel (M2e) [113], the internal protein nucleoprotein (NP) combined with matrix protein (M1) [114] or M2e [80], and NA [115]. Several vaccine constructs inducing broad or even universal influenza virus protection are currently in preclinical and clinical development [93, 110].

2.2.2 Types of influenza vaccines

Whole virus vaccines – inactivated and live-attenuated

The traditional whole disease-causing virus in a vaccine induces an immune response similar to that seen during natural infection. The inactivation of the virus is done by heating or treating it with chemical reagents, which maintains the antigenic epitopes of the virus leading to good immunogenicity. Whole-virus vaccines are considered

more immunogenic than split or subunut vaccines in previously unvaccinated populations [116]. In China, the influenza vaccines for preventing avian influenza are predominantly inactivated whole influenza virus vaccines [117].

The LAIV mimics natural infection utilizing attenuated influenza viruses and the vaccine is able to induce both cellular and humoral immunity [86, 102]. Since the 1980s LAIVs have been developed and used in Russia [118]. Trivalent LAIV FluMist® was approved in 2003 by the FDA and as of 2012 for QIV formulation [86]. The EMA approved QIV LAIV Fluenz Tetra® in 2013. LAIV is delivered via nasal spray, inducing mucosal immunity. LAIV has been reported to be more effective than TIV in children [119].

Split virion vaccines

Split virion influenza vaccines are based on whole influenza vaccines. Purified virus is inactivated, split and further purified to concentrate and increase the amounts of antigenic proteins. Compared to inactivated whole virus influenza vaccines, split influenza vaccines cause less side effects yet preserve high immunogenicity [117]. Split virus vaccines include commonly used seasonal TIV and QIV, e.g. Vaxigrip® and Fluarix®.

Subunit vaccines

The conventional subunit vaccines are based on the split-virion influenza vaccines, from which one or more antigenic proteins have been further purified and concentrated. Adjuvants are often needed to improve immunogenicity. Subunit vaccines include commonly used seasonal influenza vaccines such as Influvac® and Agrippal® [117].

Furthermore, molecular cloning techniques have been used to construct subunit vaccines [117]. The first trivalent recombinant seasonal influenza vaccine Flublok® received FDA approval in 2013 [120]. Recombinant HA for this vaccine was manufactured using baculovirus expression and purification from infected insect cell cultures. FluBlok® was formulated to contain threefold the amount of HA compared to egg grown TIV [121].

Viral vectored vaccines

A viral vector vaccine utilizes a modified viral vector to deliver genetic material encoding for a desired viral protein or antigen into the recipient's cells, leading to antigen expression and antigen-specific immune response in the host [93]. Due to

activation of host's innate immunity, vector-based vaccines induce both humoral and cell-mediated immune responses [122–124].

The adenovirus is the most commonly used vector in many different vaccines. The cell culture-based technology without the need for enhanced biosafety facilities is an advantage regarding emerging avian influenza vaccine development [125]. Clinical trials using replication-defective adenovirus vectors have been conducted with promising results [126, 127]. The pre-existing immunity against the commonly used adenovirus vector may limit the efficacy of the vaccine [127]. However, alternative adenovirus serotypes as vectors [92], or a completely different vaccine platform as a booster may elicit an optimal immune response [125].

The modified vaccinia virus Ankara is an attenuated poxvirus, which is a potential vector expressing influenza virus genes [114, 123, 124]. Other attenuated poxviruses such as Raccoonpox, Canarypox and Fowlpox have been used as viral vectors for the development of influenza candidate vaccine viruses in animals [123].

Semliki forest virus, sindbis virus and Venezuelan equine encephalitis are alphaviruses, which have been tested as influenza vaccine vectors in animal models. Furthermore, recombinant baculoviruses, vesicular stomatitis virus, several alphaherpesviruses, and paramyxoviruses such as Newcastle disease virus and parainfluenza virus 5 have been tested in animals, providing viral vectored vaccines as potential novel influenza vaccine candidates [123].

Virus-like particle vaccines

Virus-like particles (VLPs) are molecules that resemble viruses in structure and morphology. They are highly immunogenic but are not infectious since they are lacking the viral genome. Immunologically relevant proteins such as HA and NA on influenza VLPs are in a native conformation, since VLPs are not modified by fixatives or chemicals for inactivation [128]. VLPs can be produced in multiple cell culture systems to produce virus vaccines [129]. Influenza VLPs have been produced by co-expression of structural proteins in insect cells by the baculovirus expression system [130–135]. Influenza VLPs have been expressed also in mammalian cells [136–138], plants [139–141] and in Eri silkworm pupae [142]. There have been a number of preclinical studies for both seasonal and pandemic influenza VLP vaccines [143, 144] suggesting good safety and efficacy profiles [133, 145, 146].

Nucleid acid vaccines

Nucleid acid vaccines, or genetic vaccines, belong to new vaccine platforms and include deoxyribonucleic acid (DNA) and RNA vaccines [147]. A DNA vaccine is based on an antigen-encoding gene cloned into a non-replicating expression plasmid.

After vaccine delivery via intramuscular, intradermal or mucosal route, host cells take up the plasmid which is transported into the nucleus leading to expression and presentation of the corresponding protein to immune cells [148]. DNA vaccines against influenza have been investigated since the 1990s, and promising results in murine models indicated induction of both humoral and cellular immunity [149]. Favourable results with influenza A DNA vaccine was seen in clinical studies [150–153], but due to low immunogenicity in larger animal models, a wider use of IAV DNA vaccines in humans have been delayed [147, 148].

Although concerns regarding their low stability, RNA-based vaccines have certain advantages over DNA vaccines. Antigen-encoding messenger RNA (mRNA) needs only to be delivered into the cytoplasm of the host cell, where it is translated into a protein. In addition, safety concerns of potential integration of foreign DNA into the host genome are also avoided [147]. Furthermore, RNA vaccines can be cheaply mass-produced and easily updated once the genome sequence of an emerging influenza virus strain is available [154]. Recent success in RNA vaccine platforms comprises lipid nanoparticle mRNA vaccines such as the first licensed vaccine against COVID-19 [155, 156]. Two types of mRNA vaccines based on a lipid nanoparticle delivery system have been developed against influenza, both showing potential to protect animals from different influenza strains. These vaccines include non-replicative or conventional and self-amplifying or replicative RNA vaccines [157]. Clinical trials have indicated that conventional mRNA lipid nanoparticle vaccines against influenza A(H10N8) and A(H7N9) were immunogenic and well-tolerated [158, 159], yet human data concerning self-amplifying mRNA influenza vaccines are still missing [157].

Peptide vaccines

Synthetic peptides can be designed to induce influenza-specific humoral and cell-mediated immune responses to conserved epitopes [160]. A large-scale production of peptide vaccines is possible [161], and the synthesis of peptides is relatively fast, but to formulate an immunogenic peptide vaccine a suitable adjuvant or delivery system is needed [162]. Clinical trials using multi-epitope peptide-based influenza vaccines have shown an induction of vaccine-specific cellular immunity [163–165]. The vaccine also showed clinical efficacy in a human influenza A challenge study [166]. Furthermore, a prime-boost strategy combining peptide vaccine immunization with a conventional seasonal vaccine has further broadened and enhanced the immune response [167, 168].

Nanoparticle vaccines

One alternative approach to conventional vaccines is the incorporation of antigens in nanoparticles, which can be administered through the mucosal sites, or by intramuscular or subcutaneous injections. Nano-sized materials include liposomes, VLPs, polymeric nanoparticles e.g. poly(lactic-co-glycolic acid), inorganic nanoparticles e.g. gold nanoparticles, and self-assembled protein nanoparticles e.g. ferritin. Enhanced antigen presentation and strong immunogenicity can be achieved by designing for instance the shape, size, surface properties and functionality of the nanoparticles [169].

Animal experiments indicated that papaya mosaic virus nanoparticle-based vaccine combining M2e and NP viral proteins induced a broad and robust protection against two different influenza A strains [80]. Nanoparticle immunogens displaying QIV HA trimers induced broadly protective antibody responses against homologous and heterologous viruses [111]. A phase 1 clinical trial in adults who received a H2 HA-ferritin nanoparticle vaccine demonstrated both H2 responses and broadly neutralizing antibody responses against group 1 influenza viruses [170]. A phase 3 clinical trial among the elderly with Matrix-M adjuvanted quadrivalent nanoparticle influenza vaccine showed an enhanced humoral and cellular immune response compared to a standard-dose QIV [171].

2.2.3 Role of adjuvants

An adjuvant is a synthetic or biological agent, which can be added to a vaccine to enhance an immune response and to reduce the amount of antigen in the vaccine [44]. The effect of adjuvants is based on a combination of mechanisms to improve the ability of the host immune system to recognise the antigen as foreign and induce an immune response against the antigen. These mechanisms include induction of cytokines and chemokines, formation of depot, recruitment of immune cells, enhancing antigen transport to draining lymph nodes and improvement of antigen uptake and presentation [172].

Currently four adjuvants are approved to be used in inactivated influenza vaccines: aluminium salt (alum), oil-in water emulsion systems such as MF59 and AS03, and virosomes [44]. In addition, several new influenza vaccine adjuvants are in the development phase [84].

Alum, the oldest adjuvant, has been the most used adjuvant also in influenza vaccines. However, alum-adjuvant has not been very effective with pre-pandemic A(H5N1) vaccines compared to non-adjuvanted formulations [173–175].

MF59 adjuvant is an oil-in-water emulsion of squalene. It has been licensed as an influenza vaccine product Fluad® since 1997 [176] and later for pre-pandemic and pandemic H5N1 vaccines [172]. Compared to non-adjuvanted vaccine responses,

MF59-adjuvanted mono- and multivalent influenza vaccines have induced substantially higher HI titres and seroconversion rates in children [177–179]. Moreover, MF59 has been shown to significantly enhance the immunogenicity of inactivated influenza vaccines in elderly individuals [180–182].

Adjuvant System 03 (AS03) contains α-tocopherol and squalene in an oil-in-water emulsion [183]. AS03 was licensed for the pandemic flu vaccine Pandemrix® [172]. AS03-adjuvanted influenza A(H5N1) vaccine was shown to be highly immunogenic in children 6–35 months of age [184] and to enhance better antibody responses compared to a non-adjuvanted vaccine [185]. In the elderly, AS03-adjuvanted TIV has shown higher efficacy for the prevention of influenza infections compared to a non-adjuvanted TIV [186]. Although in pre-clinical and clinical studies the safety profile for AS03-adjuvanted influenza vaccines was acceptable [187], an association between Pandemrix® and narcolepsy was observed during the A(H1N1)pdm09 pandemic. So far, the mechanisms of Pandemrix-associated narcolepsy have remained elusive [188]. Immunological data suggest a possible role for the A(H1N1)pdm09 antigen rather than the AS03 adjuvant in narcolepsy. However, a multifactorial mechanism involving antigen mimicry is likely involved [187].

Virosomes and liposomes are spherical vesicles used as a delivery vehicle for administration of various substances into the cells such as drugs. The incorporation of viral membrane proteins into liposomes has been shown to potentiate the immune response to several viral glycoproteins [189]. In contrast to liposomes, virosomes contain functional viral envelope glycoproteins [190]. Influenza virosomes are VLPs reconstituted from virus envelopes retaining the receptor binding and membrane fusion activity of the native virus yet lacking the viral genetic material [191]. Virosomes activate both humoral and cellular immune responses [191, 192] and are licensed as Inflezal®V and Invivac® influenza vaccines [172].

2.2.4 Influenza vaccine efficacy and vaccine effectiveness

Vaccine efficacy

Vaccine efficacy measures the percentage of reduction in influenza disease incidence between vaccinated and unvaccinated groups. This evaluation is typically used during vaccine pre-licensing phases I–III and under optimal conditions such as controlled clinical trials [75, 193].

Vaccine effectiveness

Vaccine effectiveness (VE) measures the protective ability of a vaccine towards the influenza disease and or outcomes of interest in real life situations once the vaccine is approved for use in the general population i.e. during vaccine post-licensing phase IV [194]. The most common VE study designs used are observational studies including cohort studies and case-control studies with test-negative controls [193].

VE has been shown to be affected by influenza type and subtype [195], antigenic match between circulating and vaccine strains [195–197], time after vaccination [198], residual effects from prior vaccination [199, 200], the type of vaccine [196, 201] and host factors including age, sex, comorbidities, and preexisting immunity [202, 203].

2.3 Antibody response to influenza A virus infection and vaccination

The first line of defense against non-self pathogens is a non-specific and immediate innate immune response, which consists of physical, chemical, and cellular defenses against pathogens. The second line of defense is specific and long-lasting to the pathogen presented, called adaptive immune response. Within the adaptive immune system there are two main mechanisms: humoral and cellular immunity. Humoral immunity is also called antibody-mediated immunity, which will be focused on below.

The adaptive immune response to influenza virus infection involves both antibody and cellular responses at the systemic and mucosal levels [33]. Natural influenza infection induces usually broader and longer-lived antibody responses than antibody responses induced by vaccination [33, 204, 205], but the mechanisms behind this are partly unclear. Viral replication during natural infection provides opportunities to generate antibodies reacting with heterosubtypic influenza virus strains [206, 207], whereas the inactivated vaccine predominantly induces antibodies recognizing the globular head domain of HA. In addition, the amount of virus antigen in an infection is likely much higher than that provided by vaccination. Influenza VE is low compared with that of other viral vaccines, and the induced immune response is narrow and short-lived, yet the vaccination has been the best available countermeasure against an infection [33].

Original antigenic sin (OAS) hypothesis refers to the immunological imprint caused by the first exposure to influenza virus. A subsequent influenza infection may lead to a low-affinity response against the subsequent strain while enhancing the antibody response to the previous virus strain [208]. Natural or vaccine exposures to antigenically different influenza virus strains have been shown to boost responses to

shared epitopes between different virus strains, leading to increased antibody titers against influenza virus strains faced earlier in life [66, 209, 210]. OAS has raised discussion regarding the reduced effects of annual vaccinations with the finding that revaccination with influenza virus vaccines can lead to reduced VE [211]. However, the vaccinated individuals are usually better protected against influenza as compared to their unvaccinated counterparts [211, 212]. There is a possibility that pre-existing antibodies may mask some of the antigenic epitopes thus impairing VE [213]. The finding that the majority of the influenza-specific monoclonal antibodies had the highest affinity for the current vaccine strain suggests that OAS is not common in healthy adults receiving influenza vaccination [214].

2.3.1 B cell responses

B cells are a lymphocyte subtype of white blood cells. Different types of B cells can produce or secrete antibodies, present antigens, and secrete cytokines. B cells produce antibody molecules inserted as a part of B cell receptors. If a naïve or memory B cell is activated by an antigen, differentiation and proliferation occurs to antibody-secreting effector B cells called plasmablasts and plasma cells. In addition, cellular differentiation leads to generation of memory B cells and regulatory B cells [215]. B cell activation can take place either in a T cell-dependent or T cell-independent way [216].

Influenza infection induces the activation of naïve B cells or pre-existing memory B cells. The activation is mediated via viral antigen recognition and interaction with CD4+ T cells. A part of the activated B cells differentiates quickly into plasmablasts. These plasmablasts are short-lived, producing the first influenza virus-specific antibodies; at first IgM, then IgG or IgA [33]. In humans, the amount of plasmablasts peaks about a week after infection or vaccination if activated by memory B cells [214].

Some activated B cells migrate to B cell follicles in secondary lymphoid organs, where proliferation and affinity maturation takes place, generating both high-affinity plasma cells and memory B cells. Long-lived plasma cells migrate to bone marrow and maintain the long-term serum antibody levels [33]. Memory B cells do not secrete antibodies, but they are long-lived and specialized in immune surveillance [217, 218]. Memory B cells have been shown to have a broader capacity to recognize various antigenic epitopes [219], which may have a protective role from a severe disease [33].

