Universität Rostock Traditio et Innovatio

Aus dem Institut für Virusdiagnostik
des Friedrich-Loeffler-Institut (FLI) in Greifswald - Insel Riems
und der Professur für Tiergesundheit und Tierschutz
der Agrar- und Umweltwissenschaftlichen Fakultät

Serodiagnosis of influenza virus and Newcastle disease virus – new molecular approaches

Kumulative Dissertation

zur Erlangung des akademischen Grades
Doktor der Agrarwissenschaften (doctor agriculturae)

an der Agrar- und Umweltwissenschaftlichen Fakultät der Universität Rostock

vorgelegt von Na Zhao aus Qiqihar

Rostock, May 2023

https://doi.org/10.18453/rosdok_id00004359

Dieses Werk ist lizenziert unter einer
Creative Commons Namensnennung - Nicht kommerziell - Keine Bearbeitungen 4.0 International Lizenz.

Gutachter:

Prof. Dr. Helen Louton, Universität Rostock, AUF, Tiergesundheit und Tierschutz

Prof. Dr. Timm Harder, Friedrich-Loeffler-Institut (FLI), Institut für Virusdiagnostik

Prof. Dr. Martin Pfeffer, Universität Leipzig, FB Epidemiologie

Jahr der Einreichung: 2022

Jahr der Verteidigung: 2023

Content

List of Figures ... VI

List of Tables ... VIII

List of Abbreviations ... IX

1 General Introduction ... 1

1.1 Biology of influenza viruses ... 1

1.1.1 Influenza virus overview ... 1

1.1.2 Morphology and genome ... 5

1.2 Biology of Newcastle disease virus ... 10

1.2.1 Newcastle disease virus overview ... 10

1.2.2 Morphology and genome ... 10

1.2.3 Replication ... 13

1.3 Humoral immune response against influenza A virus and Newcastle disease virus ... 14

1.3.1 Overview ... 14

1.3.2 Antibody formation against hemagglutinin, neuraminidase and nucleocapsid proteins of influenza A viruses ... 14

1.3.3 Antibody formation against the hemagglutinin-neuraminidase (HN), fusion (F) and nucleocapsid protein (NP) of NDV ... 15

1.4 Diagnosis of influenza A virus and NDV infections ... 15

1.4.1 Virus isolation ... 16

1.4.2 Serological Assays ... 16

1.4.3 Nucleic acid-based assay ... 17

1.5 Aim of study ... 18

2 Experimental Studies ... 20

2.1 First Study: Distinction of subtype-specific antibodies against European porcine influenza viruses by indirect ELISA based on recombinant hemagglutinin protein fragment-1 ... 21

2.1.1 Introduction ... 23

2.1.2 Material and methods ... 25

2.1.3 Results 28

2.1.4 Discussion 37

2.1.5 Conclusions 39

2.2 Second Study: Engineered recombinant protein products of the avian paramyxovirus type-1 nucleocapsid and phosphoprotein genes for serological diagnosis 40

2.2.1 Introduction 42

2.2.2 Methods 44

2.2.3 Results 47

2.2.4 Discussion 58

2.2.5 Conclusions 60

2.3 Third Study: Tetraplex Fluorescent Microbead-Based Immunoassay for the Serodiagnosis of Newcastle Disease Virus and Avian Influenza Viruses in Poultry Sera 62

2.3.1 Introduction 64

2.3.2 Materials and methods 66

2.3.3 Results 71

2.3.4 Discussion 80

3 General Discussion 83

3.1 New approaches to serodiagnostic investigations for IAV and NDV infections in avian and porcine hosts 83

3.2 Recombinant viral proteins as serological diagnostic reagents: Tailoring for purpose 84

3.3 Recombinant viral hemagglutinin facilitates subtype-specific detection of antibodies against influenza A viruses in swine 85

3.4 Trimming recombinant avian orthoavulavirus nucleoproteins increases specificity of NDV serodiagnosis 87

3.5 Liquid array technology widens the scope for simultaneous detection and discrimination of generic and subtype-specific antibodies against AIV and NDV 89

3.6 Conclusion and outlook 91

References 92

Summary 110

Zusammenfassung 111

Acknowledgements 113

Scientific Curriculum Vitae ···115

List of publications ···116

List of Figures

Figure 1.1 Schematic diagram of the structure of the influenza A virus. 6

Figure 1.2 Influenza virus replication cycle... 9

Figure 1.3 Structure of NDV (Newcastle disease virus) genome and virion. 12

Figure 2.1 Detection of biotinylated recombinant HA1 hemagglutinin and nucleocapsid proteins of European porcine influenza viruses........................... 30

Figure 2.2 Specificity of an indirect ELISA based on recombinant hemagglutinin fragment HA1 of porcine influenza A viruses. 33

Figure 2.3 Development of antibodies against viral nucleocapsid and hemagglutinin fragment HA1 proteins in swine experimentally infected with porcine influenza A viruses. 35

Figure 2.4 Venn diagram of detection of NP (nucleocapsid protein)-specific antibodies by commercial blocking ELISA (hatched ellipsis) and indirect ELISA (recombinant NP, Red ellipsis) in porcine field sera (n = 207) compared with hemagglutination inhibition assays (white ellipsis). 36

Figure 2.5 Comparison of HI (hemagglutinin inhibition) titres measured against porcine avian-derived H1N1 isolate A/swine/Germany/R248/2010 and S/P ratios obtained by indirect ELISA using recombinant HA1 antigen of avian-derived H1N1 isolate A/swine/Germany/R1738/2011. 36

Figure 2.6 Schematic presentation of constructs used for generation of pET19b plasmids and T7- driven expression of NDV (Newcastle disease virus) proteins in Rosettagami *E. coli* cultures. 48

Figure 2.7 Assembled Western blot assays of monobiotinylated recombinant proteins of NDV LaSota.. 50

Figure 2.8 Use of NP and P gene products of APMV-1 and APMV-8 (NP) as diagnostic antigens in indirect ELISA compared to a commercial NDV ELISA. . 52

Figure 2.9 Assembled Western blot assays of monobiotinylated recombinant C-terminal fragments of NP proteins. 53

Figure 2.10 Antibody kinetics in NDV (Newcastle disease virus) vaccinated and challenged chickens against APMV-1 measured by different ELISA formats and HI (hemagglutinin inhibition) assay. 54

Figure 2.11 Venn diagram 223 of detection of NDV (Newcastle disease virus) NP-specific antibodies by ELISAs. 58

Figure 2.12 Schematic presentation of constructs used for the generation of pET19b plasmids and T7-driven expression of NDV (Newcastle disease virus) and AIV (avian influenza virus) proteins in Rosettagami *E. coli* cultures. 67

Figure 2.13 Assembled Western blots of recombinant, in vivo biotinylated HA1 and NP proteins of avian influenza and Newcastle Disease viruses. 72

Figure 2.14 Schematic principle of the Luminex bead-based assay utilized in this study. 73

Figure 2.15 Antibody kinetics in SPF chickens vaccinated against NDV (Newcastle disease virus) as measured by HI (hemagglutinin inhibition), commercial ELISA and 4plex iFMIA assay. 76

Figure 2.16 ROC analyses of the 4plex iFMIA for performance characteristics in sera from experimentally infected and/or vaccinated galliform poultry. 77

List of Tables

Table 2.1 Origin and properties of porcine influenza viruses used in this study for generation of recombinant proteins. 25

Table 2.2 HI (hemagglutinin inhibition) titres of porcine and ferret post infection sera used in Western blotting and indirect ELISA (homologous serum-antigen paris depicted in bold). 31

Table 2.3 Determination of cut-offs of indirect ELISAs based on recombinant NP (Nucleocapsid protein) and HA1 proteins. 32

Table 2.4 Serum avidity in indirect HA1 ELISA. 33

Table 2.5 Results of recombinant indirect ELISAs (iEIA) and HI (hemagglutinin inhibition) or commercial NP (nucleocapsid)-specific ELISA obtained with 207 porcine field sera compared on a holding base. 37

Table 2.6 Serological investigation of chickens challenged with velogenic NDV strain Herts following vaccination with live-attenuated NDV vaccine. 55

Table 2.7 Qualitative results of serological investigations (seropositive/total samples analysed per assay). 56

Table 2.8 Determination of cut-off values of indirect ELISAs based on recombinant avian paramyxovirus NP and 56

Table 2.9 Origin and characteristics of 177 NDV- and AIV-specific sera and 80 SPF sera obtained from chicken and turkeys. 75

Table 2.10 Comparison of performance characteristics (sensitivity, specificity with 95% confidence intervals) of commercial ELISAs and the 4plex iFMIA assay using the hemagglutination inhibition (HI) assay as gold standard. 75

Table 2.11 Performance characteristics of the 4plex iFMIA assay compared to commercial ELISA and HI (hemagglutinin inhibition) assay in 250 sera of field origin. 78

Table 2.12 Positive and negative control sera for assay validation 78

Table 2.13 Qualitative results of serological investigations (seropositive/total samples analysed per assay) 79

List of Abbreviations

4plex	Tetraplex
AGID	Agar gel immunodiffusion
AIV	Avian influenza virus
APMV	Avian paramyxovirus
AOAV	Avian orthoavulavirus
DIVA	Distinguish between infected and vaccinated animals
DMEM	Dulbecco's Minimal Essential Medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
F	Fusion protein
FCS	Fetal calf serum
HA	Haemagglutinin
HI	Hemagglutination inhibition
HN	Hemagglutinin-neuraminidase
HP	Highly pathogenic
HPAI	Highly pathogenic avian influenza
HPAIV	Highly pathogenic avian influenza virus
HRPO	Horseradish peroxidase
IC	Inclusion body
iFMIA	Inhibition fluorescence microsphere assay
L	Large RNA polymerase
LP	Low pathogenic
LPAI	Low pathogenic avian influenza
LPAIV	Low pathogenic avian influenza virus
M1	Matrix 1

M2	Matrix 2
mAb	Monoclonal antibody
MDCK	Madin-Darby canine kidney
mRNA	Messenger ribonucleic acid
NA	Neuraminidase
ND	Newcastle disease
NEP	Nuclear export protein
NP	Nucleoprotein
NS1	Non-structural protein 1
NS2	Non-structural protein 2
OIE	World Organization for Animal Health
ORF	Open reading frame
P	phosphoprotein
PA	Polymerase acidic
PB1	Polymerase basic 1
PB1-F2	Polymerase basic 1 - Frame 2
PB2	Polymerase basic 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RT-PCR	Reverse-transcription polymerase chain reaction
SIV	Swine influenza virus
TBST	Tris-buffered saline with Tween 20
TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone

1

General Introduction

1.1 Biology of influenza viruses

1.1.1 Influenza virus overview

Influenza commonly known as “the flu”, is an infectious disease caused by influenza viruses. Influenza viruses are enveloped RNA viruses belonging to the Orthmyxoviridae family which possess segmented, negative-sense, single-stranded RNA genomes (Cheung & Poon 2007). This family consists of four influenza virus genera (influenza A, influenza B, influenza C and influenza D) that are classified based on differences in their nucleo- (NP) and matrix (M) proteins. Influenza A virus has the broadest host range and can infect humans, pigs, birds, horses, sea mammals and other animals (Cao et al 2017, Ducatez et al 2015, Ferguson et al 2015, Fouchier et al 2005, Harder & Vahlenkamp 2010, Olsen et al 2006, Tong et al 2013, Weber et al 2007, Webster et al 1992). Influenza B virus almost exclusively infects humans. The seal is the only other species confirmed to be susceptible for influenza B virus although swine are suspected to be susceptible as well (Guan et al 2011, Osterhaus et al 2000). Influenza C virus infects humans, pigs and dogs but usually causes mild infections and is less widespread (Guo et al 1983, Manuguerra & Hannoun 1992, Matsubara et al 2004, Youzbashi et al 1996). Influenza D virus was first identified in swine with respiratory disease in 2011 and has subsequently been found in both healthy and sick cattle in France, Italy, United States, Japan and China (Chiapponi et al 2016, Ducatez et al 2015, Ferguson et al 2015, Ferguson et al 2016,

Horimoto et al 2016, Luo et al 2017, Quast et al 2015, Zhai et al 2017). Currently, this virus has not been shown to infect poultry and human beings. Influenza A and influenza B are responsible for human seasonal influenza epizootics (Hsieh et al 2006).

The influenza A viruses are further classified according to the antigenic variations of the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). It is only influenza A virus that is divided into subtypes of 18 known HA (1-18) and 11 known NA (1-11) variants while influenza B, C and D are not divided into subtypes based on the antibody responses (Fouchier et al 2005, Mehle 2014, Tong et al 2012, Tong et al 2013). All known subtypes of influenza A viruses can infect birds, except subtypes H17N10 and H18N11, which have only been found in bats (Mehle 2014, Tong et al 2012, Tong et al 2013). No evidence has been found that the latter two subtypes can naturally infect avian species.

1.1.1.1 Avian influenza virus

The term “avian influenza virus” (AIV) embraces all type A influenza viruses isolated from and adapted to avian host species. There are potentially 144 HA (HA1 to HA16) and NA (NA1 to NA9) subtype combinations but not all have been found in natural reservoir species. Waterfowl including ducks, geese and swans are considered to be the natural reservoir of these subtypes (Taubenberger & Morens 2010). However some subtypes are more common than others in a limited species range, for instance, the HA subtypes H13 and H16 are almost exclusively found in gulls (Munster et al 2007) and the subtype H1 and H3 are more often associated with mammals (H1 and H3 in swine and humans, and H3 in horses and dogs) (Poland et al 2007) although there is no clear association with host range according to the HA subtype. Similarly, virulence is not directly associated with HA or NA subtype in any of the species. Occasionally some viruses can be transmitted to non-natural hosts, and on rare occasions those isolates may evolve into adapted viral lineages in the new host species which may even become a reservoir, as was the case with swine H1N1 and equine H3N8. It is important to note that virus isolation from a species alone does not indicate that it is a natural host or a reservoir species.