2.3.2 Antibody isotype responses

An antibody, also known as an immunoglobulin (Ig), is most often a Y-shaped, heterodimeric protein composed of two heavy and two light chains. Both chains can be separated functionally into variable domains that binds antigens, and constant domains that are involved in interactions with other components of the immune system. Human immunoglobulins exist as IgA, IgD, IgE, IgG and IgM isotypes, which are further subdivided into subclasses [220]. IgA, IgG, and IgM have key roles in immunity against influenza viruses [33].

The major antibody isotype produced in humans is IgA [221], which exists in both monomeric and polymeric forms. The dimeric form is the most prevalent one, also called secretory IgA [222]. Mouse models have shown that pre-existing secretory IgA antibodies have a role in eliminating influenza virus at mucosal surfaces preventing the entry of the virus into the body [223]. IgA is the predominant immunoglobulin in most mucosal secretions, yet, in serum, the IgA levels are lower and the half-life is shorter than that of IgG [224–227]. Human IgA has two subtypes: IgA1 and IgA2, whose distribution varies between different external secretions [226]. IgA1 predominates as a monomeric form in serum, and it can be found as a dimer on mucosal surfaces of the upper respiratory tract [226, 228]. IgA2 exists mostly in a dimeric form, and it predominates in the intestinal lavage fluid [228]. Influenza virus-specific trimeric and tetrameric IgA forms have been detected in the human upper respiratory tract, showing increased neutralizing potency against seasonal and avian IAV compared with dimeric IgA [229].

IgM antibody indicates the first antibody response in viral infections. The monomeric form of IgM is expressed on B cells as the B cell antigen receptor. When secreted, multiple IgM monomers assemble to polymers, mostly pentamers or more rarely hexamers, which increases their avidity [220, 230]. An influenza B study indicated that IgM's high avidity may compensate for the loss of affinity caused by imperfect matching to altered target epitopes [231]. This suggests that IgM could neutralize a broader range of viral strains than the corresponding IgG [230]. In humans, 10% of the total serum antibodies are IgM [33]. IgM is also present in external secretions, but at markedly lower levels compared to secretory IgA [226]. Polymeric IgM is a very potent complement activator [230] and IgM has shown to have a central role in preventing uncontrolled inflammatory response and mortality in severe pandemic influenza A(H1N1)pdm09 infection [232]. Interestingly, the discovery of long-lived, antigen-specific IgM plasma cells in mice suggests that IgM plays a role also in long-term humoral immunity [233, 234].

Most of the antibodies found in serum are monomeric IgG consisting of four subtypes: IgG1, IgG2, IgG3 and IgG4 [220]. Mainly the subtype IgG1 and a smaller proportion of IgG3 antibodies target influenza virus, whereas the levels of IgG2 and IgG4 antibodies are negligible [235–238]. IgG1 has a long serum half-life and IgG3

has a shorter half-life yet both interact strongly with Fcγ-receptors, which triggers a direct virus inhibition and Fc receptor (FcR)-mediated effector functions [239]. In most human external secretions IgG is present at levels comparable to those of secretory IgM. However, some secretions such as bronchoalveolar fluid and genital tract secretions contain IgG as the dominant antibody isotype [226].

2.3.3 Antibody-based mechanisms of protection

Influenza virus infection induces a robust immune response involving the generation of both neutralizing and non-neutralizing antibodies. Antibodies may interfere with different stages of influenza virus life cycle and can be targeted to different influenza proteins leading to direct or indirect antiviral effects (Figure 4). In the upper respiratory tract, the antibody response is dominated by dimeric IgA1 [228], whereas the protection in lower respiratory tract is dominated by IgG1 [33].

Antibodies to HA

Influenza virus surface trimeric glycoprotein HA is an important and dominant target of neutralizing antibodies [240]. Antibodies to the HA head domain, which includes the receptor binding domain, block an interaction between HA and sialylated host cell receptors, leading to neutralization of the virus before its entry into the cell takes place (Figure 4b) [241]. HA head-specific antibodies typically have a narrow binding range. These antibodies can also be involved in inhibiting viral egress [33] (Figure 4d).

Antibodies to the stalk domain of HA can bind and protect against a broad range of subtypes of IAV [242]. They do not interfere with the virus entry or endocytosis [243] but they neutralize the virus at a different stage. Stalk-specific antibodies may inhibit the release of the viral nucleocapsids (genome) from the endosomes (Figure 4c) by blocking the fusion of viral and endosomal membranes [244, 245], or interfere with viral egress from the cell [245] (Figure 4d). Stalk-specific antibodies may also block the access of proteases to the HA1-HA2 cleavage site (Figure 4e), which leads to non-infectious virus particles [246]. Moreover, HA stalk-specific antibodies have been shown to activate complement [247, 248] (Figure 4g).

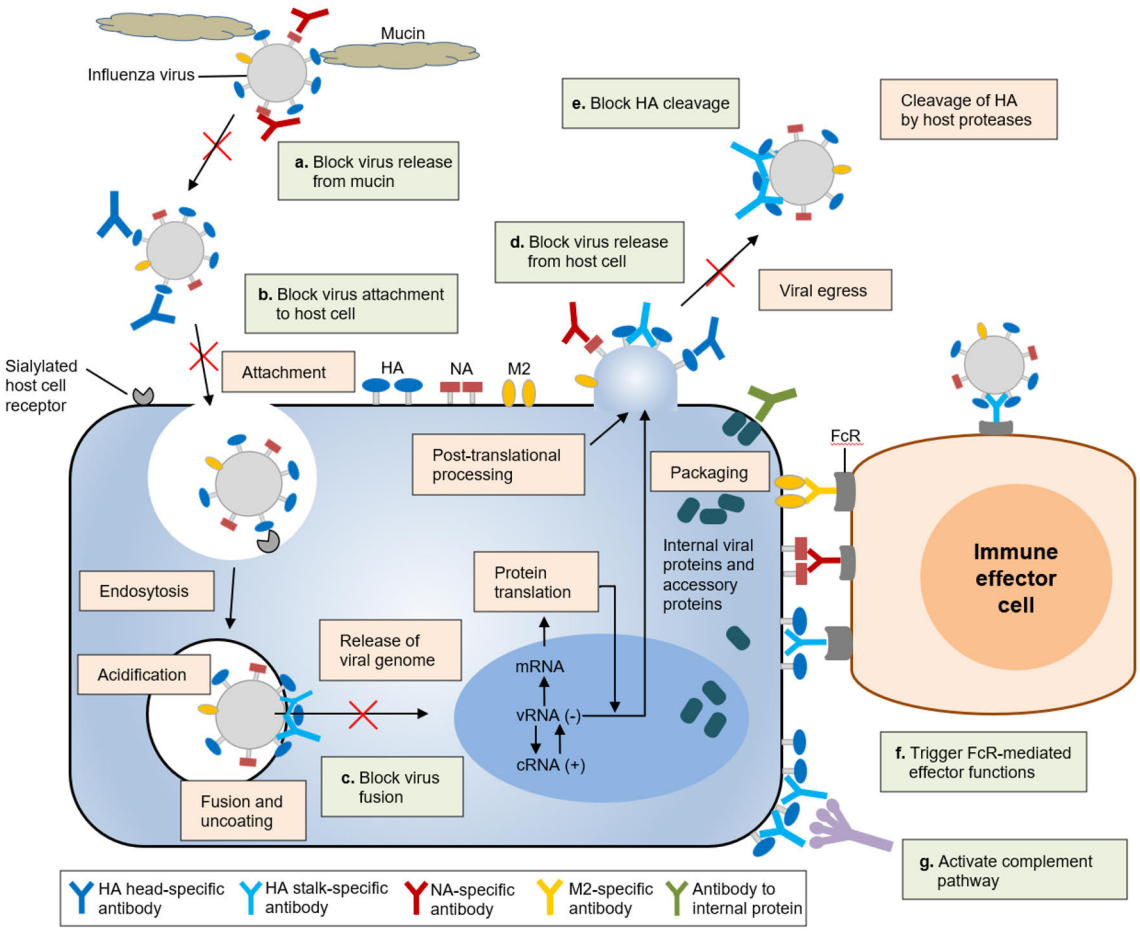

Figure 4. The life cycle of influenza virus (marked by beige retangles) and potential mechanism of action of antibodies against the virus (marked by light green retangles); modified from Krammer 2019 [33]. Antibodies may inferfere with the viral life cycle during different stages. **a.** Neuraminidase (NA)-specific antibodies block the NA activity. **b.** Haemagglutinin (HA) head-specific antibodies block the virus attachment to the host cell. **c.** HA stalk-specific antibodies block the fusion of viral and endosomal membranes. **d.** NA- and HA-specific antibodies block the virus release from the host cell. **e.** HA stalk-specific antibodies block the HA cleavage process. **f.** Antibodies to HA stalk and to NA and M2 proteins trigger Fc receptor (FcR)-mediated effector cell functions against infected cells. **g.** HA stalk-specific antibodies activate complement.

Antibodies to NA

Tetrameric NA is another main surface glycoprotein embedded in the envelope of influenza viruses. NA has various roles in influenza virus infection [249]. NA-activity frees influenza virus from decoy receptors on mucins, which NA-specific antibodies can block [250] (Figure 4a). In addition, NA is needed for the successful

detachment of budding influenza virus particles from host cells [251], which can be interfered by specific antibodies (Figure 4d). NA-reactive antibodies can also inhibit the immunomodulatory activity of NA [252].

Antibodies to M2

The third protein on the influenza virus surface is M2, which acts as an ion channel to acidify the interior of the influenza virion within host cellular endosomes, participates in virus assembly and budding, and interferes with host cell functions [253]. The M2 protein has a short and highly conserved N-terminal ectodomain, which is a universal influenza A vaccine candidate [113, 253, 254]. IAV infected subjects have shown low levels of M2e-specific antibodies [255, 256], yet the seroprevalence of anti-M2 antibodies has been reported to increase with age [256]. Antibodies against M2e are non-neutralizing and they may assist FcR-mediated effector cell functions [253].

Antibodies to internal proteins

Influenza virus infection has been shown to induce non-neutralizing antibodies to internal influenza virus proteins, e.g. NP, M1, PA-X and PB1-F2 [257–260]. Antibodies against NP may help to clear influenza infection [261, 262], and NP-specific and M1-specific antibodies have been shown to activate natural killer cells although without killing activity [258]. However, the protective role of antibodies against internal and nonstructural influenza virus proteins remains still unclear [33, 254].

Fc receptor-mediated effector functions

The tail region of an antibody, called Fc (fragment, crystallizable) binds to a FcR, which is located on the surface of immune cells (Figure 4f). Indirect antiviral FcR-mediated effector functions include antibody-dependent cell-mediated cytotoxicity, antibody-dependent cellular phagocytosis, and complement-dependent lysis [33].

Non-neutralizing antibodies have been detected following influenza infection and vaccination. They can provide additional protection via antibody-dependent cell-mediated cytotoxicity [263–265], and through activation of complement [247, 265] (Figure 4g).

2.3.4 Traditional serum antibody quantification methods

Influenza HA-specific antibodies have been traditionally quantified by measuring HI, SRH, or VN [266]. Each influenza vaccine within European Union needs to fulfil the criteria issued by the EMA to be licensed. Previously the immunogenicity criteria were solely based on officially recognized HI and SRH assays [267]. However, presently also other assays such as VN assays are encouraged to be utilised when appropriate to provide a biologically relevant potency measure [268, 269].

2.3.4.1 Haemagglutination inhibition test

The technique of haemagglutination was first described in 1941 [270]. Haemagglutination refers to the ability of influenza viruses to agglutinate erythrocytes from both mammals such as humans, guinea pigs and horses, and from some birds such as turkey and chicken [271]. Specific antibodies against influenza virus can prevent the haemagglutination of RBCs (Figure 5).

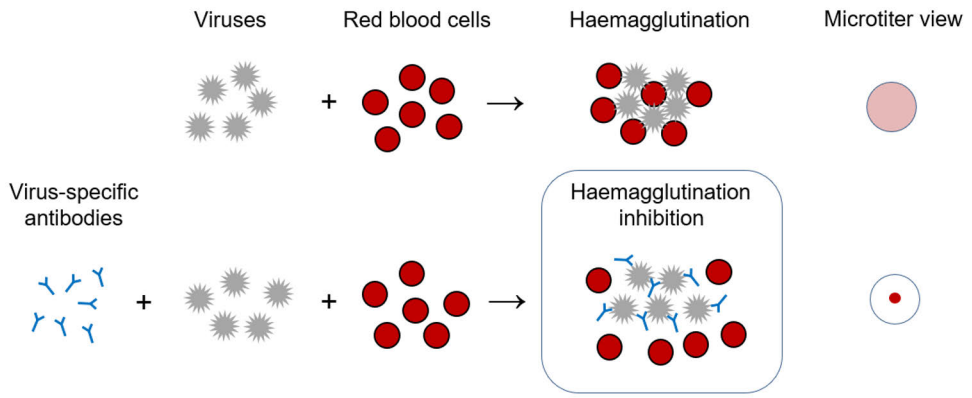

Figure 5. The basis of the haemagglutination inhibition test: influenza virus-specific antibodies prevent the attachment of the virus to red blood cells, thus inhibiting haemagglutination. Unagglutinated cells settle in a compact pellet in a well whereas agglutinated cells settle as a lattice. Author's own drawing.

In the HI test, a standardized quantity of influenza virus is mixed with serially diluted serum or antisera, enabling antibodies against a specific influenza HA protein to bind to the antigenic sites on the HA protein. RBCs are then added to assess the degree of binding of the antibody to the HA molecule. Unagglutinated erythrocytes settle in a compact pellet on the bottom of the well while agglutinated cells settle irregularly (Figure 5). The HI titer is quantified as the reciprocal of the highest serum dilution that inhibits agglutination [272].

The HI assay has been the gold standard for measuring antibody levels in influenza virus infection and after vaccination [273] as well as determining antigenic relatedness of influenza virus strains [97, 274]. Among seasonal influenza A strains, the HI test usually correlates well with VN assays. Other advantages include low cost and short turnaround time, which is appropriate for global influenza antigenic monitoring [275]. Although the inter-laboratory variability of HI assay results tend to be high [276], the harmonization of protocols and reagents effectively reduce the variability [277].

There are several technical aspects to consider in the HI test. Serum specimens often contain non-specific inhibitors of haemagglutination. The pre-treatment of the antisera with receptor-destroying enzyme (RDE) present in the filtrate from a culture of *Vibrio cholera* is needed to remove the interfering substances [272], yet some of the inhibitors may resist RDE [278]. Furthermore, the source of erythrocytes used, even batches, have an impact on the assay performance [28, 271], and the passage history of the influenza virus may have an influence on HI titres [279]. Reduced receptor binding of human A(H3N2) influenza viruses at first led to a failure to agglutinate chicken erythrocytes used in HI assays [280–283]. Later reduced virus growth capacity in eggs and MDCK cell cultures was seen [40, 69, 284]. Moreover, oseltamivir-sensitive NA-mediated haemagglutination [285] with poor haemagglutination via HA of recent A(H3N2) viruses has complicated HI testing emphasizing the use of alternative assays such as VN assays for analyzing immunity against the A(H3N2) viruses [286].

2.3.4.2 Single radial haemolysis

The SRH method is based on antibody diffusion in agar gel, leading to a passive hemolysis of RBCs which measures the antibody content of the test sera [287, 288].

For the SRH assay, erythrocytes are mixed with influenza virus allowing the coating of RBCs with a given virus. Unadsorbed viruses are removed from RBC-bound viruses by centrifugation. Virus-coated RBCs and guinea pig complement are added to an agarose solution and then spread onto plates. Serum samples are pipetted to the holes made into plates. During subsequent incubations, the zones of lysis are formed around those wells filled with serum having antibodies against the virus coating the RBCs. The area of haemolysis is induced by the antibody-virus complex and mediated by complement activation visualizing the zone by lysing the RBCs having virus-antibody complexes at their surfaces. The diameter of haemolysis is proportional to the concentration of antibodies [287, 288].