According to the severity of disease caused by experimental intravenous infections of chickens, avian influenza viruses can be divided into two groups, the Highly Pathogenic Avian Influenza (HPAI) or Low Pathogenic Avian Influenza (LPAI) viruses. The definition criteria have been harmonized by the World Organization for Animal Health (O.I.E.): In vivo testing requires an avian influenza virus isolate to be inoculated into ten 4-6 weeks old chickens by the intravenous route. In case the mortality rate reaches up to 75% within 10 days or a clinical index of > 1.2 it can be classified as being HPAIV. All other isolated viruses are considered to be LPAIV. The pathogenicity of influenza viruses of the subtypes

H5 and H7 is primarily determined by the HA protein. In particular, presence of a short sequence motif R-X-R/K-R (R-Arginine, K-Lysine, X-any amino acid) at the HA0 endoproteolytic cleavage site has been associated with an HP phenotype (Senne et al 1996, Vey et al 1992). The precursor HA0 should be cleaved by host-produced proteases into two subunits HA1 and HA2 after virus infection, only then become viral particles infectious. The HA0 protein of HPAIV possesses a multi-basic cleavage site (multiple basic amino acids R or K), so that it can be cleaved by a wide range of proteases, including ubiquitous furin-like proteases, and this allows the virus to replicate systemically in various tissues and organs causing severe clinical disease and death due to multiorgan failure. The HA of LPAIV presents a monobasic cleavage site, and infection is restricted to the respiratory and enteric tracts (Klenk et al 1977, Rott 1992) where cleavage occurs generally by trypsin-like proteases that are restricted to the respiratory and gastrointestinal tract of the host. Therefore, the nucleotide sequence at the HA0 cleavage site can be used to distinguish pathotypes, but only so for subtypes H5 and H7. There are only a few viruses isolates which were characterized as HPAIV based on the HA proteolytic cleavage site but caused very mild clinical symptoms in chickens (Lee et al 2005). Similarly, LPAIV can cause severe disease in the field but does have an IVPI >1.2, demonstrating that other factors may have an influence on AIV pathogenicity as well (Bano et al 2003, Nili & Asasi 2002, Woolcock et al 2003). To date, only H5 and H7 subtypes have been associated with HP phenotypes in poultry. Many AIV of H5 and H7 subtypes isolated from gallinaceous birds and waterfowl were LPAIV. However, they bear potential to generate HPAIV by spontaneous mutation at the HA cleavage site once transmitted to chickens or turkey. Therefore all the viruses of H5 and H7 subtypes have been identified as notifiable avian influenza (NAI) viruses due to these risks (Slemons & Swayne 1995). The time to mutation from LP to HPAIV cannot be predicted and may occur within days to weeks following infection of poultry (Pasick et al 2005, Suarez et al 2004), but also has taken months to years (Kawaoka et al 1987, Sturm-Ramirez et al 2004).

LPAIV induces a localized infection resulting in mild disease that consists of respiratory disease and decreased egg production in laying poultry (Alexander 2007, Poland et al 2007). Infection of poultry with HPAI viruses can result in severe acute disease that causes systemic infection with mortality rates reaching 100% in vulnerable species (Capua & Marangon 2007, Jennelle et al 2017). The clinical signs of HPAI virus infection include: cyanosis of unfeather skin, lacrimation, oedema of the head, severe lethargy, and nervous disorders (Webster et al 1992). There were several serious outbreaks of HPAIV in poultry in the past that resulted in devastating losses to the poultry industry and also

caused significant human health issues due to zoonotic propensity of some H5 and H7 HPAIV including the risk of generating a new pandemic in human (Alexander & Brown 2009, Krammer 2019, Subbarao 2018, Sutton 2018).

1.1.1.2 Swine influenza overview

Although wild aquatic birds are the natural reservoir of influenza A viruses, mammalian hosts including swine maintain genetic lineages of IAV as well. Swine express two common receptors to which both human and avian influenza viruses are able to bind. This facilitates bidirectional virus transmission and fosters adaptation to different hosts (Baudon et al 2017, Ma et al 2008). When swine were infected simultaneously with both human and avian influenza viruses, novel reassortant influenza viruses can be generated by reassortment of influenza viral genome segments (Ma et al 2009). Swine influenza virus (SIV) are influenza A viruses that are endemic in pigs and express distinguishable genetic and antigenic features. The first SIV has been isolated from swine by Shope in 1930 who found the virus that caused respiratory disease in pigs to be similar to human influenza (Shope 1931). The subtypes of SIV that are most frequently identified in pigs include classical H1N1, H1N2 and H3N2 (Choi et al 2004, Marozin et al 2002, Olsen et al 2002). Other subtypes that have been identified at rare occasions include reassortant rH1N7, H2N3, rH3N1, avian (av) H3N3, H4N6av, avH5N1 and H9N2av (Brown et al 1997, Kothalawala et al 2006). In the 1970s, human H3N2 influenza viruses were first transmitted to swine in Italy, and subsequently avian H1N1 influenza virus was transmitted to swine and by then these viruses became adapted in swine in Europe (Brown et al 1998, Karasin et al 2002, Kothalawala et al 2006, Scholtissek et al 1993, Sheerar et al 1989, Webby et al 2000, Webster 2002).

Swine influenza is an economically important disease of pigs. Economic losses are caused by retarded growth of fattening pigs due to influenza-induced or -aggravated respiratory disease. In addition, febrile influenza virus infections in sows may cause fertility problems (Torremorell et al 2012). Influenza viruses are an important factor in the polymicrobial respiratory syndrome of swine (Opriessnig et al 2011). In contrast to human influenza, infections in swine do not appear to be seasonal, and virus circulation, especially in larger herds, is observed year-round (Kyriakis et al 2013).

Moreover, swine influenza has been reported several times as a zoonotic infection in humans. These outbreaks and pandemics include the human Spanish flu pandemic in 1918 which was associated with H1N1, and it is estimated that 50-100 million people were killed worldwide (Patterson & Pyle 1991, Taubenberger & Morens 2006). The H1N1 virus

of the United States outbreak in 1976 were transmitted from pigs to humans and killed a person, but did not trigger a pandemic. The 2009 swine flu in America, in contrast, became pandemic and was caused by a new strain of H1N1 virus that was generated by triple reassortment of swine, human and avian influenza viruses (Schultz-Cherry et al 2013).

1.1.2 Morphology and genome

The influenza virus is an enveloped, single-stranded negative sense RNA virus, surrounded by a lipid bilayer which is derived from the host cell membrane during the viral releasing process. The viral genome is comprised of eight single-stranded RNA segments located inside the virus particle (Figure 1.1). Laboratory isolated strains of influenza are roughly pleomorphic to spherical ranging from 80 to 120 nm in diameter (Horimoto & Kawaoka 2005, Lamb & Choppin 1983).

The influenza viral surface consists of the glycoproteins hemagglutinin (HA) and neuraminidase (NA) which are presented as spikes. The surface protein HA and NA are anchored in the lipid bilayer at a ratio of four to one. The two proteins are encoded by RNA segment 4 and 6, respectively. HA protein can bind to sialic acid-containing receptors and mediates viral entry. NA protein is responsible for releasing viruses bound to non-functional receptors and helping viral spread. The matrix 2 (M2, encoded by segment 7) proteins also are presented at the viral surface and are embedded in the lipid membrane. The M2 protein forms an ion channel, which can be the target of antiviral drugs. Beneath the lipid membrane is the viral matrix 1 (M1, also encoded by segment 7) protein which forms a shell to provide rigidity for the lipid envelop.

The influenza virus interior, the virion core, comprises of the ribonucleoprotein (RNP) complex. The RNP complex is composed of eight genomic viral RNA segments covered with nucleoprotein (NP, encoded by segment 5) and the RNA-dependent-RNA polymerase (RdRp) comprised of two polymerases basic (PB1 and PB2) as well as one polymerase acidic (PA) subunit. The PB1, PB2 and PA proteins are encoded by three largest RNA segments, which are responsible for RNA synthesis and replication in infected cells.

Finally, the non-structural protein NS1 encoded by segment 8 is a virulence factor that inhibits host antiviral responses in infected cells. Apart from NS-1, the influenza virus can also produce further accessory proteins such as NEP (nuclear export protein or NS-2), PB1–F2 and PA-x which are not present in the virions. These non-structural proteins play a key role in preventing host innate antiviral responses (Krammer et al 2018).

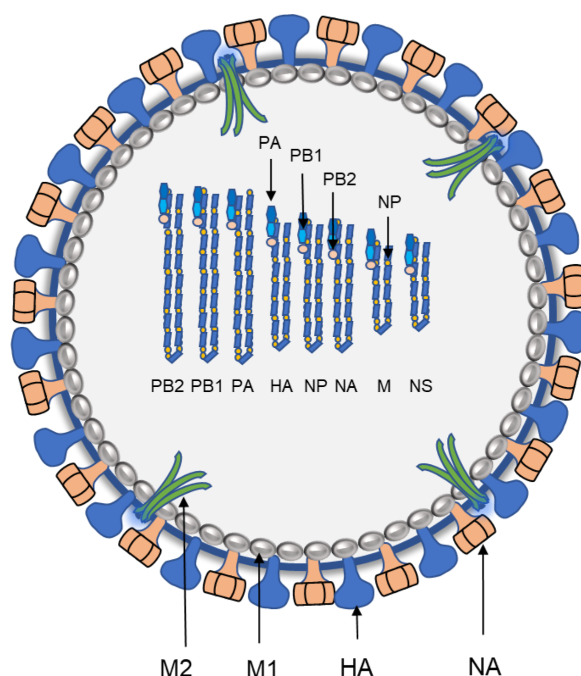


Figure 1.1 Schematic diagram of the structure of the influenza A virus. The figure represents an influenza A virus particle. Both influenza A and influenza B viruses are enveloped negative- sense RNA viruses with genomes comprising eight single- stranded RNA segments located inside the virus particle. These are the RNA polymerase subunits polymerase basic protein (PB2), polymerase basic protein 1 (PB1) and polymerase acidic protein (PA) which form the polymerase complex. The viral RNA genome is coated by the nucleocapsid protein (NP). Two RNA segments encode the viral glycoproteins haemagglutinin (HA), which mediates binding to sialic acid- containing receptors and viral entry, and neuraminidase (NA), which is responsible for releasing viruses bound to non-functional receptors and helping viral spread; the HA has distinct globular 'head' and 'stalk' structures, and also is the major immunogenic protein. RNA segments 6 and 8 encode more than one protein, namely, the matrix protein (M1) and membrane protein (M2) and the nonstructural protein (NS1) and nuclear export protein (NEP). M1 is thought to form a shell to provide rigidity for the lipid envelop and that, together with NEP, regulates the trafficking of the viral RNA segments in the cell; The M2 protein forms an ion channel, which can be the target of antiviral drugs; the NS1 protein is a virulence factor that inhibits host antiviral responses in infected cells (Krammer et al 2018).

1.1.2.1 Nucleoprotein

The NP of influenza A virus is a structural protein containing 498 amino acids in length which is abundant in arginine, glycine and serine residues. Thus, this protein has a net positive charge at neutral pH that can bind the negatively charged viral RNA segments with high affinity (Digard et al 1999). The NP is highly conserved with less than 11% amino acid difference in virus strains isolated form different hosts (Shu et al 1993), therefore it can be used to distinguish influenza virus from other pathogens. In addition, NP plays important roles including RNA synthesis (Elton et al 1999, Medcalf et al 1999), NP- viral

RNA polymerase interactions (Naffakh et al 2000), NP- M1 interactions (Ye et al 1999), as well as RNP export (Portela & Digard 2002).

1.1.2.2 The surface glycoproteins Hemagglutinin and Neuraminidase

The hemagglutinin, presented on the viral surface, is an integral membrane glycoprotein which is able to agglutinate erythrocytes (Sriwilaijaroen & Suzuki 2012). HA is a trimer of identical subunits which are linked non-covalently. The HA monomer consists of two polypeptides resulting from proteolytic cleavage of a HA precursor named HA0. The length of HA0 is approximated 560 amino acid residues (Lamb & Choppin 1983). It can be post translationally cleaved into two subunits HA1 (head) and HA2 (stalk) by host-origin trypsin-like proteases at a conserved arginine residue. The two HA subunits are held together by a disulphide bond. The cleavage of HA0 is required for virus infectivity and the spread of infection in the host. This process has been recognized as the most important determinant of pathogenicity (Liu et al 2014, Steinhauer 1999, Takahashi & Suzuki 2015). HA1 holds the globular head domain and contains the N-terminal portion of HA. HA2 holds the hydrophobic region of the C-terminal portion of HA with a stalk domain and the membrane anchor.

Sialic acid is an acidic sugar with a nine-carbon backbone that presents on the cell surface. It has been found to serve as receptor of many viruses such as adenoviruses, rotaviruses, and influenza viruses. HA1 contains the sialic acid binding site, which is responsible for binding virus to sialic acid receptors at the target cell surface causing the virus to attach. After that, the viral particles are endocytosed and transported within the endosome along microtubules. HA2 holds a fusion peptide at its N-terminus, which is hidden in the HA structure. After endocytosis of viral particles, the low pH of the endolysosomes renders the structure unfolded to expose the fusion peptide. This conformational change triggers the membrane fusion process between the viral envelope and the endosomal membrane, and the viral genetic information is released into the cytoplasm. The major antigenic epitopes are determined by HA (Hefferon 2014, Lee & Saif 2009). The sequence conservation of HA1 (34-59%) is lower than that of the HA2 (51-80%) subunit domain, thus HA1 expresses much more antigenic variation compared to HA2.