The advantages of the SRH assay include safety due to the possibility of using inactivated viruses, small volumes of sera, and the ability to simultaneously analyze a large number of serum samples without pre-treatment other than complement

inactivation [266]. Compared to the HI assay, the SRH assay has shown to be more sensitive for influenza B virus-specific antibody measurements [275, 289], yet showing comparable sensitivity for antibodies against seasonal influenza A strains [289].

2.3.4.3 Virus neutralization tests

The VN test quantifies the presence of functional antibodies capable of neutralizing the ability of the virus to enter or replicate in cells [290]. Many different options exist for the VN detection. The well format may be 6, 24 or 96, the microtiter format referred to as MNT, which enables more samples to be analyzed and reagents saved [291]. Since infective virus is used, a conventional virus-specific neutralisation test can be developed quickly upon isolation of a novel virus [292]. Figure 6 demonstrates examples of various VN assay platforms for different viruses.

In the VN tests, serially diluted, inactivated sera are pre-incubated with a standardized amount of virus prior to the addition of the virus-antibody mixture to cells - or cells to the virus-antibody mixture - to detect residual virus infectivity using different assay protocols employing different readouts. The absence of infectivity represents a positive neutralization reaction and indicates the presence of virus-specific antibodies in sera [293]. The most common cell line used in influenza VN tests is canine-derived MDCK cells [272, 291, 293, 294] and for recent A(H3N2) strains MDCK-SIAT-1 with increased α-2,6 sialic galactose moieties on the surface [295]. Also cell lines of human origin have been tested [296].

Conventional influenza VN tests require serum-free culture medium, which contains trypsin to allow influenza viruses to undergo productive replication in cells [293]. The traditional VN tests are based on directly visualising the inhibition of virus growth i.e. cytopathic effect (CPE) [266] or reduction of plaque formation (PRNT) [28], the latter generally considered as the gold standard for measuring virus neutralization [297–300]. In PRNT, each infectious virus particle multiplies by spreading cell-to-cell under solid or semisolid overlay e.g. Avicel [301], that results in a localized area of infected cells leading to visually or microscopically countable plaques.

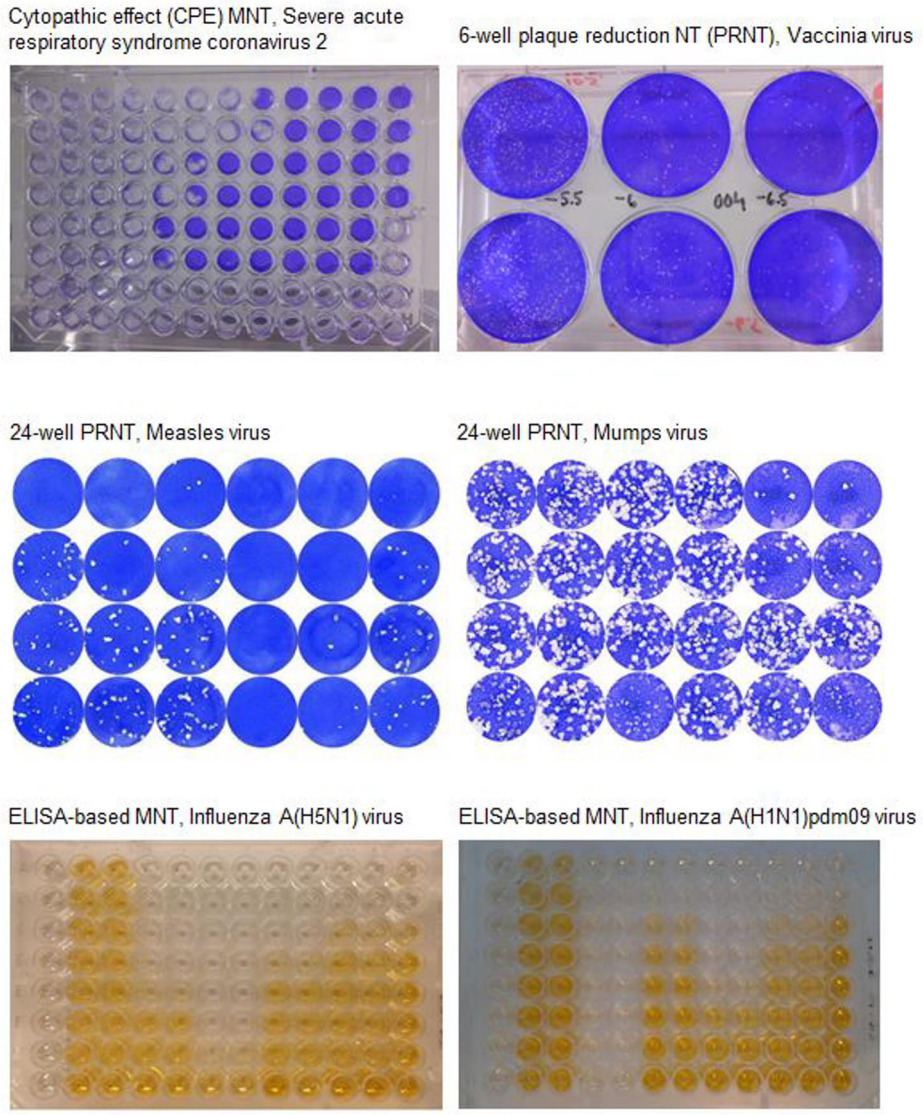

Figure 6. Examples of neutralization assays for different viruses. Conventional cytopathic effect (CPE) microneutralization test (MNT) and 6-well and 24-well plaque reduction neutralization tests (PRNT). ELISA-based MNT detects virus-infected cells and can yield results within two days. Author's own drawing.

The MNT format for residual virus infectivity counted from influenza virus foci stained by specific antibodies, called a focus reduction neutralization test (FRNT) [291], shortens the VN detection time and has been further optimized for more efficient quantification of infected cell populations with an imaging method [302]. Based on FRNT, a ViroSpot assay, utilizing automated readout of immunostained

cell monolayers [303] and high content imaging-based NT to reduce antigenic mischaracterization resulting from virus adaptation to cell culture, have also been developed [304].

A detailed protocol is available for a colorimetric ELISA-based MNT method, which combines culture and influenza NP detection by ELISA [272]. This protocol can yield results within 2 days and tend to show lower variation compared to other MNT assays [294, 305]. Other MNT readouts include the detection of released virus through haemagglutination [28, 294], and quantification of viral RNA by quantitative reverse-transcription polymerase chain reaction [306]. In addition to live-virus VN tests, pseudovirus modifications of VN represent a potential alternative to study neutralizing antibodies against HPAI viruses [307–309].

An advantage of the VN assay is that it measures antibodies which truly inhibit the infection [294]. The HA is the major influenza viral antigen inducing the neutralizing antibody response [13], yet VN assays can reflect also antigenic properties of NA [28, 290]. The MNT has shown to be more sensitive than the HI and SRH for detecting antibodies against seasonal strains and A(H5N1) viruses [289, 310]. Therefore, the MNT is recommended as a serological test for the measurement of antibodies against HPAI in humans [311]. The results usually correlate well between the MNT and the HI assays [275], and between different VN assays [294]. However, the inter-laboratory variability of VN tests appear to be even higher compared with the HI assay [276, 305, 312]. Furthermore, regardless of the development of novel VN readouts, VN tests are lower-throughput and more laborious than the HI assays [28].

2.3.5 Correlates of immunity

Serology refers to the diagnostic testing for antibodies mostly in serum, and is used to determine antibody positivity. Most vaccines are thought to mainly confer protection via induction of antibodies. Important considerations in vaccinology are how well a vaccine prevents the infection and a disease, and the determination of a protective antibody threshold in the prevention of the disease [74]. There are certain definitions to clarify the concept of correlates of immunity.

Seroconversion

Seroconversion refers to the development of specific and detectable antibodies in serum to a pathogen. Seroconversion can result in response to an infection or immunization. Prior to facing the first infection or immunization, the serological test is negative for the antibodies. After seroconversion the test is seropositive. A fourfold or greater increase in antibody titre is considered diagnostic [33, 272].

Seroprotection

The definition for a seroprotection is an antibody response regarded as being capable of preventing the disease. A subject is considered seroprotected if the level of antibody titer is equal or above a certain cut-off level, usually reflecting the antibody level at which the probability of clinical protection is expected to be moderate (50%), if exposed to an infective virus [266]. The probability of protection is higher with higher antibody levels [313]. Seroprotection rate (SR) refers to the proportion of a study group with antibody level equal or above the assay cut-off level [266].

Correlates of protection

A definition for a correlate of protection by Plotkin is "an immune response that is responsible for and statistically interrelated with protection" [314]. An absolute correlate of protection is for example a certain antibody titer that is shown to be protective, thus a threshold of a correlate. A relative correlate means that the level of response correlates variably with protection. In addition, a surrogate marker of protection is an immune marker "for the true immunologic correlate of protection, which may be unknown or not easily measurable" [314] but is significantly associated with the true correlate. Available correlates of protection facilitate the vaccine development [315].

Almost all current vaccines, including influenza vaccines, work through antibodies in serum or on mucosa that block viremia or infection [74]. Antibody levels especially against the HA have shown to correlate with protection against influenza virus infection in humans [316]. After vaccination, several factors may influence the antibody levels and thus the protection, such as individual characteristics e.g. age, genetic factors such as immunodeficiency states, or vaccine-dependent factors such as the dose of vaccine and the route of administration [74].

Antibodies measured by the HI assay have traditionally been used as the gold standard of influenza vaccine-induced correlate of protection. In 1972, an HI serum antibody titer in the range of 1:18–1:36 was shown to provide 50% protection from influenza A or influenza B virus challenge in adult volunteers [316]. Current consensus has been that in the case of inactivated vaccine, a serum HI antibody level of 1:40 against the HA indicates 50% protection in adults against IAV infection [290]. Thus, this means rather a relative than an absolute correlate of protection [314].

A SRH zone area of 25 mm^2 has been defined as a protective titre for influenza [289], however, no generally accepted correlate of protection has been established for the influenza VN tests [275]. In addition, many alternative serological correlates of protection from infection and disease have been described [315], for example for NA [317], full-length HA, and HA stalk-specific antibodies [318].

Since adaptive immune responses to influenza virus infection involve both humoral and cellular mechanisms [33], one goal of the universal influenza vaccine development is to broaden the immune response to better stimulate cell-mediated immune responses [110]. Therefore, defining the cell-mediated correlates of protection is equally important to predict the vaccine responses further [315]. Finally, without standardization of any methods, defined thresholds for correlates of protection may be questionable due to a variability of results between laboratories [315].

3 Aims of the Study

The overall aim of the study was to analyze homologous and heterologous influenza antibody responses in humans before and after different influenza vaccinations in relation to evolution of influenza A(H3N2), A(H1N1)pdm09 and A(H5N1) viruses.

Antibody levels were first measured with a traditional HI assay and with 20nM oseltamivir carboxylate (OC) for influenza A(H3N2) viruses. In this study, we optimized colorimetric ELISA-based MNT for A(H1N1)pdm09 viruses and A(H5N1) vaccine candidate viruses to further characterize the neutralizing antibody levels. The correlation between HI and MNT results was determined and a theoretical correlate of protection was estimated for influenza A virus subtypes. In addition, the effect of the genetic evolution of the influenza A HA gene was compared with antigenic changes.

The specific research objectives were:

1. To monitor antibody responses against vaccine and epidemic strains after seasonal influenza vaccination, focusing on drifted influenza A(H3N2) viruses. In addition, to demonstrate the effect of 20 nM OC on the results of the A(H3N2) virus HI assay.
2. To optimize the ELISA-based MNT for influenza A(H1N1)pdm09 viruses and use it in addition to the HI test to analyze vaccine-induced immunity in relation to the post-pandemic evolution of A(H1N1)pdm09 virus.
3. To optimize the ELISA-based MNT for influenza A(H5N1) vaccine candidate viruses to study pre-pandemic avian influenza A(H5N1) vaccine-induced serological responses against several clades of A(H5N1) vaccine candidate viruses.

4 Materials and Methods

4.1 Vaccines

4.1.1 Seasonal influenza vaccines (I–II)

In Study I, the seasonal TIV influenza vaccine administered was Vaxigrip® in 2013. In Study II, TIV influenza vaccines administered were FluarixTM and Vaxigrip® in 2010 and 2012, respectively. All TIV vaccines were inactivated, non-adjuvanted split virion vaccines and contained the three WHO-recommended influenza virus strains (Table 1).

Different TIV vaccines were used in different years due to a government funded National Vaccination Programme that provided seasonal influenza vaccines free to healthcare workers, young children, elderly and medical risk groups according to the national vaccination policy.

Table 1. Trivalent seasonal influenza vaccines administered in Studies I–II.

Seasonal vaccine viruses A(H1N1)pdm09 A(H3N2) B	**Adjuvant**	**µg HA/ dose**	**Vaccine, lot number**	**Manufacturer**	**Year**	**Study**
A/California/07/2009 A/Texas/50/2012 B/Massachusetts/02/2012	No	45 (15/ strain)	Vaxigrip®	Sanofi Pasteur MSD	2013	I
A/California/07/2009 A/Perth/16/2009 B/Brisbane/60/2008	No	45 (15/ strain)	Fluarix™, AFLUA523AA	GlaxoSmithKlein	2010	II
A/California/07/2009 A/Victoria/361/2011 B/Wisconsin/1/2010	No	45 (15/ strain)	Vaxigrip®, J8389-2 J8395-2	Sanofi Pasteur MSD	2012	II

4.1.2 Pre-pandemic A(H5N1) vaccines (III)

In Study III, the pre-pandemic influenza A(H5N1) vaccines administered were inactivated, AS03-adjuvanted A/Indonesia/5/2005-like split virion vaccine in 2009 and Vepacel®, inactivated, non-adjuvanted A/Vietnam/1203/2004-like whole virion H5N1 vaccine in 2011 (Table 2).

Table 2. Pre-pandemic influenza A(H5N1) vaccines administered in Study III.

A(H5N1) vaccine viruses	Adjuvant	µg HA/ dose	Vaccine, lot number	Manufacturer	Year	Study
A/Indonesia/5/2005	AS03	3.75	AA3BA020AA	GlaxoSmithKlein	2009	III
A/Vietnam/1203/2004	No	7.50	Vepacel®, VNV1K005A	Baxter	2011	III

4.2 Samples and ethical statements

4.2.1 Human samples

4.2.1.1 Serum samples (I–III)

In Study I–II, clinically healthy healthcare workers were recruited on a voluntary basis from the personnel of the Department of Medicine at the Helsinki University Hospital (HUS) and the Viral Infections Unit at the Finnish Institute for Health and Welfare (THL), Helsinki. In Study I, serum samples were collected before vaccination (day 0) and three weeks and six months after vaccination from a total of 79 subjects (12 men, 67 women), median age 46 years (range: 22–66). In Study II, 50 subjects (eight men and 42 women), median age 47 years (range: 19–64), were involved in the Fluarix™ vaccination trial in 2010. Two years later, at the time of the Vaxigrip® vaccination in 2012, 50 subjects (eight men and 42 women), median age 46 years (range: 24–65), participated in the study. 23 of the participants were included in both vaccinations during Study II. Serum specimens were collected before the vaccination (day 0) and three weeks after the vaccination with seasonal influenza vaccines.

In Study III, in addition to THL and HUS participants, clinically healthy subjects were recruited on a voluntary basis from the Finnish Food Authority. Sixty four subjects (11 men and 53 women), median age 40 years (range: 26–68), were involved in the 2009 vaccination trial. Two years later, at the time of the Vepacel® vaccination

in 2011, 61 subjects (13 men and 48 women), median age 39 years (range: 19–68), participated in the study. 40 of the participants were included in both vaccinations. Serum samples were collected prior to vaccination on day 0, and the post-vaccination serum specimens were collected at three weeks, six weeks, six months, one year (for 2011 vaccination only) and two years after the vaccinations.