Neuraminidase (NA) protein is an integral membrane glycoprotein which is presented on the virion surface. It is a homo-tetramer of mushroom shape. NA is also a minor antigenic molecule that undergoes antigenic variation (Shtyrya et al 2009, Sylte & Suarez 2009). The functional role of NA is to remove sialic acid from glycoproteins. The removal of sialic acid from viral proteins plays a key role in both viral attachment and viral release from the

host cell (Matrosovich et al 2004). The antiviral drugs, Tamiflu and Relenza, exploit this essential function of the NA to prevent virus spread from one cell to another (Mitnaul et al 1996).

1.1.2.3 Antigen drift and antigen shift

Antibodies conferring protection against infection directly interact with the viral surface glycoproteins to inhibit viral attachment. Influenza viruses evade antibody detection via frequent amino acid variation at the antigenic sites mainly within HA. These small changes can be accumulated over time, leading to antigenic variation. This phenomenon was defined as antigenic drift. The high mutation ratio can lead to viral escape from adaptive host immunity and antiviral drugs. Antigenic drift of the HA protein in human seasonal influenza viruses requires frequent updating of vaccines to match new antigenic variants (Neumann et al 2009).

Another type of antigenic change is called “antigen shift”. The segmented nature of the viral genome allows for reassortment. New reassortants may acquire the HA or NA segment from another influenza A subtype. Reassortment can increase genetic diversity rapidly, resulting in abrupt and major antigenic changes which may lead to pandemic spread of human-adapted viruses (Bouvier & Palese 2008, Carrat & Flahault 2007, Treanor 2004). Antigenic shift triggered the 1957 (H2N2) and 1968 (H3N2) pandemic outbreaks and the swine origin 2009 pandemic (H1N1).

1.1.2.4 Influenza virus replication

The influenza viruses enter susceptible cells through HA binding to sialic acid residues on glycoproteins or glycolipid receptors at the host cell surface (Figure 1.2). Bound virions are engulfed into endosomes. Acidification in the endolysomal compartment triggers HA1 conformational change, then exposing the fusion peptide to fuse the viral envelop and the endosomal membrane. This process results in release of the viral RNPs into the cytoplasm (Rossman & Lamb 2011). Meanwhile, the viral M2 protein functions as an ion channel to pump hydrogen ions from the endosome into the virion (Bouvier & Palese 2008, Cheung & Poon 2007).

Once released, the RNPs are transported to the cell nucleus by nuclear localisation signals presented on RNP complex proteins, where the RdRp can use the negative-sense viral RNA as a template to synthesize mRNA and, in later phases also antigenomic positive-sense RNA (Figure 1.2, steps 3a and b) (Cros & Palese 2003) as the templates to transcribe more copies of viral RNA for the genomes of progeny viruses (Kash et al

2006). The newly synthesized viral proteins, HA, NA and M2, are secreted on to the cell surface, whereas some proteins (PA, NP, PB1 and PB2) are transported back into the cell nucleus to bind viral RNA and form new viral RNP (Figure 1.2, step 7). M1 protein can inhibit nuclear import and promote nuclear export through interacting with viral RNP, while NEP is responsible for export of the RNP complex directly (Boulo et al 2007, Cheung & Poon 2007, Martin & Helenius 1991, Noda & Kawaoka 2010).

Eight viral RNA segments are packaged properly depending on packaging signals on all viral RNA segments to ensure a full genome is incorporated into the majority of virus particles (Cros & Palese 2003, Noda & Kawaoka 2010). The cytoplasmic domains of HA and NA interact with M1 to stimulate an outward bulging of the plasma membrane, resulting in membrane scission to release the virion. This process is called budding (Boulo et al 2007, Cros & Palese 2003, Noda & Kawaoka 2010, Rossman & Lamb 2011).

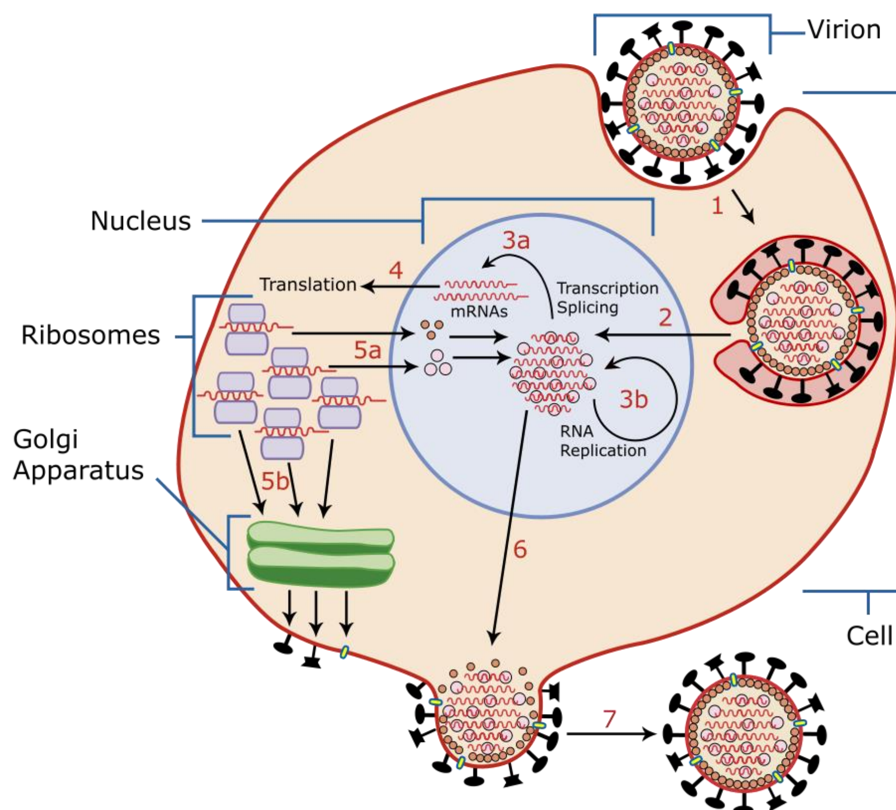


Figure 1.2 Influenza virus replication cycle.

Step 1: Binding; Step 2: Entry; Step 3: Complex formation and transcription; Step 4: Translation Step; 5: Secretion; Step 6: Assembly; Step 7: Release. This picture is taken from Wikipedia (Times 2007).

1.2 Biology of Newcastle disease virus

1.2.1 Newcastle disease virus overview

Newcastle disease virus (NDV) which designation has been changed from Avian paramyxovirus 1 (APMV-1) to Viruses of genus *Avian orthoavulavirus 1* (AOAV-1) according to a new nomenclature can cause Newcastle disease in poultry. NDV has been isolated from many species of domestic and wild bird. Its strains are divided into three pathotypes: velogenic (highly virulent), mesogenic (intermediate virulence) and lentogenic (nonvirulent). Highly virulent (velogenic) NDV strains can lead to high rates of morbidity and mortality, with huge economic losses to the poultry industries (Marks et al 2014, Umali et al 2014). Therefore, Newcastle disease (ND) together with HPAI are the two poultry diseases notifiable to OIE.

The clinical signs of ND caused by velogenic strains include respiratory distress, digestive and central nervous system symptoms. It spreads rapidly in poultry flocks and can causes up to 90% mortality. ND caused by mesogenic presents with depression, coughing, weakness and drop in egg production. Lentogenic strains do not induce clinical signs in healthy birds (Alexander 2000, Gogoi et al 2017, Hines & Miller 2012). The clinical signs of ND are not specific but are frequently seen also with other respiratory virus infections such as influenza, laryngotracheitis virus, or infectious bronchitis virus (Kapczynski et al 2013). Diagnosing the proper pathogen of infection is still a difficult issue on clinical basis.

Chickens are highly susceptible to NDV infection. Ducks and geese are also susceptible but severe disease is more rarely induced. Lentogenic viruses from ducks potentially mutate into velogenic viruses by duck to chicken transmission in nature (Lee et al 2009). Humans can be infected by NDV but no-life threatening disease is provoked (A. Ahad 2013). Instead, mild conjunctivitis, and influenza-like symptoms may be seen; human to human transmission has not been reported.

Vaccination for ND is widely practiced for poultry. Modified-live lentogenic vaccines, based on LaSota and B1 strains, are broadly used by mass application in drinking water or by spraying, which can be an effective strategy in the face of outbreaks (Kapczynski et al 2013).

1.2.2 Morphology and genome

NDV is a member of the genus Orthoavulavirus in the family Paramyxoviridae. It is an enveloped virus, containing a negative-sense, single stranded, non-segmented RNA

genome surrounded by a lipid bilayer derived from the host cell membrane (Alexander 2000) (Figure 1.3). The genome of NDV encodes six structural proteins in the order from 3' to 5': nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and the large RNA polymerase (L) (Hamaguchi et al 1983). Furthermore, two extra proteins, V and W, are expressed by mRNAs, which are derived from the P gene by RNA editing (Samson et al 1991, Steward et al 1995, Steward et al 1993). When one or two non-templated guanine (G) residue are inserted at a conserved editing site within the mRNA of P gene, the consequence is that the reading frames is changed during translation, producing V or W protein respectively (Steward et al 1993) According to this translational pattern, the P, V and W proteins possess a common N-terminal domain and unique C- terminal domains.

V protein is considered as an effector for IFN antagonism. It can efficiently inhibit the host's innate immune system via reducing interferon activity and apoptosis (Chambers & Samson 1982, Collins et al 1982, Huang et al 2003, Park et al 2003) The function of W protein is still less known (Huang et al 2003).

The HN and F are glycoproteins anchored in the lipid bilayer of the virus particle. The HN is a homo-tetrameric protein and forms spiked projections at the surface of the virion: HN has both hemagglutination and neuraminidase activities. It binds to sialic acid host cell receptors and is able to agglutinate red blood cells (RBCs). The important role of HN is to remove sialic acid from host cell receptors and virion proteins to facilitate release of virus progeny from infected cells (Nagai et al 1976, Porotto et al 2012).

The F protein is a trimeric protein and involved in fusing membranes of the virion and the host cell, as well as between two host cells to facilitate cell-to-cell spread (Lamb et al 2006). The F protein is synthesized as an inactive precursor F0, that is subsequently cleaved by host proteases into F1 and F2 subunits, which remain linked together by a disulphide bond. Properly cleaved F0 protein is a prerequisite of virion infectivity. The avirulent NDV pathotype expresses a single basic amino acid at the cleavage site for access to exogenous trypsin-like proteases of the host from the respiratory or gastrointestinal tract (Rott 1979). Virulent pathotypes, in contrast, have multiple basic amino acids at the cleavage sites which can be processed by ubiquitous proteases of the furin type. Therefore, the sequence of the F protein cleavage site is a major determinant of NDV pathogenicity in chickens (Bergfeld et al 2017, Ji et al 2017, Kim et al 2017, Mahon et al 2011).

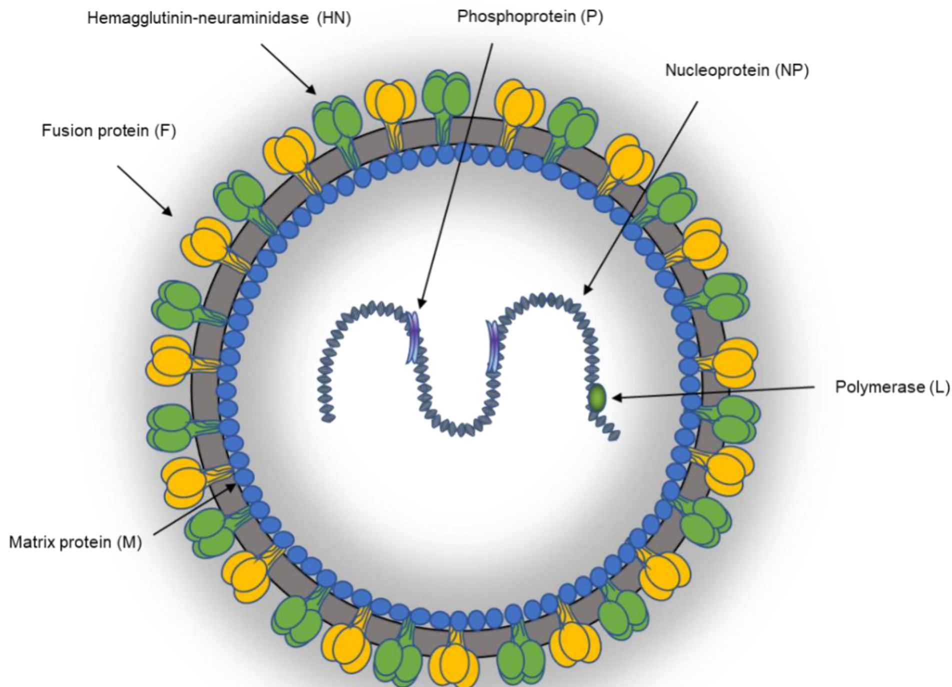


Figure 1.3 Structure of NDV (Newcastle disease virus) genome and virion. The NDV genome is a non-segmented, single strand, negative-sense RNA, approximately 15 kb in length. It contains six genes, encoding at least 7 to 8 proteins. They are capsid protein (NP), phosphoprotein (P), matrix protein (M), fusion proteins (F), hemagglutinin neuraminidase (HN), and large RNA dependent polymerase protein (L). The NP protein is a highly conserved and the most abundant viral protein expressed in infected cells, and induces a strong humoral (non-neutralizing) and cellular immune response in the infected host and also following vaccination with inactivated virus. The P protein plays an important role in the viral transcription and replication, and it is associated with the nucleocapsid in the virion. Two additional proteins, V and putative W, are predicted to be produced from P gene by mRNA editing post transcription. The product that ensues by insertion into the nascent P mRNA of one non-template G residue at position 401 (+2 reading frame) is referred to as the V protein. Addition of two untemplated G's at the polymerase slipping point of the P gene would generate a third protein species, the W protein, from the P gene. The M protein is located at the inner viral envelope surface to support the structure of the virion; the F protein is a trimeric protein and involved in fusing membranes of the virion and the host cell, as well as between two host cells to facilitate cell-to-cell spread; the HN is a homo-tetrameric protein and forms spiked projections at the surface of the virion, and it binds to sialic acid host cell receptors and is able to agglutinate red blood cells (RBCs). The important role of HN is to remove sialic acid from host cell receptors and virion proteins to facilitate release of virus progeny from infected cells.