Written informed consent was provided by all participants. The study protocols were approved by the Ethic Committee of the Department of Medicine, University of Helsinki (Table 3).

Table 3. The study protocol permissions.

Study conducted at	Permission	Study
THL, HUS	298/13/03/00/2012	I, II
THL, HUS	382/E5/07 §48/2008	II
THL, Finnish Food Authority, HUS	250/13/03/00/2011	III

4.2.1.2 Respiratory specimens (I–II)

As part of virological surveillance of influenza in Finland, a subset of influenza positive samples from sites in a sentinel influenza surveillance network and non-sentinel sites were selected throughout the influenza seasons for genetic characterisation on the basis of their geographical origin and temporal distribution. Representative viruses from different genetic groups were further isolated for serologic analysis.

Based on national Communicable Diseases Act 1227/2016, ethical permission was not required for specific microbiological diagnostics and further characterisation of detected viruses at THL. However, written informed consent was provided by sentinel surveillance participants.

4.2.2 Animal samples and immunizations

4.2.2.1 Red blood cells for haemagglutination inhibition assay (I–III)

Turkey and guinea pig RBCs were obtained from the University of Helsinki experimental animal unit. Blood was collected into an equal volume of Alsevers solution. The licenses for animal experiments were approved by the National Animal Experiment Board: permissions ESAVI/7399/04.10.03/2012 and

ESAVI/9395/04.10.07/2015. Animal use plan was accepted by the Laboratory Animal Centre of the University of Helsinki (internal license).

4.2.2.2 Guinea pig and rabbit immunizations (III)

In Study II, an inhouse rabbit antibody Ag125 made against the A/California/07/2009 A(H1N1)pdm09 whole virion vaccine antigen (GSK, London, UK) [319] was used as the primary antibody in MNT. Other two rabbit antibodies tested as primary antibodies were Ag116 and Ag117 for IAV NP and IAV M1, respectively. Rabbit immunizations were done as follows: days 0, 21, 42, and 63, and serum samples were collected before each immunization, and one week after the last immunization on day 70.

In Study III, five guinea pigs and five rabbits were immunized four times in three week intervals for AS03-adjuvanted A/Indonesia/5/2005 A(H5N1) vaccine. Serum samples from guinea pigs were collected before the first immunization (day 0) and one week after the last immunization (day 70). Rabbit immunizations were done with the same protocol described above. Serum samples were stored at -20°C and analyzed for influenza A(H5N1) virus specific antibodies by the HI test.

Immunizations of the animals and the collection of serum samples were approved by the Ethics Committee of THL, permission KTL 2008-02.

4.3 Influenza viruses used in serological tests

4.3.1 Seasonal influenza A vaccine viruses (I–II)

Seasonal influenza vaccine viruses were provided by the WHO Collaborating Centre for Reference and Research on Influenza at the Crick Worldwide Influenza Centre, London, UK (Table 4).

Table 4. Seasonal influenza A vaccine viruses included in Study I and Study II.

Virus strain	Isolate name HA segment ID	Genetic group	HA titre (turkey RBC)	HA titre (guinea pig RBC)	MNT working dilution	Study
A(H1N1)pdm09	A/California/07/2009 EPI176620	1	16–32	-	1:44	I, II
A(H3N2)	A/Texas/50/2012 EPI391247	3C.1	-	32–64	-	I
A(H3N2)	A/Switzerland/9715293/2013 EPI540526	3C.3a	-	64	-	I

The virus stocks were propagated in MDCK and MDCK-SIAT1 cells for A(H1N1)pdm09 and A(H3N2) viruses, respectively. The cell lines were regularly tested to be free of Mycoplasma contamination. Separate virus stocks were grown for HI assay and MNT. The virus growth with CPE formation was observed by light microscopy. For MNT, viruses were harvested at CPE 75% whereas virus stocks for HI tests were harvested at CPE 90–100%. The HA titration (presented in 4.4.1) for each virus stock was performed before freezing. The virus stocks were aliquoted and stored at -70 ºC.

4.3.2 Circulating influenza virus strains (I–II)

Table 5. Epidemic influenza A virus strains included in serological analysis in Studies I–II.

Virus strain	Isolate name HA segment ID	Genetic group	HA titre (turkey RBC)	HA titre (guinea pig RBC)	MNT working dilution	Study
A(H1N1)pdm09	A/Finland/554/2009 EPI182994	1	64	-	-	II
A(H1N1)pdm09	A/Finland/24/2010 EPI336836	3	32	-	1:141	II
A(H1N1)pdm09	A/Finland/124/2011 EPI322980	4	16–32	-	1:141	II
A(H1N1)pdm09	A/Finland/153/2011 EPI322983	5	32	-	1:141	II
A(H1N1)pdm09	A/Finland/142/2011 EPI322981	6A	32	-	1:445	II
A(H1N1)pdm09	A/Finland/420/2014 EPI532747	6B	16	-	-	I
A(H1N1)pdm09	A/Finland/308/2013 EPI433367	6C	16–32	-	1:44	II
A(H1N1)pdm09	A/Finland/148/2011 EPI322982	7	16–32	-	1:44	II
A(H1N1)pdm09	A/Finland/300/2012 EPI433370	7	16	-	1:141	II
A(H3N2)	A/Finland/385/2013 EPI502957	3C.3	-	16–32	-	I
A(H3N2)	A/Finland/428/2014 EPI556939	3C.3a	-	32	-	I
A(H3N2)	A/Finland/464/2014 EPI557063	3C.2a	-	16	-	I

In Study I and Study II, circulating Finnish IAVs were selected for serologic analysis (Table 5). The viruses were isolated, propagated, and aliquoted as described in chapter 4.3.1. The HA titration (presented in 4.4.1) for each virus stock was performed before freezing.

4.3.3 Pre-pandemic influenza A(H5N1) vaccine viruses (III)

Table 6. Pre-pandemic influenza A(H5N1) candidate vaccine viruses included in Study III.

Virus strain	Isolate name HA segment ID	Clade	HA titre (turkey RBC)	HA titre (guinea pig RBC)	MNT working dilution	Study
A(H5N1)	A/Vietnam/1203/2004 EPI361524	1	64	-	1:282	III
A(H5N1)	A/duck/Hunan/795/2002 EPI135862	2.1.1	32	-	1:28	III
A(H5N1)	A/Indonesia/5/2005 EPI116487	2.1.3.2	64	-	1:282	III
A(H5N1)	A/whooper swan/Mongolia/244/2005 EPI227591	2.2	16	-	1:28	III
A(H5N1)	A/Egypt/N03072/2010 EPI255379	2.2.1	64	-	1:890	III
A(H5N1)	A/Egypt/3300-Namru3/2008 EPI165072	2.2.1.1	64	-	1:282	III
A(H5N1)	A/Hubei/1/2010 EPI337231	2.3.2.1a	32	-	1:28	III
A(H5N1)	A/Anhui/1/2005 EPI101917	2.3.4	64	-	1:282	III
A(H5N1)	A/goose/Guiyang/337/2006 EPI107811	4	64	-	1:28	III
A(H5N1)	A/chicken/Vietnam/NCVD-016/2008 EPI180243	7.1	64	-	1:282	III

Pre-pandemic candidate influenza A(H5N1) vaccine viruses studied were provided by the Centers for Disease Control and Prevention, USA and Dr. Richard Webby and Dr. Ashley Webb at Division of Virology, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, USA (Table 6). The virus stocks were propagated as described in chapter 4.3.1 in MDCK cells in biosafety level 2+, aliquoted and stored at -70 ºC. The HA titration (presented in 4.4.1) for each virus stock was performed before freezing.

4.4 Serological methods for antibody detection

4.4.1 Haemagglutination inhibition assay (I–III)

The HI tests were performed according to WHO guidelines [272] with minor differences.

Erythrocytes

Blood was collected into an equal volume of Alsever's solution, centrifuged, washed twice and resuspended as follows: one volume of RBC to nine volumes of phosphate-buffered saline (PBS) with penicillin-streptomycin (PeSt). For subsequent HA and HI testing, turkey erythrocytes (0.5%/vol) were used for influenza A(H1N1)pdm09 and A(H5N1) viruses, and guinea pig erythrocytes (0.7%/vol) for A(H3N2) viruses.

Serum pre-treatments

To inactivate nonspecific inhibitors of haemagglutination, one volume of serum samples were pretreated with three volumes of RDE from *Vibrio cholerae* filtrate (Denka Seiken, Tokyo, Japan) at +37 °C for 18–20 hours. This reaction was stopped at +56 °C for 30 minutes and then one volume of PBS was added to obtain 1:5 serum dilution.

In addition, to remove non-specific inhibitors and agglutinins 20 volumes of RDE-pretreated serum samples were incubated with one volume of packed (100%) erythrocytes at +4°C for one hour; gently mixing every 15 minutes. After centrifugation the pretreated serum samples were transferred to new tubes.

Haemagglutination (HA) titration of viral isolates

Haemagglutination (HA) titration was performed for all virus stocks before freezing to determine their HA titers. After freezing HA titration was done for HI test to determine the amount of virus needed to agglutinate an equal volume of a standardized RBC suspension, i.e. HA unit. Standardized antigens were adjusted to a HA titre of 4 HA units per 25 µl.

For HA titration, two-fold serial dilutions of virus was done in U-shaped 96-well plates in 50 µl volumes of the diluent PBS with bovine serum albumin (BSA). Fifty µl of corresponding RBCs were added followed by incubation at room temperature (RT) for 30 minutes and for a maximum of one hour for turkey and guinea pig erythrocytes, respectively. For influenza A(H3N2) viruses, the HA titration was performed in the presence of 20nM OC (Roche, Switzerland).

The HA titre was determined after incubation. Complete haemagglutination was considered to have occurred when the RBCs were still in suspension. Non-agglutinated turkey RBCs formed a compact pellet on the bottom of the wells and the absence of haemagglutination was confirmed by tilting the plates to observe the settled RBCs forming a teardrop. Non-agglutinated guinea pig erythrocytes appeared as a halo or circle of settled RBCs on the bottom of the wells.

Haemagglutination inhibition (HI) tests

Two-fold serial dilutions of pretreated sera, starting at a dilution of 1:10, were done in U-shaped 96-well plates in 25 µl volumes of the diluent PBS-BSA. Standardized viral antigen in 25 µl was added to serum plates and incubated for 30 minutes to one hour at RT. Fifty µl of corresponding RBCs were added to the antigen-serum solutions followed by incubation at RT for 30 minutes and a maximum of one hour for turkey and guinea pig erythrocytes, respectively. Control wells for the RBCs alone, serum+RBCs without the virus, and a positive control serum were included in each analysis.

After incubation, the highest dilution of sera causing a complete inhibition of haemagglutination, i.e. HI titre, was analyzed. The principle of defining the haemagglutination and the absence of it, i.e., HI, was defined as described above.

In the case of influenza A(H3N2) viruses, the HI assay was performed in the presence of 20nM OC to inhibit any potential NA-dependent agglutination. For comparison, some viruses were tested also without the presence of OC.

4.4.2 ELISA-based microneutralization test

The ELISA-based MNTs used in Study II and Study III were done based on the WHO Global Influenza Surveillance Network manual [272]. However, two MNT protocols used were further optimized, as described in chapter 5.2.

During the test, serum-neutralizing antibodies to influenza virus HA inhibit the infection of MDCK cells by the virus. Serially diluted serum samples were pre-incubated with a standardized amount of virus before the addition of MDCK cells for 18–20 hours. The cells were fixed and the presence of influenza A virus in infected cells was detected by ELISA.

Tissue culture infectious dose 50%

To find out a standardized amount of virus enabling comparisons between virus strains, a 50% tissue culture infectious dose ($TCID_{50}$) was determined for each virus stock separately. The titration of viruses was performed in quadruplicate with a

starting dilution of 1:100 in MNT medium: OptiPro™ SFM (Gibco, USA), supplemented with 0.2% BSA, non-essential amino acids and PeSt. Virus dilutions ½ log_{10} were performed as described [272]. Cell control (CC) wells were included. The plates were placed in a 37 ºC 5% CO2 incubator for one hour.

MDCK cells were detached, counted and diluted in MNT medium. 2.5 x 10^4 cells were added to each well for an 18–20-hour incubation at 37 ºC with 5% CO_2. Wells were fixed within cold 80% acetone for 10 minutes.

A standard ELISA protocol was followed with modifications indicated below (4.4.2.1 and 4.4.2.2). The mean absorbance (OD_{492}) of the CC wells was calculated and any test well with an OD_{492} greater than twice that of the CC wells was scored positive for virus growth. $TCID_{50}$ was calculated by the Reed-Muench method [272] to determine virus working dilution (Tables 4, 5 and 6).

Serum inactivation

To inacticate complement, all serum samples were heat-inactivated at 56 ºC for 30 minutes before MNT.

Microneutralization

Two-fold serial dilutions of heat-inactivated sera, starting at a dilution of 1:10, were done in 96-well tissue culture plates in 50 µl volumes of the diluent MNT medium. Serum dilutions were mixed with a diluent containing 100 $TCID_{50}$ of influenza viruses. Control wells with the virus and the diluent (VC), the diluent alone (CC) and an internal positive control were included in each plate. After incubation for one hour at 37 ºC with 5% CO_2, 2.5 x 10^4 MDCK cells in 100 µl were added to each well for an 18–20-hour incubation at 37 ºC with 5% CO_2. Wells were washed with PBS, fixed with cold 80% acetone for 10 minutes and presence of influenza antigens in fixed cells was detected by ELISA (Figure 7).

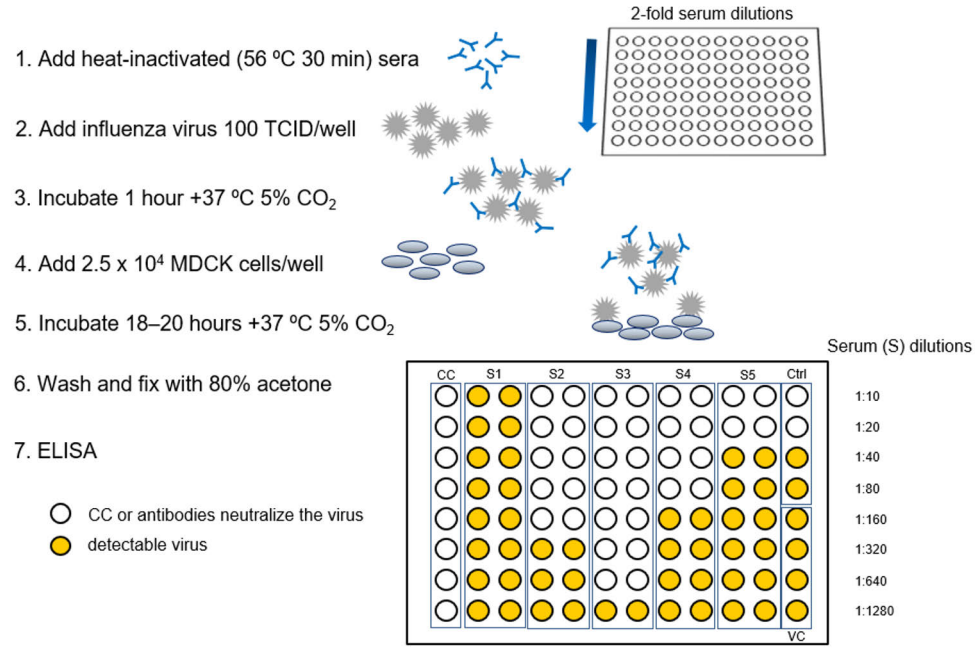

Figure 7. Overview of the ELISA-based microneutralization assay. Author's own drawing. CC cell control; VC virus control; S serum.

4.4.2.1 A(H1N1)pdm09 microneutralization test (II)

Addition of primary antibody

The fixed plates were washed once with a washing buffer: PBS containing 0.05% Tween 20. An in-house rabbit antibody Ag125 against the whole virus (A/California/07/2009 whole virion vaccine antigen, GSK, London, UK) was used as the primary antibody, at a 1:4000 dilution in PBS containing 5% milk. An aliquot of 80 µl of antibody dilution was added and incubated at RT for one hour followed by washing three times with the washing buffer.

Addition of secondary antibody

A total of 80 µl of a 1:2000 dilution of polyclonal horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulins (Dako, Denmark) was added to each well and incubated at RT for one hour followed by washing three times as above.

Addition of substrate

Precisely 100 µl freshly prepared substrate o-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, USA) was added into each well and incubated at RT for 20 minutes. The reaction was stopped with 100 µl 1 M sulfuric acid. Absorbances were measured at 492 nm and 620 nm.

4.4.2.2 A(H5N1) microneutralization test (III)

Addition of HRP-labeled influenza A antibody

The fixed plates were washed twice with washing buffer PBS containing 0.05% Tween 20. A HRP-labeled influenza A NP antibody (7304-HRP, Medix Biochemica, Finland) was diluted 1:500 in PBS containing 5% milk. A total of 80 µl of antibody dilution was added and incubated at RT for one hour followed by washing six times with the washing buffer.

Addition of substrate

Precisely 100 µl freshly prepared substrate OPD was added into each well and incubated at RT for one hour. The reaction was stopped with 100 µl 1 M sulfuric acid. Absorbances were measured at 492 nm and 620 nm.

4.5 Serological data analysis (I–III)

The HA titration endpoint was defined as the highest dilution of virus that still caused complete haemagglutination. The HA titre was the reciprocal of this dilution. The HI test endpoint was defined as the highest dilution of sera that was able to cause complete inhibition of haemagglutination. The HI titre was the reciprocal of this dilution.

The neutralizing endpoint was determined as previously described [272]. The OD_{492} value x representing the cut off for virus neutralization antibody 50% titre was calculated as follows:

$$x = \frac{\text{(average OD of VC cells)} - \text{(average OD of CC wells)}}{2}$$

The VC consisted of infected cells without the addition of serum and the CC involved non-infected cells. All wells with an OD_{492} below or equal to x were

considered positive for neutralization activity. In addition, the VC and the CC were acceptable when an OD_{492} value of >0.8 and <0.2, respectively.

For statistical analyses in all studies, serum specimens with HI and MNT titres <10 were assigned a titre value of 5 and MNT titres greater than 1280 were assigned a titre value of 2560.

Geometric mean titres (GMT) with 95% confidence intervals (CI) and seroprotection rates (SR) for each virus were calculated. Statistical differences between the groups were calculated using Student's t-test (paired, two-tailed) and the statistical significance level of difference was set to $p<0.01$.

HI and MNT results were correlated and compared with Pearson and t-tests. Log-transformed titres were performed by a linear regression analysis using Microsoft Excel 2010 software.

4.6 Phylogenetic analyses of the haemagglutinin gene (I–III)

The phylogenetic analyses of the HA genes of viruses and reference strains were performed as previously described [320]. Molecular Evolutionary Genetics Analysis software version 5 [321] or 7 [322] was used in amino acid sequence comparison and the construction of the phylogenetic tree. The Neighbor-joining method [323] with the maximum composite likelihood model [324] was used to generate the phylogenetic tree. Bootstrapping was performed with 1000 replicates [325]. Reference virus sequences for the phylogenetic tree were obtained from Global Initiative on Sharing Avian Influenza Data EpiFlu™Database.

5 Results and Discussion

5.1 Oseltamivir carboxylate in A(H3N2) haemagglutination inhibition test (I)

The recent influenza A(H3N2) viruses isolated and propagated in MDCK cells have gained the ability to agglutinate RBCs through interactions between NA and sialic acid [285, 326]. The addition of 20nM OC to A(H3N2) HI assays performed with guinea pig RBCs have been used to prevent this NA-dependent agglutination [285, 327].

Table 7. Influence of 20nM oseltamivir carboxylate (OC) to seroprotection rates (SR) and geometric mean titres (GMT) of three influenza A(H3N2) virus strains using haemagglutination inhibition test.

OC	A(H3N2) strain	**Day 0** n=79		**Day 21** n=77		**Day 182** n=72		Study
		SR%	**GMT (95% CI)**	**SR%**	**GMT (95% CI)**	**SR%**	**GMT (95% CI)**	
+	A/Texas/50/2012 (vaccine)	61	33 (27–41)	87	70 (58–86)	86	51 (40–60)	I
-	A/Texas/50/2012 (vaccine)	47	25 * (21–30)	82	54 * (43–64)	58	32 ** (25–38)	UD
+	A/Finland/385/2013	49	25 (21–31)	78	47 (38–56)	54	27 (22–32)	I
-	A/Finland/385/2013	7.6	9.3 **** (7.8–11)	26	15 **** (12–18)	14	10 **** (8.4–12)	UD
+	A/Finland/428/2014	8.9	8.6 (7.3–10)	27	14 (12–18)	9.7	8.7 (7.5–10)	I
-	A/Finland/428/2014	3.8	7.7 (6.7–8.9)	22	13 (12–16)	5.6	9.0 (7.7–11)	UD

* p<0.05; ** p<0.01; **** p<0.0001; difference between the presence of OC per strain
CI Confidence interval
UD Unpublished data

The influence of 20nM OC to HI titres was tested in connection with Study I. Three influenza A(H3N2) virus strains, which resulted in different serum HI titre levels before and after vaccination, were tested with and without 20nM OC: the vaccine strain A/Texas/50/2012 (group 3C.1) and two circulating strains A/Finland/385/2013 (group 3C.3) and A/Finland/428/2014 (group 3C.3a). Table 7 shows the effects of 20nM OC to HI titres and SRs.

The addition of 20nM OC to the HI assay protocol predominantly raised the serum GMTs and SRs for all three viruses studied. The most prominent difference was observed with A/Finland/385/2013, indicating a strong NA-dependent agglutination. The OC also increased serum HI titres and SRs against the MDCK-propagated homologous vaccine strain A/Texas/50/2012. In contrast, OC effect was not seen when egg-propagated A/Texas/50/2012 was tested against post-infection ferret antisera raised against 2011–2012 A(H3N2) viruses [302]. This difference reflects MDCK-SIAT1 propagation-induced mutations due to adaptation [328–330], and demonstrates the influence of different passage history to HI titres [279]. Interestingly, NA-binding associated mutations induced during A(H3N2) MDCK-SIAT1 propagation were possible to reduce or change back to a clinical sample-like NA sequence after a single passage in human airway epithelial cells [330].

NA-dependent, oseltamivir-sensitive agglutination has a pronounced influence on A(H3N2) HI assay; however, it does not affect virus neutralization [285]. MNT is a commonly used method for additional estimation of the antigenic properties of A(H3N2) viruses. The MNT assay findings may further support the results obtained from the HI assays, and concurrently indicate those functional neutralizing antibodies that interfere with infection [331].

5.2 Microneutralization test optimization (II–III)

Although the ELISA-based MNT protocol was based on the WHO Global Influenza Surveillance Network manual [272], optimization of various parameters was yet needed. The objective was to adjust different parameters to achieve optimal signal-to-noise ratio between infected and uninfected cells.

5.2.1 Amount of MDCK cells

One of the most critical aspects of the MNT is the target cell [310]. The cells used should be of low passage, free of mycoplasma contamination and in log-phase growth for maximum virus sensitivity [272]. For Study II, cell concentrations between 1.5 x 10^4 to 9.075 x 10^4 cells/well were tested using three A(H1N1)pdm09 virus strains as shown in Table 8.

Table 8. Different MDCK cell concentrations were tested for the microneutralization assay using three A(H1N1)pdm09 virus strains A/California/07/2009, A/Finland/142/2011, and A/Finland/153/2011, and two A(H5N1) virus strains A/Anhui/1/2005 and A/chicken/Vietnam/NCVD-016/2008. Background absorbance values were followed to optimize the signal-to-noise ratio. Antibody concentrations were 1:2500, 1:2000 and 1:500 for the primary anti-A/California/07/2009 (H1N1pdm09) antibody (Ag125), secondary antibody polyclonal horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin and 7304-HRP, respectively.

Cells/well	Virus strain	Background absorbance 492 Rabbit Ag125	Background absorbance 492 7304-HRP	Study
15000	A/California/07/2009	0.148	0.072	UD
	A/Finland/142/2011	0.111	nd	UD
	A/Finland/153/2011	0.132	nd	UD
	A/Anhui/1/2005	nd	0.052	UD
	A/chicken/Vietnam/NCVD-016/2008	nd	0.061	UD
20000	A/California/07/2009	0.118	0.072	UD
	A/Finland/142/2011	0.119	0.071	UD
	A/Finland/153/2011	0.116	0.074	UD
25000	A/California/07/2009	0.106*	nd	II
	A/Finland/142/2011	0.118*	nd	II
	A/Finland/153/2011	0.106*	nd	II
	A/Anhui/1/2005	nd	0.057	UD
	A/chicken/Vietnam/NCVD-016/2008	nd	0.059	UD
30000	A/California/07/2009	0.147	0.070	UD
	A/Finland/142/2011	0.172	nd	UD
	A/Finland/153/2011	0.146	nd	UD
35000	A/Anhui/1/2005	nd	0.064	UD
	A/chicken/Vietnam/NCVD-016/2008	nd	0.063	UD
55250	A/California/07/2009	0.177	0.080	UD
	A/Finland/142/2011	0.147	nd	UD
	A/Finland/153/2011	0.165	nd	UD
90750	A/California/07/2009	nd	0.065	UD
	A/Finland/142/2011	0.206	nd	UD
	A/Finland/153/2011	0.239	nd	UD

* Values behind the published data, primary antibody concentration 1:4000
Ag antigen
nd not done
UD Unpublished data

MDCK cells are able to multiply quickly and the overgrowth of the cell monolayers may introduce background variation [296]. Both background (Table 8) and virus-infected cell signal absorbance values (Figure 8) for in-house polyclonal rabbit Ag125 antibody were found to increase in connection with rising cell concentrations.

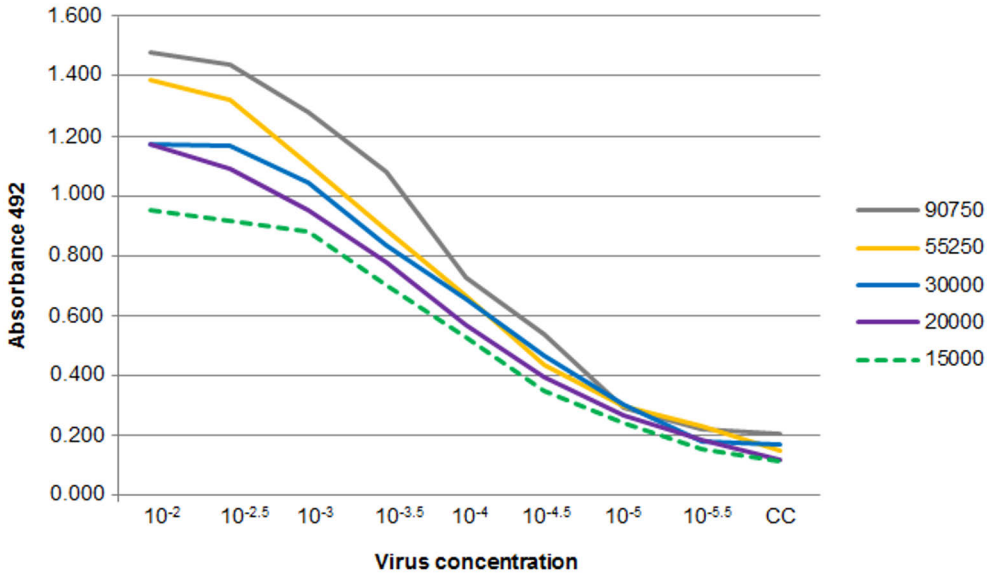

Figure 8. MDCK cell concentrations of 15000–90750 cells/well were tested for A(H1N1)pdm09 microneutralization test using virus strain A/Finland/142/2011. Absorbances 492 for ½ log10 dilutions of the virus are shown as an average of three to four wells and for background (CC) as an average of six to 12 wells. Antibody concentrations were 1:2500 and 1:2000 for primary antibody Ag125 and secondary antibody polyclonal horseradish peroxidase -labeled goat anti-rabbit immunoglobulin, respectively. Green dashed line shows the cell concentration recommendation by WHO. Author's own drawing. Unpublished data.

For Study III, two A(H5N1) vaccine virus strains were tested using cell concentrations 1.5 x 10^4 to 3.5 x 10^4 cells/well (Table 8). An increasing cell amount had no influence on background values with commercial HRP-conjugated antibody, and overall the background values were notably lower compared to those obtained with polyclonal rabbit antiserum Ag125 (Table 8). Growing cell concentrations were associated with increasing signal absorbances also with the A(H5N1) vaccine viruses (Figure 9). Of note, the A(H5N1) vaccine virus dilution curve differed from that seen for A(H1N1)pdm09 viruses suggesting suboptimal virus growth or cell damage when the highest A(H5N1) virus concentrations were used.

Although the WHO manual suggests 1.5 x 10^4 cells/well for MDCK cell concentration [272], the MDCK cell concentration for Studies II–III was chosen to be 2.5 x 10^4 cells/well to ensure better signal for MNT and acceptable background values for A(H1N1)pdm09 MNT.

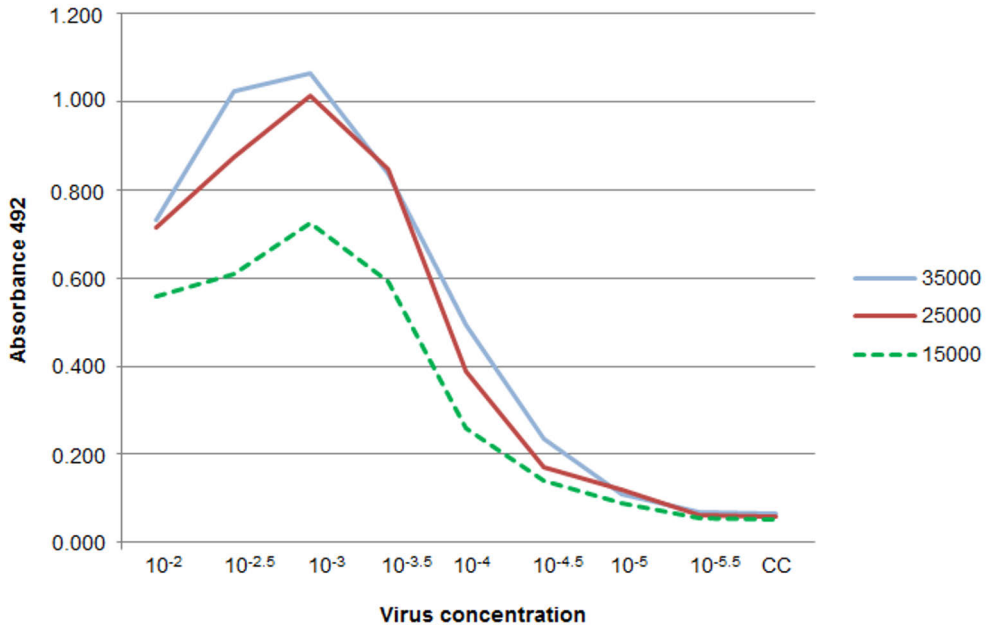

Figure 9. MDCK cell concentrations of 15000–35000 cells/well were tested for A(H5N1) microneutralization test using a candidate vaccine virus strain A/Anhui/1/2005. Absorbances 492 for ½ $\log_{10}$ dilutions of the virus are shown as an average of three wells and for background (CC) as an average of six wells. Antibody concentration was 1:500 for 7304-HRP. Green dashed line indicates the cell concentration recommendation by WHO. Author's own drawing. Unpublished data.