The M protein is located at the inner viral envelope surface to support the structure of the virion (Kai et al 2015). The NP, P and L proteins form the virus ribonucleoprotein (RNP) complex, also called nucleocapsid. The NP protein is the most abundant viral protein and is involved in controlling genomic replication. The P protein is engaged in virus replication and transcription (Qiu et al 2016). The L protein is the least abundant viral protein and represents the viral RdRp associated with methylation, capping, phosphorylation and polyadenylation activities of RNA (Cheng et al 2016, Rout & Samal 2008, Yu et al 2017).

1.2.3 Replication

NDV replication events are confined to the host cell cytoplasm. First, the viral glycoprotein HN attaches to the sialic acid receptors on the host cell surface. After attachment, the F glycoprotein fuses the viral envelope with the cellular plasma membrane. Fusion events result in pore formation allowing the viral helical nucleocapsid to enter the cytoplasm of the host cell (Deng et al 1999, Morrison 2003, Smith et al 2009, Stone-Hulslander & Morrison 1997, Tong & Compans 1999a). After penetration, the P and L proteins initiate transcription and replication of the viral genome RNA template (Peeters et al 2000).

Three different types of RNA are generated during NDV replication, termed genomic RNA, full length antigenomic RNA and mRNA. The newly translational NP, P and L proteins together with replicated genomic RNA assemble into nucleocapsid complexes in the cytoplasm. The other proteins, HN and F, are synthesized and processed in the exocytic pathway and are transported to the cell membrane for virion formation. The mechanism of viral glycoprotein folding and conformational maturing is complicated and requires numerous enzymes and the aid of molecular chaperones.

The M protein plays a key role in recruiting the assembled nucleocapsid complex to the proper region on the cell membrane. It specifically interacts with the cytoplasmic tails of the F and HN glycoproteins which are critical for proper virion assembly and budding. The newly assembled viral particles are released by budding via the infected cell membrane which forms the new viral envelope (Bissonnette et al 2009, Morrison 2003, Tong & Compans 1999b).

1.3 Humoral immune response against influenza A virus and Newcastle disease virus

1.3.1 Overview

Influenza A virus and Newcastle disease virus usually infect their hosts via the respiratory and the intestinal tracts (A. Ahad 2013, Baigent & McCauley 2003). Within hours after initial infection, the immune system of infected hosts triggers a series of events by its innate arm to limit viral replication and eliminate the virus. This is the first barrier against invading pathogens. When the innate host defences have been bypassed, evaded or overwhelmed, an adaptive immune response is required and triggered (Iwasaki & Pillai 2014).

The adaptive immune response is based on antigen-specific humoral and cellular immunity. Upon an encounter with viral antigen, in combination with $CD4^{+}$ T cells, B lymphocytes are activated to secrete viral antigen-specific antibodies. Antibodies that specifically recognize and bind to the antigens on the viral surface circulate in the bloodstream as well as permeate to other body fluids, and, depending on the Ig class, may reach also epithelial surfaces. The interaction of antibodies and viral surface antigens blocks viral entry, thereby inhibiting viral infectivity. Thus, viral specific antibodies play a key role to prevent virus infection (Abdallah & Hassanin 2015, Erf 2004, Moser & Leo 2010, Swayne & Kapczynski 2008).

Specific antibodies first appear approximately from six days after infection or vaccination. In the early phase, IgM is produced which is followed by IgG (mammalian) or IgY (avian). The peak of titer and protection is detected at three weeks after infection or vaccination, then it gradually decreases if there is no reinfection or boost (Abdallah & Hassanin 2015, Hill et al 2016).

1.3.2 Antibody formation against hemagglutinin, neuraminidase and nucleocapsid proteins of influenza A viruses

In the course of an influenza A virus infection, the humoral immune system produces antibodies specifically against HA, NA, NP and M proteins (DiLillo et al 2014). Antibodies against the HA protein contribute to virus neutralization as the hemagglutinin is the major viral surface protein and essential to the entry process by binding to receptors on the target cells (Brandenburg et al 2013, Hamilton et al 2012, Oh et al 2010). The HA1 subunit forms a large, membrane distal, globular head domain and harbours the immune-

dominant antigenic regions recognized by neutralizing antibodies which interfere with receptor binding (Gerhard et al 1981, Wiley & Skehel 1987). The HA2 subunit forms a stalk domain that is anchored in the viral membrane; in contrast to HA1, HA2 is highly conserved among the different subtypes of influenza A viruses.

Antibodies against NA also possess protective potential to a certain degree by interfering with NA's enzymatic activity (Kreijtz et al 2011, Mozdzanowska et al 1999). However, such NA specific antibodies do not neutralize virus infectivity directly but inhibit newly formed virus particles spread (Kreijtz et al 2011). Additionally, the NA specific antibodies may induce antibody-dependent cell-mediated cytotoxicity (ADCC) to eliminate virus-infected cells (Mozdzanowska et al 1999).

NP is a highly conserved protein of influenza A virus strains (Portela & Digard 2002, Shu et al 1993). It is a core antigen located within viral particles as well as in nuclei of infected cells. It is highly immunogenic but NP-specific antibodies are not protective. The NP is widely used as the target in ELISA format for both virus and antibody detection.

1.3.3 Antibody formation against the hemagglutinin-neuraminidase (HN), fusion (F) and nucleocapsid protein (NP) of NDV

The cell surface glycoproteins HN and F are both important for virus infectivity and pathogenicity (Kumar et al 2011), and both can induce NDV-specific neutralizing antibodies in infected animals (Chumbe et al 2017). NP protein accumulates in cytoplasm of the infected cells (Collins & Hightower 1982), and as it is part of the interior of the virion particle is not exposed to immunological pressure compared to viral surface glycoproteins HN and F. Antibodies against NDV NP neither neutralize infectivity nor protect from infection (Al-Garib et al 2003, Rabu et al 2002). Furthermore, NP protein is also a relatively conserved protein of NDV, especially the globular amino-terminal part (about 80% of the whole protein) while the carboxy-terminal domain harbours a highly variable region (Ye et al 1999). Based on this, NP is widely used in commercial kits for serodiagnosis of NDV infection or to control the effect of vaccination.

1.4 Diagnosis of influenza A virus and NDV infections

The clinical symptoms and pathological manifestations may help clinicians in their diagnosis of infectious diseases but often these signs are insufficient for a definitive etiological diagnosis. For effective and etiologic disease diagnosis and surveillance, a number of diagnostic techniques have been developed for influenza A virus and NDV in

animals or humans. The most common diagnostic approaches focus on virus isolation, molecular diagnostic tests, and serology.

1.4.1 Virus isolation

Influenza A and ND viruses can be isolated from excretions and tissues of infected hosts. The clinical specimens are inoculated into specific pathogen-free (SPF) embryonated chicken eggs or selected avian and mammalian cell lines. For up to five days inoculated eggs or cells are monitored daily for embryo mortality or cytopathic effect (CPE) (Spackman 2014). The amnio-allantoic fluid (AAF) from embryos or cell culture supernatants are harvested for detecting virus infection by the hemagglutination (HA) assay for final confirmation. In previous years, virus isolation was considered a gold standard for the diagnosis of influenza A and ND virus infections (Vemula et al 2016).

1.4.2 Serological Assays

1.4.2.1 Hemagglutination assay and hemagglutination inhibition assay

The influenza A hemagglutinin glycoprotein as well as the NDV HN protein, presented on the viral surface, are able to bind avian red blood cells (RBCs) resulting in hemagglutination. In the HA assay, harvested AAF or cell culture supernatants are serially diluted two-fold in a U-bottom 96 well plate, RBCs are added to incubate for approximately 30 min. HA is verified by the absence of formation of a teardrop pattern of sedimented RBCs in the tilted plate. As all HA subtypes of IAV, NDV serotypes, several other virus species and even some bacteria can agglutinate RBCs, this is a non-specific assay which sometimes leads to false positive result (Zeynalova et al 2015).

The hemagglutination inhibition (HI) assay is based on the ability of HA- or HN-specific antibodies to prevent the agglutination of RBCs by viral antigen since these antibodies sterically block the proteins' receptor binding pockets. This assay is used to detect (i) NDV-specific antibodies, (ii) the specificity of antibodies to various influenza A virus HA subtypes as well as to (iii) confirm the HA subtype of influenza A virus isolates by a panel of the 16 distinct standard antisera (Jeeninga et al 2006, Manual 2012, OIE 2012).

1.4.2.2 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a widely used serological techniques especially cherished for its high-throughput features which enables automated testing of thousands of serum samples in a short time. Currently, numerous commercial kits are available for antibody detection against influenza as well as ND viruses.

A number of those kits are based on an indirect ELISA format (Woolcock & Cardona 2005). The ELISA is performed in a 96-well flat-bottomed plate coated with purified viral antigen. Adding the sample containing anti-AIV or NDV antibodies achieves antigen-antibody binding; a second, species-specific anti Ig enzyme-linked antibody is added to the well. Extra or unbound reagents can be removed by each washing step. Finally, enzymatic substrate is added to generate a visible signal which can be measured colorimetrically.

Alternatively, competitive/blocking ELISA formats are available. Antigen in competitive/blocking ELISA is bound to the plate similar to indirect ELISA, however, an enzyme-linked monoclonal antibody binding to the target antigen and competing with antibody from the sample serum is used as a secondary antibody. This approach is species-independent compared to the indirect ELISA which requires species-specific secondary antibodies (Chiu et al 2012, Das & Kumar 2015, Jin et al 2004, Kodihalli et al 1993, Zhang et al 2006, Zhao et al 2013, Zhou et al 1998).

As with any diagnostic assay, the quality of specimens is extremely important to achieve accurate and reliable results. The ELISA tests are compatible with several sample types including sera, plasma, and egg yolk, which have been demonstrated to be effective for the viral specific antibody detection (Spackman 2008).

1.4.3 Nucleic acid-based assay

In the past decades, nucleic acid testing based on polymerase chain reaction (PCR) techniques have been broadly used for virus genome detection in routine surveillance, outbreak assessment and scientific research. This approach detects virus-specific DNA or RNA sequences via PCR amplification instead of viral antigens or infectivity that were targeted by more classical methods. PCR techniques feature high sensitivity, high specificity, and faster detection in clinical samples compared to the classical methods such as HA or HI test.

Reverse-transcription-PCR (RT-PCR) nowadays has become the primary and most reliable application to detect AIV and NDV by targeting relatively conserved matrix (M) or nucleoprotein (NP) gene segments (Berinstein et al 1999, Berinstein et al 2001, Di Trani et al 2006, Lee et al 2001, Munch et al 2001). In conventional RT-PCR, the product is analyzed by gel electrophoresis. Real time RT-PCR (rRT-PCR) takes advantage of fluorescence emittance during the reaction in real time, which is generated by hydrolysis of a specific probe that binds to the amplified target sequence. Hydrolysis is achieved by the exonuclease domain of the Taq polymerase. Use of fluorescent probes eliminates the contamination-prone and insensitive gel electrophoresis step of conventional RT-PCR

and adds a further level of specificity. The advantages of rRT-PCR are its high reproducibility (Abdelwhab el et al 2010, Di Trani et al 2006), 10-100 times increased sensitivity and lowered cross-contamination risks compared to conventional RT-PCR (Di Trani et al 2006). rRT-PCR is also widely used to detect and differentiate AIV subtypes such as the notifiable H5 or H7 (Di Trani et al 2006, Hoffmann et al 2007, Li et al 2008, Munch et al 2001, Sidoti et al 2010, Slomka et al 2007b, Spackman et al 2008, Spackman et al 2003, Spackman et al 2002, Starick et al 2000). Furthermore, the virulence of NDV can be identified via examining by rRT-PCR the F gene cleavage site (Berinstein et al 2001). Thus, rRT-PCRs applications are now considered as routine methods for surveillance, sub- and pathotype characterization of AIV and NDV (Arafa et al 2010, Slomka et al 2007a).

Multiplex-RT-PCR refers to using multiple primers (and probes, in case of rRT-PCRs) to perform PCR amplification for several targets in a single reaction simultaneously, which produces amplicons different sizes (for conventional PCRs) and specificity (for probe detection in rRT-PCR). This technique has been developed and used for detection and differentiation of AIV, NDV and other poultry respiratory pathogens (Rashid et al 2009), as well as for discrimination of subtypes of AIV (Chaharaein et al 2009, Choi et al 2013, Li et al 2008, Saberfar et al 2007, Stockton et al 1998, Xie et al 2006). This approach offers a rapid, accurate screening for viral infectious disease. However， complexity is increased compared to conventional RT-PCR system, and it is more expensive if using two or three fluorescent labelled probes.

1.5 Aim of study

The task of this thesis firstly is to establish modern ELISA systems for improved detection of serological responses to influenza A virus and NDV infections in animals. While virological surveillance in general has benefited greatly from newly designed multiplex real time RT-PCR techniques, serological techniques seem to lack behind.

Here, a number of assays are designed for detection of generic and subtype-specific antibodies against European swine influenza viruses which improve the serological subtype- and lineage-specific antibody differentiation in influenza virus infected swine using liquid multiplexed assays (experimental study 2.1). Secondly, using less conserved portions of the NDV nucleocapsid (NP) and phosphor (P) proteins as antigens serotype-specific antibodies in avian species can be detected in an indirect ELISA. Additionally, we also studied the possibility of the V protein as a target antigen in the indirect format to

distinguish between infected and vaccinated animals (DIVA) strategy (experimental study 2.2). The last part is dedicated to establish for use in avian species rapid, multiple liquid multiplexed serological assays for distinguishing NDV and AIV infection, as well as the subtype H5 and H7 simultaneously (experimental study 2.3).

2

Experimental Studies

2.1 First Study: Distinction of subtype-specific antibodies against European porcine influenza viruses by indirect ELISA based on recombinant hemagglutinin protein fragment-1

Na Zhao[1], Elke Lange[2], Sybille Kubald[3], Christian Grund[1], Martin Beer[1] and Timm C Harder[1]

[1] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Suedufer 10, Greifswald 17493, Germany

[2] Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Suedufer 10, Greifswald 17493, Germany.

[3] Institute of Immunology, Friedrich-Loeffler-Institut, Suedufer 10, Greifswald 17493, Germany.

Correspondence: timm.harder@fli.de

This article has been published in Virol J. 2013 Jul 30;10:246. doi: 10.1186/1743-422X-10-246

This is an Open Access article distributed under the terms of the Creative Commons Attribution License
Received: 2 April 2013 Accepted: 22 July 2013 Published: 30 July 2013

Abstract

Serological investigations of swine influenza virus infections and epidemiological conclusions thereof are challenging due to the complex and regionally variable pattern of co-circulating viral subtypes and lineages and varying vaccination regimes. Detection of subtype-specific antibodies currently depends on hemagglutination inhibition (HI) assays which are difficult to standardize and unsuitable for large scale investigations. The nucleocapsid protein (NP) and HA1 fragments of the hemagglutinin protein (HA) of five different lineages (H1N1av, H1N1pdm, H1pdmN2, H1N2, H3N2) of swine influenza viruses were bacterially expressed and used as diagnostic antigens in indirect ELISA. Proteins were co-translationally mono-biotinylated and refolded in vitro into an antigenically authentic conformation. Western blotting and indirect ELISA revealed highly subtype-specific antigenic characteristics of the recombinant HA1 proteins although some cross reactivity especially among antigens of the H1 subtype were evident. Discrimination of antibodies directed against four swine influenza virus subtypes co-circulating in Germany was feasible using the indirect ELISA format. Bacterially expressed recombinant NP and HA1 swine influenza virus proteins served as antigens in indirect ELISAs and provided an alternative to commercial blocking NP ELISA and HI assays concerning generic (NP-specific) and HA subtype-specific sero-diagnostics, respectively, on a herd basis.

Keywords: Pandemic influenza, Swine, Mixing vessel, Subtype specificity, Sero-diagnostics

2.1.1 Introduction

Swine influenza is an economically important disease of pigs caused by infections with influenza A viruses (IAV). Economic losses are caused by retarded growth of fattening pigs due to influenza-induced or -aggravated respiratory disease. In addition, febrile influenza virus infections in sows may cause fertility problems (Torremorell et al 2012). Influenza viruses are an important factor in the polymicrobial respiratory syndrome of swine (Opriessnig et al 2011). In contrast to human influenza, infections in swine do not appear to be seasonal, and virus circulation, especially in larger herds, is observed year around (Kyriakis et al 2013). Control of swine influenza is difficult and requires strict zoosanitary measures and herd vaccination programs. Licensed inactivated whole virus vaccines are available but high and continuous vaccine coverage within herds is required (Chen et al 2012).

In addition to being affected by disease, pigs are considered to be an integral part of the wider epidemiology of influenza, bridging the avian influenza world to mammalian influenza. The porcine respiratory epithelium is lined by cells which express the two sialic acid glycan receptor structures to which avian- or mammalian-adapted IAV bind (Trebbien et al 2011). As such, pigs can be infected by IAV of human and of avian origin and this provides opportunities for reassortment between these viruses (Hass et al 2011, Ma et al 2009). Following historic transspecies transmission events of IAV from human or avian sources to pigs some of these viruses have established stable circulating lineages in swine populations worldwide. These porcine lineages continue to reassort amongst each other and with other IAV of more recent human or avian origin. In Europe, this scenario has led to current presence of at least four distinct lineages of swine influenza viruses in pig populations (Brown 2013, Starick et al 2012).

Since the late 1970s IAV of subtype H1N1 of purely avian origin (H1N1av) dominate the influenza epidemiology in swine in many European countries including Germany. This lineage, referred to as H1N1av, has fully adapted to swine and can be distinguished genetically and antigenically from current avian-adapted H1N1 viruses. Viruses of this lineage have sporadically been detected also in humans and turkeys in Europe due to single transmission events from infected pigs (Harder TC 2011, Starick et al 2011b). A second porcine lineage consists of viruses of subtype H3N2. The progenitor of the currently circulating porcine H3N2 strains originated from human-adapted H3N2 viruses which had caused the Hong Kong flu pandemic in 1968 (Brown 2013). In the early 1980s the descendants of this virus reassorted with H1N1av and, apart from the hemagglutinin

H3 and the neuraminidase N2 segments, all further six genome segments were replaced with those of H1N1av (Brown 2013). In the early 1990s, a new porcine triple reassortant virus, H1N2, arose from reassortment events between human seasonal H1N1 and H3N2 and porcine H1N1av viruses. This porcine H1N2 virus carried hemagglutinin (HA) and neuraminidase (NA) of human origin and the cassette of six further segments of H1N1av (Brown 2013). These three lineages continue to co-circulate at varying prevalence in different European countries.

In 2009, a new human pandemic H1N1 strain (H1N1pdm) emerged. This virus carried reassorted gene segments from several American and Eurasian swine influenza lineages and was rapidly introduced from the human population to pigs (Brown 2013). Pigs proved to be highly susceptible to this virus and stable transmission chains were easily maintained (Brookes et al 2010). To date H1N1pdm appears to circulate independently from the human population in swine in several countries worldwide. Recently we and others found evidence for the emergence of reassortants between H1N1pdm and authentic porcine influenza virus lineages in Germany. In particular, a reassortant lineage of subtype H1pdmN2 which carried seven segments of the H1N1pdm virus and a neuraminidase of subtype 2 that was derived from different porcine or human HxN2 lineages, circulated stably (Starick et al 2011a, Starick et al 2012).

Measures aiming at control of swine influenza must be based on subtype-specific virological and serological diagnosis. Real-time RT-PCR (RT-qPCR) has provided ample applications for rapid and sensitive molecular virological diagnosis (Detmer et al 2013). Serology in swine influenza has been found useful for retrospective epidemiological investigations e.g., (Sreta et al 2013), estimation of disease incidence e.g., (Loeffen et al 2003), and control of vaccination success e.g., (Loving et al 2012). Several commercial ELISAs for detection of generic IAV nucleocapsid protein (NP)-specific antibodies in pigs are available and recommended for use e.g., (Ciacci-Zanella et al 2010). With regard to detection of antibodies at the subtype-specific level the hemagglutination inhibition assay (HI) is still held gold standard despite several draw-backs of this method including time and labour-consuming performance and dependence on labile and difficult-to-standardize components (viral antigen, erythrocytes). Furthermore, due to interference of antibodies against the viral neuraminidase component interpretation of HI-results are particularly difficult (Katz et al 2011, Wood et al 2011). Antibodies to the IAV hemagglutinin protein (HA) become detectable by HI assay from the second week post infection on and HI titers correlate with protection from clinically overt disease (Kim et al 2006, Lang C 2007).

Despite mentioned problems of HI assays very few swine influenza ELISA applications aiming at subtype differentiation at the antibody level have been reported and the current commercially available assays for subtypes H1 and H3 have not superseded HI assays, at least in Europe (Barbe et al 2009). The reported lack of sensitivity of these assays may be related to the American origin of the IAV isolates used which are antigenically distinct from those circulating in Europe. Low specificity of these assays may be caused by use of whole virion preparations which contain group specific antigens such as the nucleocapsid protein.

Here we show that recombinant HA1 antigen of European swine influenza viruses which was bacterially expressed and refolded in vitro can be used in indirect ELISAs for detection and differentiation of subtype-specific antibodies in porcine sera.

2.1.2 Material and methods

Virus and cell culture

Influenza A viruses were propagated in serum-free MDCK cell cultures in the presence of TPCK-trypsin as detailed elsewhere (Harder TC 2011). Isolates were obtained from the virus repository maintained at the Friedrich-Loeffler-Institut. Molecular characteristics of recent porcine field isolates have previously been reported (Starick et al 2012). A list of viruses used in this study for production of recombinant proteins is provided in Table 2.1.

Table 2.1 Origin and properties of porcine influenza viruses used in this study for generation of recombinant proteins.

Identification	Subtype	HA[a] Lineage	Sequence HA	Sequence NP[b]
A/Germany/R26/2011	H1N1pdm	Pandemic 2009	EPI356430[c]	n.d.
A/swine/Germany/R1738/2010	H1N1	Eurasian avian-like	EPI411955	EPI426141
A/swine/Germany/R1931/2011	H1N2	Eurasian avian-like reassortant	EPI412039	n.d.
A/swine/Germany/R1207/2010	H1N2	Eurasian human-like reassortant	EPI411941	n.d.
A/swine/Germany/R2035/2011	H1pdmN2	Pandemic 2009 reassortant	EPI356453	n.d.
A/swine/Germany/R96/2011	H3N2	Eurasian human-like reassortant	EPI411978	n.d.
A/swine/Germany/R76/2011	H3N2	Eurasian human-like reassortant	EPI411965	n.d.

[a] HA - hemagglutinin.

[b] NP - nucleocapsid.

[c] Sequence accession number in EpiFlu database.

n.d. - not determined

Bacterial expression, in vivo biotinylation and purification of influenza virus HA1 and NP proteins

The HA1 fragments of the viral hemagglutinin open-reading frames (ORF) were cloned into the pET19b vector by a target-primed technique using Phusion polymerase amplification and Dpn I digested amplification (Stech et al 2008). Sequences of primers are available on request. Expressed sequences stretched from the first amino acid of the mature protein to the arginine residue immediately proximal to the first glycine residue of the HA2 fusion peptide. Downstream of this arginine residue an Avi-Tag consensus sequence (Rigaut et al 1999) was inserted. The central lysin residue of the 15 amino acid Avi-Tag sequence provides an acceptor site for covalent linkage of D-biotin which is specifically catalysed by the bacterial biotin transferase BirA (Beckett et al 1999).

HA1-pET19b expression constructs were co-transformed into Rosettagami *E. coli* with plasmid pBIRAcm (Avidity, Aurora, CO, U.S.A.) for overexpression of BirA. Dually transformed cells were selected using ampicillin and chloramphenicol (CM). Since CM is also required to maintain the genotype of Rosettagami *E. coli* cells, presence of both plasmids in selected colonies had to be confirmed by plasmid/insert specific PCRs (primer sequences available on request). TYH medium supplemented with D-biotin at a concentration of 50 µg/ml was used for expression of co-translationally mono-biotinylatied Avi-tagged recombinant protein. The full length ORF of the nucleocapsid gene of the porcine influenza virus isolate R1738/10 was cloned and expressed similarly. However, the Avi-Tag was placed at the N-terminus of the protein. Lysates of Rosettagami cells transformed with plasmid pBIRAcm and an empty pET19b vector were used as a negative expression control.

Monobiotinylated bacterially expressed recombinant proteins were purified from inclusion bodies (IBs) by centrifugation and washing steps as previously described (Khurana et al 2011). Proteins sequestered in purified IBs were then subjected to solubilization in 6 M guanidin-HCl and refolding using a panel of up to 30 different primary and up to nine secondary buffer conditions in a stepwise solubilization strategy using the ProteoStat kit (Enzo, Lörrach, Germany). Protein solubilization and reactivity were screened with conformation-dependent monoclonal antibodies and specific polyclonal sera in ELISA to sort out optimal refolding conditions for each of the recombinantly expressed proteins. Here, refolding conditions were used which had been validated using avian influenza virus H5 HA1 protein and two conformational dependent monoclonal antibodies, 3H12 and 5 F3 (see (Postel et al 2011b), for properties of monoclonal antibodies). Final concentrations

of recombinant proteins in appropriate refolding buffers were measured using a Coomassie protein assay kit (ThermoScientific, Rockford, IL, U.S.A.).

Production of subtype-specific antisera

Pigs or ferrets were experimentally infected by the orinasal route with 10^6 $TCID_{50}$ of MDCK cell culture-grown influenza viruses in 1 ml cell culture medium using a nebulizer device (Wolfe Tory Medical, Salt Lake City, Utah) as previously described (Lange et al 2009). All experiments had received legal approval by an ethics commission (LALLF M-V/TSD/7221.3-2.5-004/10). Prior to infection animals were tested seronegative for influenza NP-specific antibodies in a commercial blocking ELISA (ID.Vet). Virus isolates used for infection are listed in Table 2.2. Blood samples used in further serological studies were obtained on day 21 post inoculation (p.i.). Reactivity of post infection sera in hemagglutination inhibition assays is detailed in Table 2.2.

Origin of field sera

Porcine field sera from 12 swine holdings in Germany were submitted for routine diagnostic procedures. The history of these holdings for vaccination against influenza and/or clinical episodes of influenza virus infection was not documented.