5.2.2 Cell culture medium

Optimal growth conditions are essential for cell-based methodologies. In Study II, the A(H1N1)pdm09 MNT was performed using OptiPro™ SFM, which is a serum-free, animal/human-origin free medium [332], designed for growth of several kidney-derived cell lines such as MDCK.

Before Study III, to find out whether there was a possibility for any savings in reagent expenses, comparative $TCID_{50}$ titrations were performed using OptiPro™ SFM and Dulbecco's modified Eagle's medium (DMEM) (Sigma D7777 + $NaHCO_3$ 3,7mg/ml) with the same supplements: 0.2% BSA, non-essential amino acids and PeSt. In addition, DMEM supplemented as above and also with 1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 0.2% L-glutamine (DMEM+) was included in the analysis. Two A(H5N1) candidate vaccine viruses A/Anhui/1/2005 and A/chicken/Vietnam/NCVD-016/2008 were used for testing. Figure 10 visualizes the superiority of the OptiPro™ SFM cell culture medium compared to DMEM or DMEM+. Furthermore, no difference between DMEM and

DMEM+ were seen. The influence of the cell culture medium was more pronounced in the growth of A/chicken/Vietnam/NCVD-016/2008. Therefore, despite OptiPro™ SFM culture medium being more expensive compared to DMEM, it was used also in Study III.

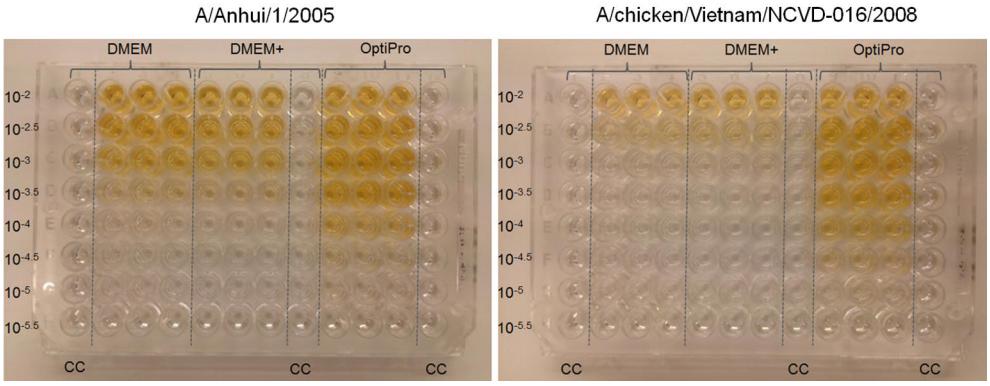

Figure 10. Three cell culture media for optimal growth conditions were tested using $TCID_{50}$ titration method with A/Anhui/1/2005 and A/chicken/Vietnam/NCVD-016/2008 H5N1 vaccine virus strains. Author's own drawing. Unpublished data. CC Cell control.

5.2.3 Antibody dilutions and incubation times

HRP-labeled influenza A nucleoprotein antibody

An anti-influenza A NP monoclonal antibody is recommended for the primary antibody in ELISA-based MNT [272]. The NP is a highly conserved protein among the different subtypes of IAVs, despite differences in evolutionary pathway, subtypes, and host species [333–335]. In direct ELISA, the detection antibody is directly conjugated to an enzyme, such as HRP. The detection is faster than other ELISA techniques because fewer incubation steps are required [336]. In Study III, an HRP-labeled influenza A NP antibody (7304-HRP) was succesfully used for all A(H5N1) vaccine virus strains studied using a dilution of 1:500 and a one-hour incubation at RT.

For Study II, the 7304-HRP was tested for A(H1N1)pdm09 viruses using the vaccine virus and four epidemic strains (Table 9). The 7304-HRP was found to effectively detect the vaccine virus A/California/07/2009 with a low background level and a high signal. However, the 7304-HRP did not recognize NP well enough (an OD_{492} value of >0.8) for any circulating A(H1N1)pdm09 viruses studied, as shown in Table 9, suggesting changes in NP of epidemic A(H1N1)pdm09 viruses compared to the A/Puerto Rico/8/34 backbone, used in vaccine viruses.

Table 9. A horseradish peroxidase (HRP)-labeled influenza A nucleoprotein antibody (7304-HRP) with a dilution of 1:500 was tested for A(H1N1)pdm09 viruses. Absorbances 492 for ½ $\log_{10}$ dilutions of the viruses are shown as an average of three wells and for background as an average of six wells. The amount of cells/well in this experiment was 20000. Unpublished data.

Virus	A/California/07/2009 (vaccine)	A/Finland/ 124/2011	A/Finland/ 142/2011	A/Finland/ 148/2011	A/Finland/ 153/2011
10^{-2}	1.726	0.227	0.188	0.207	0.207
$10^{-2.5}$	1.593	0.167	0.172	0.137	0.146
10^{-3}	1.270	0.131	0.162	0.109	0.125
$10^{-3.5}$	0.730	0.090	0.106	0.078	0.088
10^{-4}	0.368	0.074	0.082	0.076	0.079
$10^{-4.5}$	0.205	0.095	0.095	0.099	0.096
10^{-5}	0.121	0.085	0.087	0.095	0.089
$10^{-5.5}$	0.077	0.063	0.060	0.063	0.058
Background	0.079	0.074	0.071	0.076	0.074

Primary antibody for A(H1N1)pdm09 MNT

For A(H1N1)pdm09 MNT used in Study II, three in-house rabbit antibodies were tested as possible primary antibodies: Ag116, Ag117, and Ag125 for IAV NP, IAV M1, and A/California/07/2009 whole virion vaccine, respectively. The first analysis with three viruses A/California/07/2009, A/Finland/142/2011, and A/Finland/153/2011 using antibody dilution 1:1000 indicated the strongest signal for Ag125 and the lowest background for Ag116 (data not shown). Ag116 and Ag125 were tested further.

The subsequent Ag116 testing with dilutions 1:500, 1:1000 and 1:1500 indicated a too weak signal compared to the background value (data not shown). Additional testing of Ag125 using dilutions 1:1000, 1:2000, 1:2500 and 1:4000, indicated a decreasing background in connection with increasing dilutions, still maintaining a reasonable signal (Table 10). Finally, Ag125 with the dilution 1:4000 was chosen as the primary antibody for an indirect ELISA.

Secondary antibody for A(H1N1)pdm09 MNT

Dilutions 1:1000 and 1:2000 of polyclonal HRP-labeled goat anti-rabbit Igs were tested for a secondary antibody (Table 10). Although both dilutions worked well in this experiment with three A(H1N1)pdm09 viruses using 20000 cells/well, there was

a trend for increasing background absorbances when using the secondary antibody dilution of 1:1000 with 25000 cells/well. Mean background absorbances 492 calculated as an average of 12 wells using a cell amount of 25000/well were found to be 0.150, 0.139, 0.144, 0.175, and 0.200 for A/Finland/24/2010, A/Finland/124/2011, A/Finland/142/2011, A/Finland/300/2012, and A/Finland/308/2013, respectively. Therefore, the secondary antibody dilution 1:2000 was chosen for subsequent analyses.

Table 10. Optimization of primary antibody (Ab) Ag125 and secondary antibody polyclonal horseradish peroxidase -labeled goat anti-rabbit immunoglobulin for A(H1N1)pdm09 microneutralization test. Absorbances 492 for ½ $\log_{10}$ dilutions of the viruses are shown as an average of three wells and for background as an average of six wells. The amount of cells/well in this experiment was 20000. Unpublished data.

Virus	**A/California/07/2009**			**A/Finland/142/2011**			**A/Finland/153/2011**		
Primary Ab	1:2500	1:4000	1:4000	1:2500	1:4000	1:4000	1:2500	1:4000	1:4000
Secondary Ab	1:2000	1:1000	1:2000	1:2000	1:1000	1:2000	1:2000	1:1000	1:2000
10^{-2}	1.174	1.050	0.944	1.222	1.306	1.028	1.178	1.203	0.943
$10^{-2.5}$	0.733	0.692	0.582	1.129	1.138	0.909	0.967	0.954	0.742
10^{-3}	0.501	0.428	0.377	0.934	0.978	0.794	0.753	0.717	0.607
$10^{-3.5}$	0.324	0.245	0.254	0.731	0.735	0.587	0.538	0.487	0.389
10^{-4}	0.198	0.160	0.162	0.501	0.494	0.444	0.341	0.314	0.254
$10^{-4.5}$	0.164	0.129	0.137	0.369	0.344	0.281	0.222	0.210	0.169
10^{-5}	0.131	0.109	0.109	0.243	0.205	0.189	0.158	0.140	0.118
$10^{-5.5}$	0.100	0.091	0.091	0.176	0.136	0.134	0.116	0.107	0.105
Background*	0.111	0.096	0.083	0.101	0.100	0.083	0.094	0.088	0.077

* Background absorbance 492 was calculated as average of six wells.

5.2.4 Substrate incubation times

For A(H1N1)pdm09 MNT, the substrate incubation time for 15, 20 and 30 minutes was tested. There was a trend for increasing background absorbance with increasing incubation time (data not shown). The 20 minutes substrate incubation time was found to be optimal for A(H1N1)pdm09 MNT.

For A(H5N1) MNT, the substrate incubation time for 30 minutes and one hour was tested. No differences between background absorbances were detected (Table

11). However, the signal was stronger with longer incubation time; thus one hour incubation was selected.

5.2.5 Washings

For A(H1N1)pdm09 MNT, plates were washed once before addition of primary antibody and three times before the addition of the secondary antibody and the substrate.

Table 11. Optimization of substrate incubation time and washing protocol for A(H5N1) microneutralization test. Absorbances 492 for ½ $\log_{10}$ dilutions of the viruses are shown as an average of four wells and for background as an average of 12 wells. Unpublished data.

Virus	**A/Anhui/1/2005**				**A/chicken/Vietnam/NCVD-016/2008**			
Substrate	60 min	60 min	30 min	30 min	60 min	60 min	30 min	30 min
Washings	2+6	1+3	2+6	1+3	2+6	1+3	2+6	1+3
10^{-2}	0.687	0.756	0.634	0.539	0.828	0.808	0.692	0.706
$10^{-2.5}$	0.884	0.865	0.731	0.680	0.938	0.880	0.774	0.734
10^{-3}	0.816	0.717	0.661	0.598	0.704	0.663	0.622	0.586
$10^{-3.5}$	0.397	0.358	0.460	0.486	0.406	0.328	0.323	0.367
10^{-4}	0.146	0.140	0.159	0.149	0.187	0.161	0.158	0.184
$10^{-4.5}$	0.101	0.103	0.074	0.100	0.122	0.112	0.114	0.095
10^{-5}	0.077	0.085	0.058	0.083	0.092	0.079	0.091	0.072
$10^{-5.5}$	0.050	0.051	0.048	0.049	0.062	0.063	0.058	0.057
Background	0.062	0.066	0.061	0.064	0.059	0.057	0.062	0.050

For A(H5N1) MNT, two washing protocols were tested. Plates were washed once or twice before addition of the 7304-HRP and three or six times before addition of substrate for protocols 1+3 and 2+6, respectively. No major differences were seen between the protocols (Table 11). However, during the one hour incubation the wells appeared to show slightly higher absorbance values with the washing protocol 2+6, therefore, it was chosen for subsequent analyses.

5.3 Comparison between microneutralization tests and haemagglutination inhibition assays (II–III)

The HI assay has traditionally been used for identification of antigenic variants and the selection of influenza viruses for vaccine production based on antigenic characterisation of the HA of circulating viruses [337], and for the detection of infection-induced or vaccine-induced antibodies [33]. In Studies II and III an ELISA-based MNT was used as an additional serological method to assess the presence of vaccine-induced neutralizing antibodies for A(H1N1)pdm09 viruses and A(H5N1) vaccine viruses, respectively.

Although the MNT is more laborious to conduct than the HI test, the MNT has certain advantages over the HI test. The MNT may detect a wider range of antibodies than the HI test, thus the MNT likely elicits more comprehensively the antigenic differencies or similarities between the viruses [302]. Studies II and III indicate that MNT titres against both A(H1N1)pdm09 viruses and A(H5N1) vaccine viruses tend to be higher than those obtained in the HI test. This is in accordance with previous studies [302, 312, 338] suggesting preferable sensitivity of the MNT over the HI assay [338–342], particularly in serum specimens that show low anti-viral antibody levels [312].

A strong correlation was detected between the antibody titers of the HI test and the ELISA-based MNT for the vaccine viruses A/California/07/2009 and A/Vietnam/1203/2004. Among A(H1N1)pdm09 viruses, the HI test usually has a good correlation with virus neutralization assays [275, 338, 343, 344]. In addition, neutralizing A(H5N1) antibody responses have usually confirmed the trend seen in the HI test [345–347], however, the results do not always correlate. The correlation has been reported to be lower for A/Vietnam/1194/2004 and A/Hubei/1/2020 strains [348]. Based on positive correlation, an A/California/07/2009 HI titer of 40 was found to correspond to an MNT titre of 160 among A(H1N1)pdm09 viruses, in line with another study analyzing A(H1N1)pdm09 viruses [341]. An A/Vietnam/1203/2004 HI titre of 40 was considered to be equivalent to an MNT titre of 80 for A(H5N1) vaccine viruses. Of note, an MNT titre of 80 has been defined as a serological cut-off for influenza A(H5N1) infection [311]. However, the data on the surrogate protective value of neutralizing antibody titres against influenza virus varies considerably depending on the method and viruses used in the assays [305, 338, 341, 342, 344].

Although MNTs have displayed higher interlaboratory variability than the HI assay [276, 305, 312], a good correlation has been shown between the results of different MNTs [204, 338, 349]. Moreover, the ELISA-based MNTs tend to show lower variation [312]. MNT is recommended as a serological test for the measurement of antibodies against HPAI in humans [311], due to the generally insensitive HI assay for the detection of antibodies to avian strains [310]. Recent

challenges in the performance of the HI assay with A(H3N2) viruses also favors the analyses of anti-influenza immunity of A(H3N2) viruses with MNT, which is not significantly affected by NA-mediated agglutination [302].

5.4 Follow-up of influenza vaccine-induced antibody responses in relation to evolution of influenza A viruses

In publications I–III, influenza vaccine-induced antibody responses were investigated in clinically healthy adults. Figure 11 provides a general description of the study design, number of participants, serum sampling intervals and assay methods.