Indirect ELISA

Bacterially expressed proteins in refolding buffer were adjusted to a concentration of 5 µg/ml using TRIS buffered saline (TBS). A total of 100 µl per well was used for binding to streptavidin-coated plates for 2 hours at room temperature or overnight at 4°C. Each plate included different recombinant antigens for each row with following strains: A:SIV/R1738/10 (H1N1),B: SIV/ R1207/11 (H1N2), C: –SIV/ R26/11 (H1N1pdm) D: SIV R2035/11 H1pdmN2, E: SIV/– R1931 /11 (H1N2), F: R76/11 (H3N2), G: R96/11 (H3N2), H: R1738/10 nucleocapsid protein. After washing wells were blocked using 5% fat free milk-TBS containing 0.05% Tween 20 (TBST) (see Postel et al., 2011) for 2 hours at room temperature and then washed four times with TBST. Individual sera (100 µl per well, prediluted 1:200 in sample dilution buffer [ID.Vet, Montpellier, France]) were pipetted into columns (1A-1H) of the microtiter plate. This procedure assured that the reactivity of each serum against all antigens was measured in the same plate. Sera were incubated at room temperature for 1 hour. Wells were washed again four times with TBST before 100 µl of appropriately diluted goat-anti-swine IgG peroxidase conjugate (Dianova) was added for one hour at room temperature. Antibody was removed and after a final washing cycle with TBST, 50 µl of chromogenic TMB substrate was added. OD450 values were measured

after 10 minutes of incubation and addition of 50 µl of 1 N H_2SO4 to each well. Results were calculated and expressed in S/P units:

$$\frac{\text{OD Test - OD Background}}{\text{OD Positive control - OD Background}} \times 100 \quad = \text{S/P}$$

Avidity measurement of sera by indirect ELISA

The indirect ELISA was performed as described above. However, after incubation of sera in the wells a washing step using urea in TBST was carried out. Different urea concentrations (0.5 and 2 M) and incubation times were evaluated, Final assays were carried out with 6 M urea for ten minutes at room temperature. Consecutive washing steps and conjugate incubation were carried out with TBST without urea. The sera were tested in parallel with and without the urea-buffer washing step and an avidity index (AVI) was calculated:

$$\frac{\text{OD sample without urea}}{\text{OD sample with urea}} \times 100 \quad = \text{AVI}$$

Generic nucleocapsid protein blocking ELISA (NP-bEIA)

For detection of group specific antibodies a commercial NP-bEIA was purchased (ID.Vet, Montpellier, France) and used according to recommendations of the manufacturer. Accordingly, samples were considered positive if the S/N (sample OD450/negative-control OD450 × 100) ratio was less than 45%, negative if the S/N ratio was more than 50%, and doubtful if the S/N ratio was between 45% and 50%.

Hemagglutination inhibition assay (HI)

HI assays were performed according to O.I.E. recommendations essentially as described by (Lange et al 2009). Four hemagglutinating units of cell culture-grown influenza viruses were used throughout. All porcine and ferret sera were heat-inactivated for 30 minutes at 56°C and treated with receptor-destroying enzyme (neuraminidase from Bacillus subtilis).

2.1.3 Results

Bacterial expression of antigenic influenza HA1 protein

The HA1 protein fragments of seven recent swine influenza virus isolates (Table 2.1) were bacterially expressed (pET19b expression vectors) and co-translationally

monobiotinylated by overexpressed bacterial BirA biotin ligase (pBIRAcm vector). In addition, the full-length nucleocapsid protein of one of the seven isolates was expressed similarly. The recombinant proteins sequestered into bacterial inclusion bodies. Purified bacterial inclusion body proteins were subjected to SDS-PAGE under reducing conditions for detection by Western blot analysis (Figure 2.1). Using an anti-biotin monoclonal antibody, recombinant proteins of expected molecular weights (HA-1 38 +/− 3 kD; NP ca, 56 kD) are depicted in Figure 2.1A. No further protein bands were identified and no biotinylated proteins were detected in a control which consisted of a clarified lysate of Rosettagami *E. coli* cells which had been co-transformed by an empty pET19b expression vector and pBIRAcm. The NP protein showed liability to proteolytical degradation as shown by a few and weak bands of lower molecular weight (Figure 2.1 A, lane 8). Thus, the chosen bacterial co-expression system specifically produced biotinylated recombinant HA1 and NP proteins which could be successfully purified from inclusion bodies.

The antigens reacted also with sera from IAV infected pigs and ferrets (Figure 2.1 B-E; Table 2.2). The NP-antigen, although derived from a porcine H3N2 virus, was recognized by sera raised against four porcine IAV lineages (H1N1pdm, H1N1av, H1N2, and the homologous H3N2) as shown each in lanes 8 of Figure 2.1, panels B – D. A porcine serum raised against H1N1pdm was specific for the HA1 proteins of H1N1pdm and the reassortant H1pdmN2 (Figure 2.1B, lane 1 and 2). Serum from a ferret experimentally infected by an H1N1av isolate strongly reacted with homologous H1av HA1 proteins (Figure 2.1C, lane 4) but cross-reacted weakly also with other H1 HA1 recombinant proteins. An H1N2-specific porcine serum (Figure 2.1D) similarly showed strong specific staining with the homologous H1N2 HA1 (lane 3) and produced weaker signals with other recombinant HA1 antigens (e.g., lanes 2, 5). A ferret anti-H3 serum proved to be subtype-specific (Figure 2.1E, lanes 6 and 7).

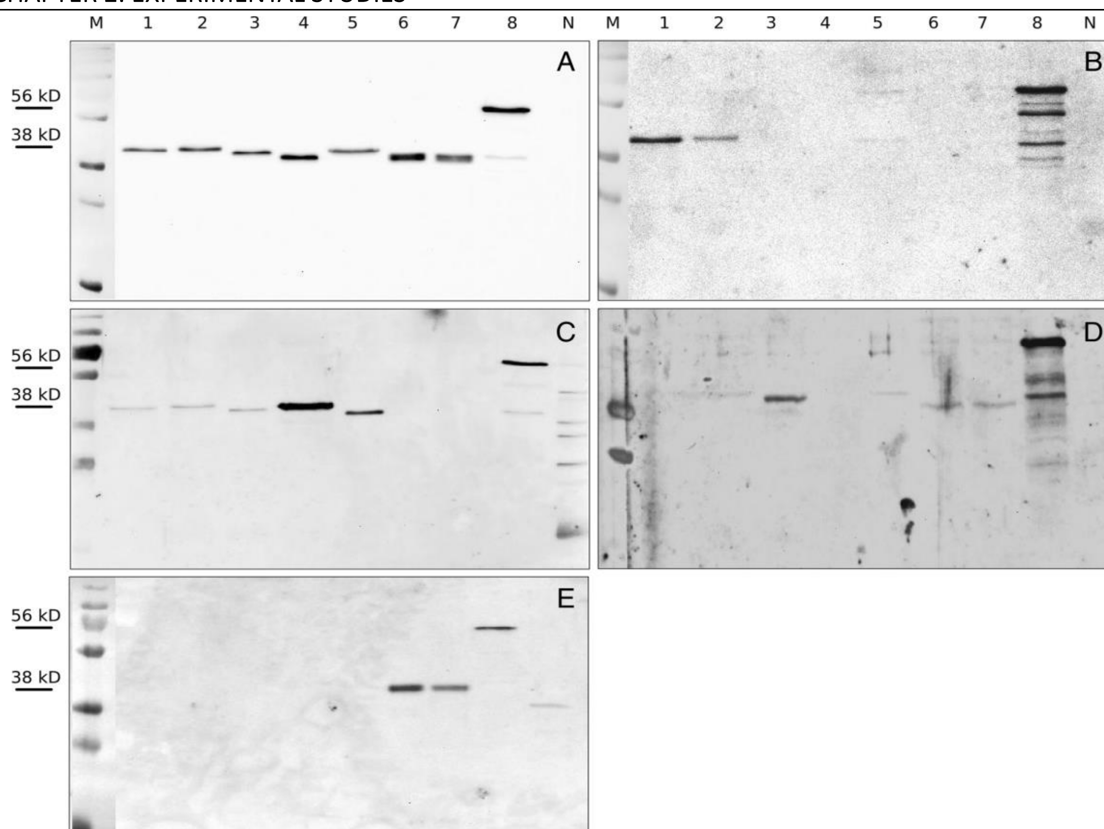


Figure 2.1 Detection of biotinylated recombinant HA1 hemagglutinin and nucleocapsid proteins of European porcine influenza viruses. An amount of approximately 20 µg of recombinant protein was loaded onto each lane and subsequently probed by an anti-biotin monoclonal antibody (A) H1N1pdm (Bavaria/74) specific porcine serum OM46 (B) H1N1av (R681/11) specific ferret serum EL1 (C) H1N2 (Bakum/1800) specific porcine serum OM1 (D) H3N2 (R655/12) specific ferret serum EL2 (E) as described in materials and methods. Recombinant antigens in lanes: 1 – R26/11 H1N1pdm, 2 – R2035/11 H1pdmN2, 3 – R1207/10 H1N2, 4 – R1738/11 H1N1av, 5 – R1931/11 H1N2, 6 – R76/11 H3N2, 7 – R96/11 H3N2, 8 - nucleocapsid protein of A/swine/Germany/R96/2011 (H3N2), N - Bacterial lysate of Rosettagami cells carrying the BirA plasmid and an empty pET19b vector. The approximate molecular weight of recombinant HA1 (38 kD) and NP (56 kD) is indicated.

Determination of cut-off values for indirect ELISAs using recombinant NP and HA1 proteins

Using the biotin residue of the recombinant NP and HA1 proteins, streptavidin ELISA plates were coated with 0,5 µg of recombinant protein per well and then blocked. Sera to be tested were prediluted 1:200 and species-specific anti IgG antibodies conjugated with horseradish peroxidase were used to detect bound antibodies in this indirect ELISA format. A total of 50 pigs were examined to determine cut-off values. These pigs originated from the influenza-negative minipig breeding cluster at FLI (n = 10) and from a swine farm which tested continuously seronegative against IAV. None of these sera tested positive in a commercial NP-specific blocking ELISA (ID.Vet, France). Mean extinctions were

measured and standard deviations were calculated for the indirect ELISAs using recombinant NP and HA1 (Table 2.3). A porcine serum derived from a pig after multiple vaccination with four subtypes of European swine IAV possessed high HI titres against H1av, H1N2, H1pdm and H3N2 (Table 2.2) and was chosen as standard positive control. Likewise, a negative porcine field serum with OD values close to the upper cut-off value of the set of negative sera was chosen as standard negative control. Based on these control sera (Table 2.3) S/P ratios were calculated for the set of 75 negative sera and the S/P mean plus 2 SD (recombinant HA1) or 3 SD (recombinant NP) were chosen as cut-offs in the examination of porcine field sera by indirect ELISAs. The exact cut-off values for each assay are presented in Table 2.3.

Table 2.2 HI (hemagglutinin inhibition) titres of porcine and ferret post infection sera used in Western blotting and indirect ELISA (homologous serum-antigen paris depicted in bold).

			Hemagglutination inhibiting titre				
Serum raised against Subtype Host	Subtype	Host	H1N1av[a]	H1N2[b]	H1N1pdm[c]	H1pdmN2[d]	H3N2[e]
A/swine/Belzig/2/2001	H1N1av	Swine (OM8)	4*	1	2	1	1
A/swine/Germany/ R681/2011	H1N1av	Ferret (EL1)	6	1	6	2	2
A/swine/Bakum/1832/ 2000	H1N2	Swine (OM1)	1	5	1	1	1
A/swine/Bayern/74/ 2009	H1N1pdm	Swine (OM46)	4	1	6	5	1
A/swine/Germany/ R2035/2011	H1pdmN2	Swine (OM15/4)	1	3	5	6	1
A/swine/Belzig/54/ 2001	H3N2	Swine (R4)	1	1	1	1	6
A/swine/Germany/ R655/2012	H3N2	Ferret (EL1)	1	1	2	2	9
Unknown, field serum	Pan	swine	6	6	5	5	6
Unknown, field serum	None	swine	1	1	1	1	1

Viruses that had been used for production of recombinant HA1 proteins were also used in HI assays:

[a] – A/swine/Germany/R1738/2010.

[b] – A/swine/Germany/R1207/2010.

[c] – A/Germany/R26/2011.

[d] – A/swine/Germany/R2035/2011.

[e] – A/swine/Germany/R96/2011.

* – Log2 titre (starting dilution 1:10).

Table 2.3 Determination of cut-offs of indirect ELISAs based on recombinant NP (Nucleocapsid protein) and HA1 proteins.

Serum samples	Values	Recombinant antigens					
		NP	H1N1av	H1N2	N1N1pdm	H1pdmN2	H3N2
Negative sera	Mean	0,11	0,03	0,06	0,03	0,04	0,14
N = 75	SD	0,08	0,03	0,1	0,08	0,07	0,14
	X + 2SD	0,35*	0,08	0,25	0,19	0,18	0,41
Positive standard	Mean	0,42	0,94	0,61	0,92	0,54	0,58
	SD	0,02	0,01	0,01	0,01	0,02	0,02
Negative standard	Mean	0,12	0,06	0,15	0,18	0,16	0,15
	SD	0,02	0,01	0	0,01	0,03	0,01

Viruses used for production of recombinant proteins:
NP, H1N1av – A/swine/Germany/R1738/2010.
H1N2 – A/swine/Germany/R1207/2010.
H1N1pdm – A/Germany/R26/2011.
H1pdmN2 – A/swine/Germany/R2035/2011.
H3N2 – A/swine/Germany/R96/2011.
* - For NP a cut-off of 3 SD was used.

Specificity of HA1-specific indirect ELISAs

Subsequently recombinant HA1-antigens were used to test sera obtained from experimentally infected pigs at day 21 after inoculation (Figure 2.2). HI assays confirmed a high specificity for these sera but also revealed minor cross reactivities especially between sera raised against subtype H1 viruses (Table 2.2). This was reflected in indirect ELISA in which the highest S/P ratios (set to 1.0) were consistently obtained with the homologous serum recombinant HA1 protein pair. However, cross reactions were also evident by indirect ELISA, particularly among proteins of the H1 lineages. In general, cross reactions resulted in S/P ratios well below 0.5 although a serum raised against pandemic H1 could not distinguish pandemic H1 from reassorted H1pdmN2 and vice versa.

In order to test whether the observed serum cross reactivity was due to low-affinity antibody species, avidity assays were performed. Indirect ELISA assays were extended by a urea washing step for 10 minutes at room temperature before second antibody conjugate was added. As shown in Table 2.4, urea treatment diminished antibody reactivity against heterologous recombinant HA1 but had a significant negative influence on homologous HA1-serum reactions as well. Thus, urea treatment grossly decreased sensitivity with no apparent benefit on specificity. In further assays therefore urea washing steps were omitted.

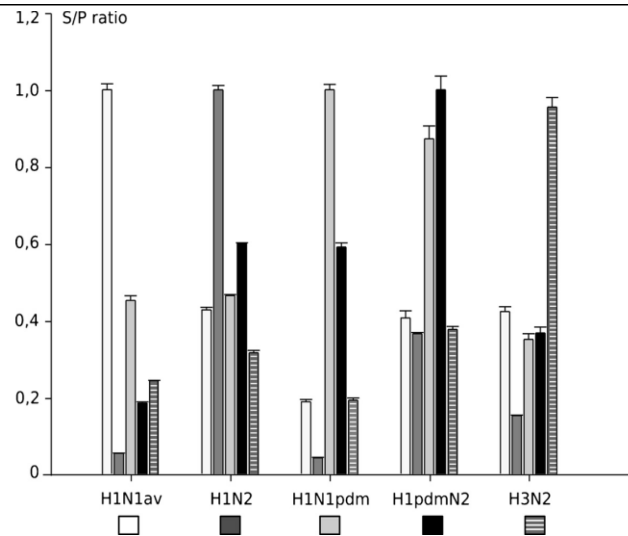


Figure 2.2 Specificity of an indirect ELISA based on recombinant hemagglutinin fragment HA1 of porcine influenza A viruses. Sera were obtained at day 21 of infection experiments in swine using the following viruses for inoculation: H1N1av – Porcine serum OM8 against A/swine/Belzig/2/2001, H1N2 – Porcine serum OM1 against A/swine/Bakum/1832/2000, H1N1pdm – Porcine serum OM46 against A/Bayern/74/2009, H1pdmN2 – Porcine serum OM15/4 against A/swine/Germany/R2035/2011, H3N2 – Porcine serum R4 against A/swine/Belzig/54/2001. The recombinant HA1 antigens were produced from the following viruses: H1N1av A/swine/Germany/R1738/2010; H1N2 – A/swine/Germany/R1207/2010; H1N1pdm – A/Germany/R26/2011; H1pdmN2 – A/swine/Germany/R2035/2011; H3N2 – A/swine/Germany/96/2011.

Table 2.4 Serum avidity in indirect HA1 ELISA.

Recombinant HA	Serum specificity								
	H1N1pdm– A/Bayern/74/2009[a]			H1N1av – A/swine/Belzig/2/2001			H3N2 – A/swine Belzig/54/2001		
	No urea	Urea	AVI (%)	No urea	Urea	AVI (%)	No urea	Urea	AVI (%)
A/Germany/R26/2011 (H1N1pdm)	2,332[b]	1,273	55	1,011	0,163	16	0,286	0,108	38
A/Swine/Germany/R2035 /2011 (H1pdmN2)	1,359	0,266	20	0,292	0,109	37	0,306	0,099	32
A/Swine/Germany/R1738 /2010 (H1N1av)	0,209	0,112	54	1,241	0,312	25	0,291	0,107	37
A/swine/Germany/R1207/ 2010 (H1N2)	0,176	0,16	91	0,078	0,061	78	0,087	0,06	69
A/swine/Germany/R76/20 11 (H3N2)	0,182	0,111	61	0,159	0,121	76	0,768	0,147	19

[a] Subtype and designation of influenza A virus isolate used to raise the respective serum.

[b] Bold face indicates homologous rec HA1 – serum pair.

AVI (%) - percentage avidity index

Detection of influenza-specific antibodies in sera of experimentally infected swine

In a next step the dynamics of antibody development after experimental influenza virus infections of pigs was examined. Experimental infections had been carried out previously using a pandemic H1 virus (A/sw/Germany/R708/ 2010), an H1pdmN2 reassortant (A/sw/Germany/R2035/2011), and an H1N1av isolate (A/sw/Germany/R248/2010). Sera obtained at different time points post infection were available for testing by a commercial NP blocking ELISA (ID.Vet, France), the indirect ELISAs specific for recombinant NP and for the homologous HA1 proteins, respectively. In addition, HI titer measured against the homologous virus were compared. Results are presented in composite Figure 2.3. The data show that antibody dynamics in experimentally infected swine can be accurately followed using the indirect ELISA formats. The results compare favourably with the commercial NP blocking ELISA and homologous HI.

Detection of influenza-specific antibodies in porcine field sera

Finally, reactivity of a collection of 207 porcine field sera was compared by testing sera in HI assays, commercial NP blocking ELISA and indirect ELISAs with recombinant NP and HA1- antigens. These sera represented daily routine diagnostic submissions. As regards the HI assay, a serum was rated qualitatively positive for influenza A specific antibodies if it showed an HI titre of ≥ 40 (≥ 3 log2, starting dilution 1:10) against at least one of the four antigens (H1N1av, H1N2, H1N1pdm, H3N2) used for testing. Depicting the results of the commercial NP blocking ELISA, the indirect NP ELISA and the sum of the HI assays using a Venn diagram (Figure 2.4 (Rodgers P 2010)), an overall high degree of agreement was visually evident. The majority of results was backed by all three or at least two of the assays. However, in the HI assays, a comparatively high number of 26 sera revealed positive results just above the threshold (3 or 4 log2) which were not supported by other assays (Figure 2.4).

Inter-rater agreement (Fleiss JL 1969) was used to evaluate results of indirect ELISAs using recombinant HA1 compared to HI titres. While excellent and good agreements were seen for the H3 (κ_{R96} = 0,8255 [95% CI 0,6997-0,9513]) and the pandemic H1 antigens (κ_{R26} = 0,7772 [0,6917-0,8627]; κ_{R2035} = 0,661 [0,5643-0,7577]) respectively, only moderate agreement was signalled for the H1av (κ_{R1738} = 0,5722 [0,4401-0,7043]) and H1N2 (κ_{R1207} = 0,5083 [0,3359- 0,6807]) antigens. A graphical analysis revealed that correlation between HI titres and S/P ratios was stronger for higher but less tight for sera with lower HI titres (Figure 2.5, exemplified for recombinant H1av HA1 antigen). A good

agreement ($\kappa_{HOLDING7}$ = 0,625 [0,374-0,876]) across all indirect ELISAs was seen when results were compared based on holding level instead of individual sera (Table 2.5).

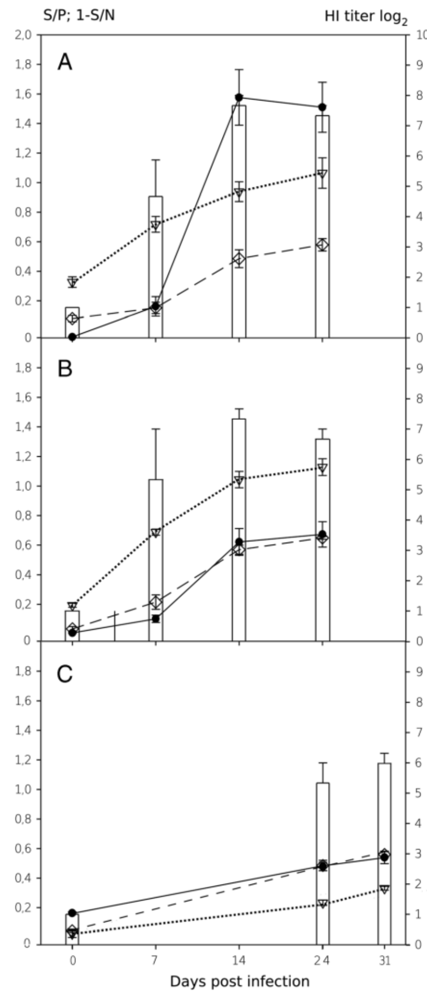


Figure 2.3 Development of antibodies against viral nucleocapsid and hemagglutinin fragment HA1 proteins in swine experimentally infected with porcine influenza A viruses. Experimental infection each of three pigs by A – A/sw/Germany/R708/2010 (H1pdmN1), B – A/sw/Germany/R2035/2011 (H1pdmN2), C – A/sw/Germany/R248/2010 (H1N1av). Columns – HI (hemagglutinin inhibition) titer against homologous virus antigen; open triangle – indirect recombinant NP ELISA; black dot – indirect recombinant HA1 ELISA (homologous antigen); open diamond – commercial blocking NP ELISA (ID.Vet). Samples of days 7 and 14 were unfortunately not available for experiment "C".

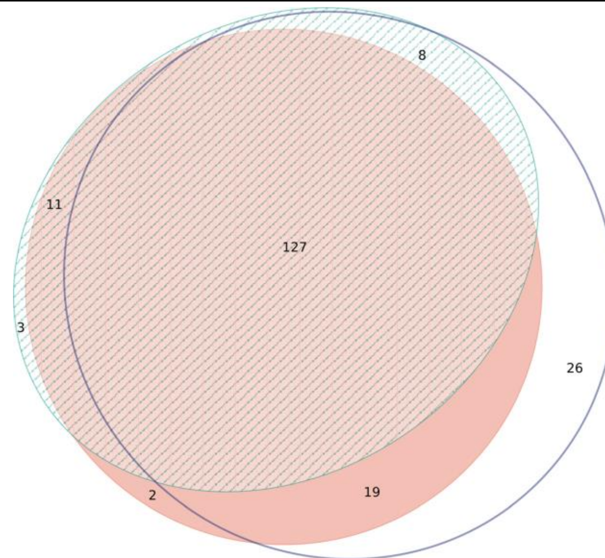


Figure 2.4 Venn diagram of detection of NP (nucleocapsid protein)-specific antibodies by commercial blocking ELISA (hatched ellipsis) and indirect ELISA (recombinant NP, Red ellipsis) in porcine field sera (n = 207) compared with hemagglutination inhibition assays (white ellipsis). Eleven sera produced congruently negative results in all three tests.

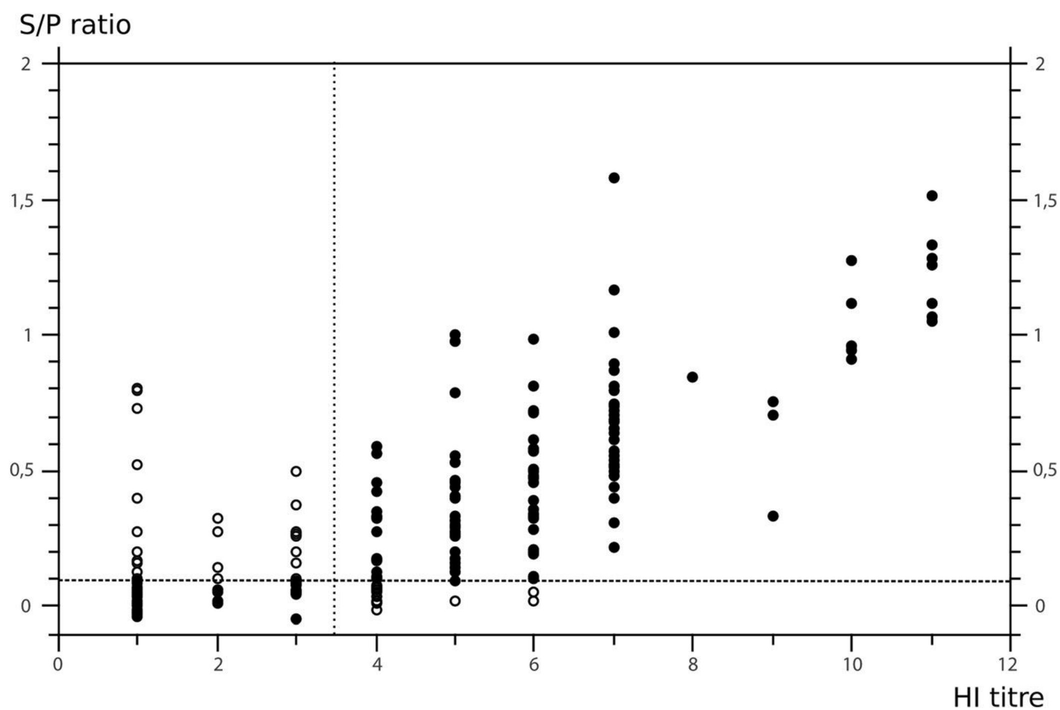


Figure 2.5 Comparison of HI (hemagglutinin inhibition) titres measured against porcine avian-derived H1N1 isolate A/swine/Germany/R248/2010 and S/P ratios obtained by indirect ELISA using recombinant HA1 antigen of avian-derived H1N1 isolate A/swine/Germany/R1738/2011. Black dots – congruent qualitative result; white dots – incongruent qualitative result; HI titre shown as log2 series (starting dilution 1 = 1:10); dotted lines represent cut-off values.

Table 2.5 Results of recombinant indirect ELISAs (iEIA) and HI (hemagglutinin inhibition) or commercial NP (nucleocapsid)-specific ELISA obtained with 207 porcine field sera compared on a holding base.