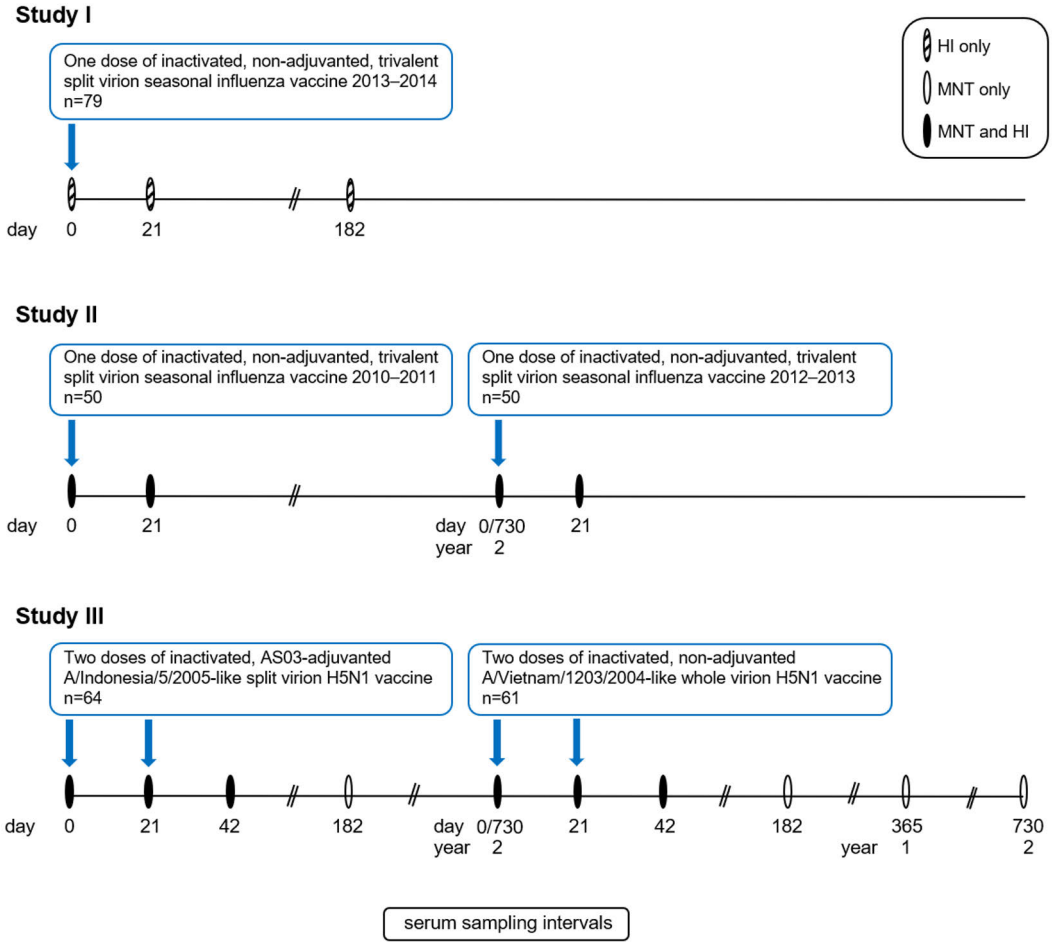

Figure 11. The study design of influenza vaccine-induced antibody responses in clinically healthy adults (Studies I–III). Modified from Original publication III.

Study I and Study II determined the antibody responses against circulating seasonal influenza strains compared to vaccine viruses used at the same period of time during 2009–2015. Study III analyzed the antibody responses against influenza A(H5N1) candidate vaccine viruses isolated in 2002–2010, whereas the serum samples were collected between 2009–2013.

5.4.1 Seasonal influenza A (I–II)

Figure 12 visualizes the variablility and the proportion of laboratory confirmed influenza A and B findings reported to the Finnish National Infectious Diseases Register between the influenza seasons 2010–2011 and 2019–2020, and the dominant IAV subtype within the season.

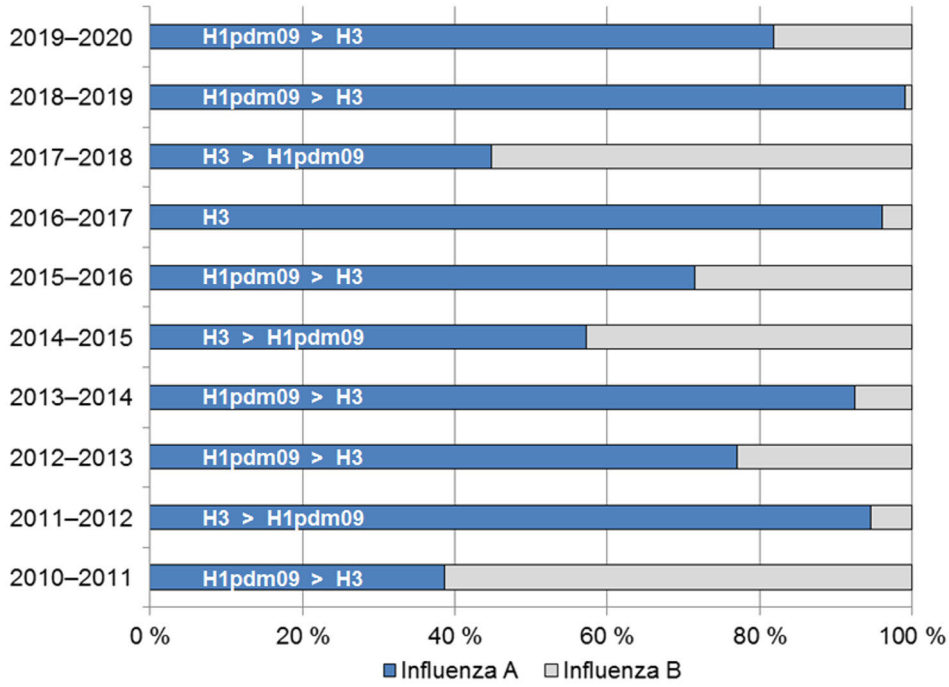

Figure 12. The proportion of influenza A and B findings reported to the Finnish National Infectious Diseases Register between influenza seasons 2010–2011 and 2019–2020. Notifications from weeks 40–20 were included per season. As part of virological surveillance of influenza in Finland, influenza A subtypes were analyzed and a subset further characterized by the National Influenza Center at the Finnish Institute for Health and Welfare. Modified from Ikonen et al. 2019 [350].

The decade of 2010 of influenza seasons started with the second wave of the Swine flu influenza pandemic in Finland in 2010–2011 caused by A(H1N1)pdm09 virus [351] and ended with the first wave of COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2, that started to spread throughout the world in 2020. The first case of severe acute respiratory syndrome coronavirus 2 was reported in Finland in January 2020 [292]. The COVID-19 pandemic resulted in globally diminished influenza cases concurrently with increased influenza vaccination and infection control measures, such as mask wearing, social distancing, handwashing, travel restrictions and closing schools [101, 352].

Studies I and II were implemented between the influenza seasons 2010–2011 and 2014–2015. Within that period of time, the second wave of A(H1N1)pdm09 pandemic dominated Finnish IAV findings in season 2010–2011, however, during which influenza B infections were more common than influenza A infections [351]. The seasons 2012–2013 and 2013–2014 were dominated by A(H1N1)pdm09 viruses [353, 354], whereas the seasons 2011–2012 and 2014–2015 were dominated by A(H3N2) viruses concomitant with a mismatch between the A(H3N2) vaccine virus and circulating A(H3N2) strains [355, 356].

A(H1N1)pdm09 virus

In Finland, the first wave of influenza A(H1N1)pdm09 pandemic happened in a period of May 2009 to March 2010 [357]. The second wave started in October 2010 [351], at the time when the first sample collection for Study II was performed (season 2010–2011). During the A(H1N1)pdm09 pandemic, young adults and children were substantially more affected compared to low morbidity and mortality reported in older populations [45]. The HA gene of A(H1N1)pdm09 virus has shown to be phylogenetically more closely related and antigenically somewhat similar to the virus of the 1918 Spanish influenza [46], which likely gives an explanation of pre-existing cross-reactive neutralizing antibodies in the elderly [46] as well as infection-induced cross-reactive antibodies [47].

After a monovalent pandemic vaccine [358, 359], the A/California/07/2009 -like virus was included in seasonal influenza vaccines as the A(H1N1) component for seven sequential Northern hemisphere influenza seasons (Table 12). This provided an opportunity to analyse vaccine-induced immunity and cross-reactivity against circulating A(H1N1)pdm09 virus strains in relation to the post-pandemic evolution of the A(H1N1)pdm09 virus.

Table 12. WHO recommendations on the composition of egg-based influenza virus vaccines A(H1N1)pdm09 component for Northern hemisphere influenza seasons between 2010–2011 and 2019–2020.

Influenza season	A(H1N1)pdm09 vaccine strain	Genetic group	Reference
2019–2020	A/Brisbane/02/2018	6B.1A	[360]
2018–2019	A/Michigan/45/2015	6B.1	[361]
2017–2018	A/Michigan/45/2015	6B.1	[362]
2016–2017	A/California/07/2009	1	[363]
2015–2016	A/California/07/2009	1	[364]
2014–2015	A/California/07/2009	1	[365]
2013–2014	A/California/07/2009	1	[366]
2012–2013	A/California/07/2009	1	[367]
2011–2012	A/California/07/2009	1	[368]
2010–2011	A/California/07/2009	1	[369]

* Different A(H1N1)pdm09 strain recommended for cell- or recombinant-based vaccines.

Since 2009, the HA genes of A(H1N1)pdm09 viruses have evolved compared to the A/California/07/2009 strain. Eight genetic groups have been designated, of which group 6 viruses further fall into subgroups [370]. During Study periods I–II, influenza A(H1N1)pdm09 viruses evolved continuously with multiple substitutions in the HA gene [371], however the majority of the epidemic viruses in Europe remained antigenically well conserved [372]. Consistent with this, antibody responses during Studies I and II indicated antigenic similarity between vaccine and epidemic A(H1N1)pdm09 strains in Finland at least up to influenza season 2013–2014, and substantial vaccine-induced immunity against circulating viruses representing the genetic groups 1, 3, 4, 5, 6, or 7. A(H1N1)pdm09 viruses have been discovered to carry more substitutions in the stem region of the HA molecule than in the antigenic sites, suggesting pandemic virus adaptation to the human host [371]. The overall adaptation rate differs between influenza subtypes and has been shown to be weaker for human A(H1N1) than A(H3N2) [11].

Pre-existing immunity from before the booster dose against both vaccine and circulating A(H1N1)pdm09 viruses was found to increase both HI and MN titers, and corresponding SRs (Table 13), which reflects the history of sequential TIV vaccinations in the study group or natural infections by A(H1N1)pdm09 viruses. HA-binding antibody responses after A(H1N1)pdm09 virus infection have shown broad specificity [373] and infection-induced HI antibody levels were maintained at

titers >1:40 for many years [374]. Higher pre-vaccination antibody levels have been reported in previously vaccinated subjects compared to those with no previous vaccination [375–377]. On the other hand, high levels of pre-existing antibodies may have a negative effect on boosting, leading to lower antibody responses after repeated influenza vaccinations [185, 376], likely due to reduced antibody-affinity maturation to the HA1 domain [378]. While repeated annual vaccinations have been reported to enhance pre-existing antibody titers, the protective antibodies to A(H1N1)pdm09 viruses were reported to increase after each vaccination and persist over time [343]. This is consistent with the findings in Studies I–II showing a significant and expected increase in the GMTs of the antibody response to all A(H1N1)pdm09 strains studied three weeks after TIV delivery.

Table 13. Seroprotection rates against vaccine and circulating strains of influenza A(H1N1)pdm09 virus in Study I and Study II.

Year Strain	2010–2011 vaccine	2012–2013 vaccine	2013–2014 vaccine	2010–2011 epidemic	2012–2013 epidemic	2013–2014 epidemic
HI / day 0	39	58	57	33–49	38–64	58
HI / day 21	89	84	86	76–90	74–84	90
MNT / day 0	34	54	nd	36–52	44–56	nd
MNT / day 21	92	84	nd	82–94	78–86	nd
Study	II	II	I	II	II	I

HI Haemagglutination inhibition test.
MNT Microneutralization test.
nd not done

Study I demonstrates a significant decrease in A(H1N1)pdm09 antibody levels of 39–46% at six months. This waning immunity after influenza vaccination or infection stands for the process by which immune reactivity such as antibody titer declines with time in the absence of booster stimulation [6]. Waning immunity leads to reduced VE with increasing time following vaccination [379, 380]. However, there are also studies which reported no change in VE against influenza A(H1N1)pdm09 over time after vaccination between the seasons 2010–2011 to 2014–2015 [381, 382]. This is in line with the findings in Study I where the HI titres as a whole decreased at six months, but yet the GMT for the A(H1N1)pdm09 epidemic strain remained above the theoretical protection titre of 1:40.

A(H1N1)pdm09 viruses, representing eight influenza genetic groups, had been detected globally already within the year 2011 [383]. During the season 2012–2013

in Finland, the most epidemic viruses represented group 6 viruses, yet also group 7 viruses were detected [353]. The viruses of group 6 further evolved into three subgroups, 6A–6C, of which representatives from subgroup 6B were detected during the season 2013–2014 in Finland [354]. In the course of influenza season 2015–2016, two sub-clades within the 6B clade emerged: 6B.1 and 6B.2. HI and VN assays using ferret antisera indicated that most A(H1N1)pdm09 viruses were still antigenically similar and closely related to the vaccine virus A/California/07/2009 [363]. The A(H1N1)pdm09 vaccine strain was changed to A/Michigan/45/2015 and A/Brisbane/02/2018 strains for the 2017–2018 and 2019–2020 Northern hemisphere vaccines, respectively [360, 362]. Both these changes were based on immunogenicity analyses in humans rather than traditional experimental data obtained in ferrets. While HI assays with ferret antisera indicated that almost all recent A(H1N1)pdm09 viruses were antigenically indistinguishable from the used vaccine virus, some of the epidemic viruses were poorly inhibited by post-vaccination human sera. Thus the distinct HI profiles of animal and human antisera need further interpretation. HA-reactive antibodies of ferrets are mostly affected by site Sa, whereas those of humans are preferentially affected by both sites Sa and Sb [384].

A(H3N2) virus

Since the emergence in 1968 in humans, influenza A(H3N2) viruses have evolved continuously to escape host immune pressure [331]. Seasonal epidemics dominated by A(H3N2) virus typically lead to higher rates of infection, hospitalization, and mortality especially among the elderly compared with the outbreaks dominated by other subtypes [385, 386]. HA and NA genes of A(H3N2) viruses evolve faster than those of A(H1N1) [11] and new antigenic A(H3N2) clusters appear on an average every 3.3 years [387].

Since 2009, seven genetic groups based on the HA gene have been defined for A(H3N2) viruses, of which group 3 has been further divided into several subgroups [388]. Genetic groups 1–2 represent the A/Perth/16/2009-lineage, which was replaced by the A/Victoria/208/2009-lineage as the dominant lineage, including genetic groups 3–7 [389]. This antigenic drift was related to a reduced A(H3N2) VE in the 2011–2012 season [355, 390, 391]. WHO has recommended several A(H3N2) vaccine strain updates due to rapid antigenic drift as shown in Table 14.

Table 14. WHO recommendations on the composition of egg-based influenza virus vaccines A(H3N2) component for Northern hemisphere influenza seasons between 2010–2011 and 2019–2020.

Influenza season	A(H3N2) vaccine strain	Genetic group	Reference
2019–2020	A/Kansas/14/2017	3C.3a	[360]
2018–2019	A/Singapore/INFIMH-16-0019/2016	3C.2a1	[361]
2017–2018	A/Hong Kong/4801/2014	3C.2a	[362]
2016–2017	A/Hong Kong/4801/2014	3C.2a	[363]
2015–2016	A/Switzerland/9715293/2013	3C.3a	[364]
2014–2015	A/Texas/50/2012	3C.1	[365]
2013–2014	A/Texas/50/2012	3C.1	[366]
2012–2013	A/Victoria/361/2011	3C.1	[367]
2011–2012	A/Perth/16/2009	1	[368]
2010–2011	A/Perth/16/2009	1	[369]

* Different A(H3N2) strain recommended for cell- or recombinant-based vaccines.

During the 2013–2014 epidemic season, representatives of influenza A(H3N2) groups 3C.2, and mostly 3C.3 were found in Europe. Since February 2014, two new genetic subgroups, 3C.2a and 3C.3a, emerged in these clusters [388]. In Finland, infections caused by A(H3N2) genetic subgroup 3C.3a viruses were detected between February and April 2014, however, genetic subgroup 3C.2a viruses emerged only during the 2014–2015 season. Drifted influenza A(H3N2) viruses dominated the 2014–2015 influenza season, leading to little or no vaccine-induced protection against influenza disease due to predominant and substantially mismatched A(H3N2) viruses [392, 393]. The majority of genetically characterised 2014–2015 circulating viruses in Finland belonged to group 3C.2a, yet 3C.3b and 3C.3 viruses were also detected [356], and in Europe also 3C.3a [394].

Subgroup 3C.2a and 3C.3a viruses carry specific amino acid substitutions that drifted from the corresponding main groups [388]. Both subgroups have a substitution at position 159, which has shown to be one of seven positions responsible for the major antigenic changes seen between 1968 and 2003 A(H3N2) viruses [395].

The strains recommended by the WHO for inclusion in the 2014–2015 TIV for the northern hemisphere [364] remained the same as those in 2013–2014 [366]. Serum specimens for Study I were collected before and after TIV delivery (Figure 11) during the 2013–2014 season, which enabled evaluation of the seroprotection levels against the A(H3N2) viruses included in TIV and a comparison to vaccine-induced cross-

protection towards circulating virus strains both from the 2013–2014 and 2014–2015 seasons. Serum A(H3N2) antibody levels were analyzed using the HI test with guinea pig RBCs in the presence of 20nM OC. A significant increase was seen in the GMTs of the antibody response against A(H3N2) viruses three weeks after TIV delivery, and a significant GMT decrease of 24–42% at six months, reflecting the waning immunity that is more pronounced for A(H3N2) viruses [198, 379, 381, 396]. The group 3C.3 virus remained antigenically similar to the TIV vaccine virus. In line with other studies, the GMTs and SRs were found to be significantly lower against the A(H3N2) subgroup 3C.2a and 3C.3a viruses compared to the homologous A/Texas/50/2012 vaccine virus, reflecting mismatch of the vaccine virus.

A(H3N2) viruses have provided several challenges for the surveillance of circulating influenza virus strains and for the selection of appropriate vaccine strains. Since the early 1990's, most of the A(H3N2) isolates have failed to agglutinate chicken RBCs used in the HI assay, yet upon passage in tissue culture most strains will adapt to avian RBC agglutination [281]. Variable agglutination of RBCs from guinea pigs, turkeys and humans [388], the loss of the ability of viruses to agglutinate any of the these RBCs [337], and NA-mediated agglutination discussed in chapter 5.1 have further complicated the antigenic characterization. In the Netherlands, poorly agglutinating influenza A(H3N2) isolates were reported to increase from about 5% to 51% between 2003 and 2012 [283]. Furthermore, virus growth capacity has been reduced in eggs and MDCK cell cultures [40, 69, 284], and egg propagation-derived mutations in A(H3N2) vaccine strains may contribute to the antigenic mismatch [78, 195, 397]. A poor growth of A(H3N2) isolates has been improved and higher virus titers obtained, when isolation and growth of A(H3N2) viruses has been performed in currently recommended MDCK-SIAT1 cells [398], which overexpress the α-2,6-linked sialic acid receptor, compared to MDCK cells [399], yet compensatory mutations occur also in MDCK-SIAT1 cells [330].

5.4.2 Pre-pandemic A(H5N1) influenza vaccine-induced antibody responses (III)

A concern of a potential avian influenza pandemic was raised in 1997, when influenza A(H5N1) virus infected 18 humans in Hong Kong, including six fatal cases [400]. The HA gene of the viruses was genetically similar to that of A/goose/Guangdong/1/96, whereas the other genes showed more similarity to other avian IAVs, suggesting one or more different progenitors among avian species [401]. These human A(H5N1) virus isolates did not show evidence of adaptive changes within the HA and the NA genes [400]. Since 2003 as of June 2022, a total of 865 laboratory-confirmed cases of A(H5N1) human infections from 20 countries have been reported, including 456 deaths [402], giving a case fatality rate of about 53%.

Influenza A(H5N1) viruses have been divided into ten genetically distinct HA-based clades (0–9) and several subclades [403]. Viruses from clades 0, 1, 2 and 7 have caused sporadic infections in humans, most commonly by clade 2 [404], which includes several subclades [403]. Since 2008, subclade 2.3.4.4. A(H5) viruses emerged in China, of which eight genetic groups (a–h) have been recognised so far [104, 402]. The HA gene of A(H5) HPAI viruses has undergone reassortments with internal genes and NA from other viruses to replace the N1 gene segment by N2, N3, N4, N5, N6, N8 or N9 [405–409]. Humans have been infected with the subtypes H5N6 [410,411] and H5N8 [412]. Transmission of avian IAV strains between birds occurs generally via the faecal-oral route, whereas transmission of avian IAV to humans is suggested to occur mostly via direct contact between human respiratory mucosa and infected bird secretions [27].

Continuous global influenza surveillance, pandemic risk assessment tools and influenza research are important to minimise the impact of a human influenza pandemic [413]. WHO coordinates the development of zoonotic influenza candidate vaccine viruses for pandemic preparedness [104]. During the year 2006, the health officials in Finland purchased an avian IAV vaccine prototype of the A/Indonesia/5/2005 strain sufficient to vaccinate all residents of the country. The vaccines were received in 2008 and they would have been used if the HPAI A(H5N1) strain of avian IAV had been causing a pandemic in humans. An advance reservation contract was also made to buy a more targeted avian influenza vaccine developed in a potential pandemic situation.

In Study III, serum antibody levels against 10 influenza A(H5N1) vaccine viruses representing four genetic clades and several subclades were measured by MNT and HI assays before and after vaccination with two different pre-pandemic A(H5N1) vaccines (Figure 11). After two doses of an inactivated AS03-adjuvanted A/Indonesia/5/2005-like split virion A(H5N1) vaccine in 2009, neutralizing antibody titers increased significantly against all but one strain. The greatest SR logically was detected for the homologous vaccine strain. Antibody responses to heterologous strains were much lower than to the homologous vaccine strain and the titers varied considerably according to the virus strain tested. Two vaccine doses and the use of adjuvants have been recommended with conventional A(H5N1) vaccines due to low immunogenicity of inactivated vaccines against avian influenza [174, 185, 414–419]. AS03 adjuvant was reported to enable the use of a lower dose of H5N1 antigen [420] and enhance cross-clade A(H5N1) virus neutralizing titres [419]. The response after the first immunization with the AS03-adjuvanted A/Indonesia/5/2005-like split virion A(H5N1) vaccine was found to be directed to the conserved HA stem region by recruiting pre-existing memory B cells and naïve B cells into the response. The second immunization induced a plasmablast response to the highly variable HA head region [185].

To analyze whether multiple immunizations would enhance vaccine-induced antibody levels and broaden the cross-reactivity against different vaccine A(H5N1) virus strains, rabbits and guinea pigs were immunized up to four times with the AS03-adjuvanted A/Indonesia/5/2005 A(H5N1) vaccine. Very high heterologous HI titres were detected after four immunizations. However, rabbit antibody responses suggested that three vaccine doses were sufficient to induce maximal antibody levels in vaccinated animals. Higher antibody titres were observed in guinea pigs than rabbits, which may reflect differences between animal species and indicate reported dose-response effects [348, 421] as the size of immunized animals was different.

The second pre-pandemic vaccine studied in 2011 was an inactivated nonadjuvanted Vero cell-derived A/Vietnam/1203/2004 whole virion A(H5N1) vaccine, for which the seroneutralization evaluation criteria by EMA were modified and was based on a MNT cut-off titre of 1:20 [422]. Studies performed by conventional MNT and with a seroneutralization threshold of 1:20 have reported a significant homologous antibody rise after two doses of this vaccine [423, 424]. In contrast, the same vaccine in Study III induced only weak homologous HI-based SRs after two vaccine doses in naïve seronegative subjects (Figure 13). Study III criteria for seroprotection was based on a HI titre of 1:40, thus different assays and seroprotection correlates may provide different interpretation of the data.

However, a single dose of the nonadjuvanted A/Vietnam/1203/2004 vaccine was able to induce robust booster effect and heterologous response for subjects primed with the AS03-adjuvanted A/Indonesia/05/2005 vaccine two years earlier (Figure 13). Vaccine-induced neutralizing antibodies against all viruses studied were significantly higher even two years after the nonadjuvanted booster vaccination. SRs for all viruses waned but stayed at elevated levels, except against the phylogenetically more distinct strains as observed with rabbit and guinea pig HI titres. Also two subjects with a history of two doses of the MF59-adjuvanted A/Vietnam/1194/2004 H5N1 vaccine (Novartis) 6–7 months prior to the AS03-adjuvanted A/Indonesia/5/2005 booster vaccine in 2009 developed very strong heterologous antibody responses. Priming with one or two doses of the AS03-adjuvanted A(H5N1) vaccine has been shown to give rise to heterologous AS03-adjuvanted boosting after 12 months [346]. Consistent with Study III data, the highest cross-reactive humoral as well as cell-mediated immune responses were observed, when two doses were followed by a heterologous booster vaccine dose [346]. The booster effect has been suggested to be the highest two years after the two-dose priming compared to the booster response seen at six or 12–15 months after primary immunization [424]. The results of Study III further confirm earlier observations that heterologous prime-boost vaccinations induce more cross-reactive responses which may give rise to a much broader protection against novel A(H5N1) virus strains [347, 425]. Cross-protective neutralizing antibodies in patients severely

infected by A(H5N1) viruses were shown to persist longer than two years while those of asymptomatically infected individuals showed a disappearance of neutralizing antibodies within a year [426].

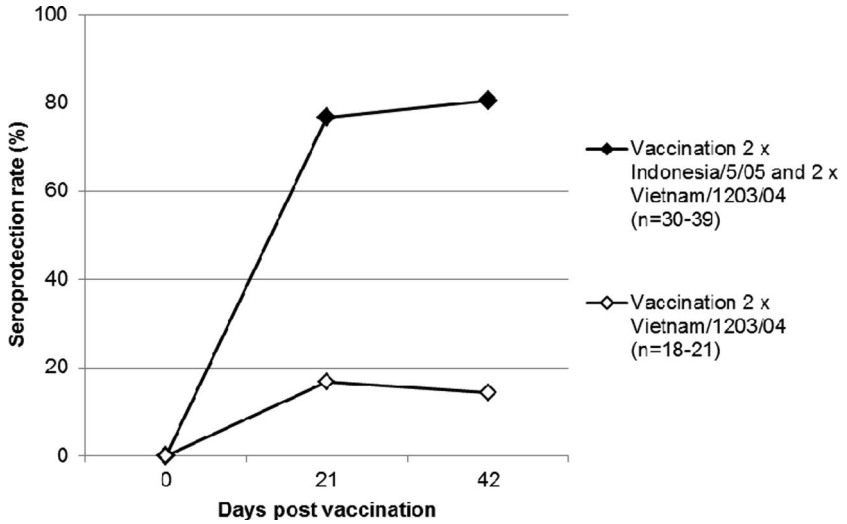

Figure 13. Seroprotection rates against A/Vietnam/1203/2004 determined by haemagglutination inhibition (HI) test before and after vaccination of 60 subjects with two doses of non-adjuvanted A/Vietnam/1203/2004-like H5N1 vaccine in 2011. 21 subjects received their first A(H5N1) vaccination (Vietnam strain only; white squares) and 39 subjects had been immunized also earlier in 2009 (black squares) with two doses of AS03-adjuvanted A/Indonesia/5/2005-like H5N1 vaccine. Seroprotection rate was defined as the percentage of vaccinees with a HI titer ≥40. Reprinted with permission from Original publication III.

HPAI A(H5) viruses have become enzootic in many countries, infecting wild birds and causing outbreaks in poultry and sporadic infections in mammals, including humans [427]. Since 2014, the subclade 2.3.4.4 has dominated European outbreaks [428]. The persistence of HPAI A(H5) virus in wild bird populations since 2020–2021 suggests these viruses may be endemic also in Europe. Of note, the 2021–2022 epidemic season included the highest number of HPAI cases observed in Europe so far [402]. HPAI A(H5) viruses currently circulating in Europe belong to subclade 2.3.4.4b, which has been connected with a growing number of transmission events of A(H5) viruses to wild mammals, including A(H5N1) virus detection in otter, lynx and two foxes in Finland [402]. Subclade 2.3.4.4b viruses have also been linked to the two latest human infections with a A(H5N1) virus in December 2021 in England [429] and in April 2022 in the USA, emphasizing the continuous risk of avian IAVs to be able to infect humans, and that these viruses may adapt further to mammals [402].

6 Summary/Conclusions

Influenza virus infection is a global One Health concern. Currently circulating human IAVs carry the history of earlier pandemics, which have originated from reassortations with avian, swine and human influenza viruses. Global surveillance of influenza viruses has a key role in monitoring human, animal, and zoonotic infections, updating vaccines, and tracking the emergence of drug-resistant viruses. The genetic analysis of viral sequence data is a key method to determine the evolutionary history and molecular epidemiology of viruses. In addition, by monitoring the genetic as well as antigenic evolution among vaccine and circulating influenza strains the efficacy of vaccines can be estimated (Figure 14).

Vaccines have been the most effective method to mitigate the morbidity and reduce the mortality associated with influenza virus infections. However, also public health measures and travel restrictions effectively suppressed influenza circulation during the COVID-19 pandemic. The goal of vaccinations is to induce protective immunity. However, clinical protection against a severe disease may be a more relevant goal, at least for the risk groups, including the elderly. The vaccine efficacy of seasonal inactivated influenza vaccines is continuously monitored, and it can be improved by the use of vaccines with e.g. higher antigen content or adjuvants.

The adaptive immune response against influenza virus involves antibody and cellular responses at both mucosal and systemic levels. Most antibodies raised by influenza viruses by natural infection or vaccination target the globular head domain of HA, which has an important role in virus entry into cells. Pre-existing neutralizing antibodies induced by previous infections or vaccinations provides one of the first line defenses against influenza infection.

Influenza A(H3N2) virus isolation and characterization of antigenic properties by traditional HI assay has faced several challenges. The MNT instead, is a functional assay, more biologically relevant and likely more sensitive than the HI test, although also more laborious. *In vitro* measured seroprotection of these methods may not be identical with clinical protection, yet suggests a moderate to high probability of clinical protection.

The main conclusions are as follows:

1. The titres of the ELISA-based MNTs were found to correlate well with the titres of a traditional HI test.
2. The MNT titre for a theoretical correlate of protection was subject to variations of the influenza A subtypes and the MNT methods used.
3. Depending on the influenza strain studied, the presence of 20nM OC was confirmed to have a notable influence on the HI assay titres of A(H3N2) viruses due to NA-dependent agglutination of the virus.
4. The theoretical protection against drifted influenza A(H3N2) 3C.2a and 3C.3a virus variants measured by the HI test was suboptimal during influenza season 2014–2015. However, at the same time the theoretical seroprotection against A(H1N1)pdm09 remained at the same level also against drifted viruses.
5. Despite the rapid accumulation of structural changes in the HA after the 2009 pandemic among circulating influenza A(H1N1)pdm09 viruses, the antigenic changes were shown to be negligible for several years.
6. Heterologous pre-pandemic influenza A(H5N1) prime-boost vaccination induced robust and long-lasting cross-reactive neutralizing antibody responses, indicating that priming with one type of A(H5N1) strain may give rise to a much broader protection against novel A(H5N1) virus strains.

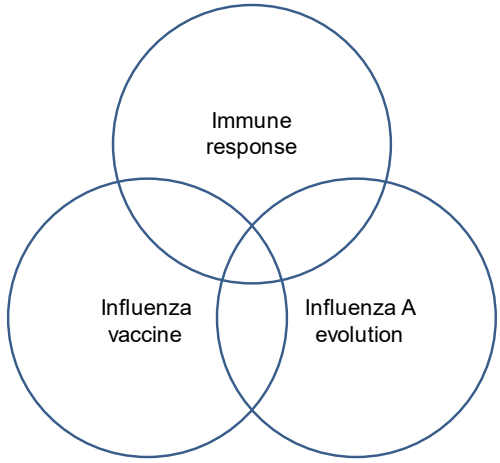

Figure 14. The relationship between influenza A evolution, immune response and influenza vaccine. Author's own drawing.

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Askola, September 2022
Anu Haveri

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