Holding	Sample	NP		H1av		H1N2		H1pdm		H1pdmN2		H3	
		cEIA IDVet	iEIA	HI	iEIA	HI	iEIA	HI	iEIA	HI	iEIA	HI	iEIA
1	6[a]	5[b]	4	5	6	1	0	4	4	n.d.	2	0	0
2	16	15	12	14	16	3	0	13	16	n.d.	16	6	6
3	9	7	7	7	7	9	2	3	6	n.d.	4	5	3
4	7	7	7	7	7	7	7	7	7	n.d.	7	7	7
5	5	5	5	5	5	5	5	5	5	n.d.	5	5	5
6	30	30	28	30	29	1	0	28	26	n.d.	20	0	3
7	30	24	26	22	22	3	1	17	15	n.d.	14	0	0
8	30	13	18	8	14	0	0	1	3	n.d.	3	0	0
9	30	18	28	28	26	0	1	26	21	n.d.	15	0	0
10	23	3	12	9	7	3	0	0	0	n.d.	0	1	0
11	13	11	12	13	13	3	1	12	11	n.d.	6	0	0
12	10	10	10	10	6	1	1	5	3	n.d.	3	0	0

[a] Number of samples obtained in holding.

[b] Number of samples rated positive in the respective assay.

n.d. Not determined.

2.1.4 Discussion

Serodiagnosis of porcine influenza virus infections in Europe and elsewhere is significantly challenged by cocirculation of several subtypes, and use of multivalent vaccines further complicates this situation. While generic antibodies directed against the well conserved influenza virus NP protein can be detected by commercial blocking ELISAs, the differentiation of subtype-specific antibodies requires use of the fastidious HI assay. Subtype-specific ELISA assays suitable for high throughput investigations of porcine sera would aid in promoting more intense studies on porcine influenza seroepidemiology.

Aiming to develop such assays we have successfully expressed recombinant full-length NP and HA1 fragments in bacteria and refolded proteins in vitro. Recombinant proteins were co-translationally mono-biotinylated which facilitated purification and binding to solid, streptavidin coated supports. Previous work by Khurana (Khurana et al 2011) has shown that bacterially expressed HA proteins can be refolded to acquire native conformation. Recombinant proteins representing recent isolates of the major subtypes and lineages of porcine influenza virus currently circulating in Germany were recognized by specific

porcine or mustelid immune sera in Western blotting and indirect ELISA. Dynamics of generic and subtype-specific antibody development in experimentally infected swine as measured by recombinant indirect ELISAs fully paralleled results obtained by a commercial NP blocking ELISA and homologous HI assays.

However, despite use of the less conserved HA1 section of the HA glycoprotein as diagnostic antigen residual cross reactivity between the different subtypes was noticed in both Western blot (Figure 2.1) and indirect ELISA (Figure 2.2) even when using experimental post infection sera which were reasonably discriminatory between subtypes in HI assays (Table 2.2). Thus, a number of conserved epitopes exists between the different subtypes in the HA1 which is not detected by the functional HI assay. Yet, a considerable number of subtype-specific epitopes must have been represented in the recombinant HA1 proteins as well since, in indirect ELISA, the homologous HA1- serum pairs always resulted by far in the highest signal intensity (Figure 2.2). Human pandemic H1 HA1 represented by isolate R26/11 and that of the porcine-adapted variant H1pdmN2 R2035/11 were indistinguishable by Western blot and indirect ELISA although slight antigenic differences have been reported when using an HI assay (Starick et al 2012).

The comparative examination of porcine field sera showed a variable agreement for the different recombinant antigens when individual sera were compared. In particular, ELISAs based on recombinant H1av and H1N2 revealed only moderate agreement when compared to HI. Fewer sera scored positive in the indirect ELISAs compared to HI assays. The majority of sera missed by the indirect ELISA showed low HI titres (3 or 4 log2) while reasonable correlation was seen between S/P ratios and HI titres for higher-titred sera (Figure 2.5). The blurring of results obtained with low-titred HI-positive sera may be due to low sensitivity of the indirect ELISAs but could as well have been caused by lack of specificity of the HI assays; problems with reproducibility and standardization of HI assays are notorious, especially when testing low-titred sera (Wood et al 2011). In addition, the HI assays here were carried out with antigens selected and used in routine diagnosis while the recombinant antigens were produced from more recent circulating viruses and differed slightly in HA1 amino acid sequences (not shown). This may have introduced further discrepancies as observed for individual sera. However, when results were compared on a herd basis a full match between HI and indirect ELISA results was evident (Table 2.5). Herds found to be seropositive by HI for a certain subtype were similarly positive in the respective HA1 indirect ELISA.

Moreover, herds negative by HI for a certain subtype tested negative by the corresponding indirect ELISA. This indicates that on herd base the HI assay may be replaced, without loss of diagnostic quality, by the indirect ELISAs.

2.1.5 Conclusions

Due to the ongoing antigenic diversification of porcine influenza viruses worldwide and new reassortant lineages springing up here and there serological diagnosis of porcine influenza becomes ever more demanding. Subtype and lineage-specific assays suitable for high throughput analysis will be required to cope with such diagnostic challenges. The recombinant mono-biotinylated HA1 proteins presented here as diagnostic antigens in indirect ELISAs provided an interesting alternative in this respect to HI assays. Further refinements of this strategy, e.g., by using lineage-specific monoclonal antibodies for competition or an immune-complex binding assay format, should be further investigated to replace HI assays.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ZN, CG and TCH conceived the study and drafted the manuscript. ZN carried out the molecular and serological work, and analysed samples. SK participated in the molecular and serological work. EL carriy out animal experiments and provided samples. MB conceived the study, provided funds and edited the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Z.N. is recipient of a fellowship of the China Scholarship Council (CSC). The study has been supported through FSI projects 2.1.1 and 3.2 by the German Federal Ministry for Food, Agriculture and Consumer Protection, and by a grant from vaxxinova, Münster, Germany. The FLI is an associated member of the EU-FP7 project ESNIP-3.

2.2 Second Study: Engineered recombinant protein products of the avian paramyxovirus type-1 nucleocapsid and phosphoprotein genes for serological diagnosis

Na Zhao, Christian Grund, Martin Beer and Timm C Harder

Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Suedufer 10, Greifswald 17493, Germany

Correspondence: timm.harder@fli.bund.de

This article has been published in Virology Journal (2018) 15:8 DOI 10.1186/s12985-018-0924-8
This is an Open Access article distributed under the terms of the Creative Commons Attribution License
Received: 24 November 2017 Accepted: 8 January 2018 published online: 11 January 2018

Abstract

Virulent Newcastle disease virus (NDV, avian Avulavirus-1, APMV-1) induces a highly contagious and lethal systemic disease in gallinaceous poultry. APMV-1 antibody detection is used for surveillance and to control vaccination, but is hampered by cross-reactivity to other subtypes of avian Avulaviruses. Data are lacking concerning the applicability of NDV V proteins as differential diagnostic marker to distinguish vaccinated from virus-infected birds (DIVA strategy). Full length and C-terminally truncated nucleocapsid (NP) protein, and the unique C-terminal regions of the phospho- (P) and V proteins of the NDV LaSota strain were bacterially expressed as fusion proteins with the multimerization domain of the human C4 binding protein, and used as diagnostic antigens in indirect ELISA. When used as diagnostic antigen in indirect ELISAs, recombinant full-length proved to be a sensitive target to detect seroconversion in chickens after APMV-1 vaccination and infection, but revealed some degree of cross reactivity with sera raised against other APMV subtypes. Cross reactivity was abolished but also sensitivity decreased when employing a C-terminal fragment of the NP of NDV as diagnostic antigen. Antibodies to the NDV V protein were mounted in poultry following NDV infection but also, albeit at lower rates and titers, after vaccination with attenuated NDV vaccines. V-specific seroconversion within the flock was incomplete and titers in individual bird transient. Indirect ELISA based on bacterially expressed recombinant full-length NP compared favourably with a commercial NDV ELISA based on whole virus antigen, but cross reactivity between the NP proteins of different APMV subtypes could compromise specificity. However, specificity increased when using a less conserved C-terminal fragment of NP instead. Moreover, a serological DIVA strategy built on the NDV V protein

was not feasible due to reduced immunogenicity of the V protein and frequent use of live-attenuated NDV vaccine.

Keywords: Newcastle disease virus, Recombinant protein, Subtype-specific serology

2.2.1 Introduction

Newcastle Disease (ND) is caused by infection of chickens with virulent strains of the avian paramyxovirus serotype-1 (APMV-1, syn. ND virus, NDV). ND, together with highly pathogenic avian influenza, are among the most dreaded viral infections of gallinaceous poultry worldwide (Alexander et al 2012). Rapid spread of a highly lethal, pantropic disease ensues in affected flocks after incursion of virulent (syn. velogenic) NDV. Consequently, ND is an O.I.E.-notifiable disease of poultry. The virus is enzootic in many regions of the world and continues to threaten industrial production as well as backyard poultry holdings. Vaccination with inactivated adjuvanted or, alternatively, live virus vaccines featuring attenuated (lentogenic) APMV-1 strains such as LaSota or B1 is widely used to prevent ND (Kapczynski et al 2013, Senne et al 2004).

Avian avulaviruses are members of the Avulavirus genus within the Paramyxoviridae family. To date at least fifteen serotypes (APMV-1 to APMV-15) have been identified (Briand et al 2012, Goraichuk et al 2016, Karamendin et al 2016, Lee et al 2017, Miller et al 2010, Terregino et al 2013, Thampaisarn et al 2017, Thomazelli et al 2017). The virus possesses a single stranded, negative-sense, non-segmented RNA genome, which encodes six structural proteins in following order: nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and the large (L) polymerase protein (Krishnamurthy & Samal 1998, Lamb 2007). The NP protein is a highly conserved and the most abundant viral protein expressed in infected cells, and induces a strong humoral (non-neutralizing) and cellular immune response in the infected host and also following vaccination with inactivated virus (Ahmad-Raus et al 2009, Kapczynski et al 2013, Makkay et al 1999, Panshin et al 2000).

The P protein plays an important role in the viral transcription and replication, and it is associated with the nucleocapsid in the virion (Errington & Emmerson 1997, Kho et al 2004). Two additional proteins, V and putative W, are predicted to be produced from P gene by mRNA editing post transcription (Hausmann et al 1999, Jordan et al 2000, McGinnes et al 1988, Samson et al 1991, Steward et al 1993). The product that ensues by insertion into the nascent P mRNA of one non-template G residue at position 401 (+2 reading frame) is referred to as the V protein (McGinnes et al 1988). Addition of two untemplated Gs at the polymerase slipping point of the P gene would generate a third protein species, the W protein, from the P gene (McGinnes et al 1988, Steward et al 1993). Thus, all three P gene-derived proteins have a common N-terminus, but vary at their carboxyl termini both in length and amino acid composition. The V protein of NDV

harbours 106 amino acids in its unique C-terminal part (LaSota strain). Similar to other viruses in the paramyxoviridae family, the V protein is found to be a zinc-finger domain protein and appears to function as a virulence factor by antagonizing, in a strain-specific manner, components of the host innate immunity, in particular the interferon system (Alamares et al 2010, Huang et al 2003). However, very little is known about the immunogenicity of the V protein.

Serological assays to detect ND-specific antibodies can be used for demonstration of lack of exposure of a flock to NDV, and for assessment of vaccination efficacy. The hemagglutination inhibition (HI) test is a standard method and widely used for NDV antibody detection, although it may lack in sensitivity and is time-consuming (Czifra et al 1996, Hauslaigner et al 2009, Xu et al 1997). Several ELISA formats have been developed as an alternative for conventional HI test in flock screening approaches. Their sensitivity, and easy standardization make them suitable for high throughput screening (Chaka et al 2013, Das & Kumar 2015, de Oliveira et al 2013, de Sousa et al 2000, Hauslaigner et al 2009, Hemmatzadeh & Kazemimanesh 2017, Kho et al 2000). ELISA formats based on whole virus and recombinant viral proteins expressed in baculovirus or bacterial systems as the coating antigen have been reported (Das & Kumar 2015, Errington et al 1995, Hauslaigner et al 2009, Miers et al 1983, Mohan et al 2006, Rivetz et al 1985). Recombinant NP protein in particular has been used for the development of indirect ELISAs (iELISA) (Errington et al 1995, Makkay et al 1999). However, the NP protein is highly conserved among avian paramyxoviruses, and serologic assays building on recombinant NDV NP may be compromised by cross reactions between various APMV serotypes.

To overcome limitations of full length NDV NP as the antigen in ELISA format, we hypothesized that use of less conserved NP and P protein fragments would enable a more serotype-specific distinction. The objective of this study was to explore the suitability of truncated NP and P proteins as diagnostic antigens for detection and differentiation of avian paramyxovirus-specific antibodies. Moreover, assuming that a humoral immune response against the V protein would be enhanced by active viral replication in the host and expression of V in infected cells, V protein-specific antibodies might be used to distinguish infected birds from those that are seropositive after vaccination with inactivated or attenuated vaccines. Therefore, another objective was to explore and evaluate the diagnostic feasibility of using the unique portion of V protein in an indirect ELISA-based DIVA (differentiating infected from vaccinated animals) strategy.

2.2.2 Methods

Virus propagation

APMV-1 vaccine strain LaSota (LS; class II, genotype 2) (Diel et al 2012) and APMV-8 strain goose/Delaware/1053/76 were grown in the allantoic cavity of embryonated chicken eggs as detailed elsewhere (Hauslaigner et al 2009).

Cloning and bacterial expression of recombinant proteins

The pET19b vector and Rosettagami *E. coli* cells (both Novagen, Darmstadt, Germany) were used for expression of recombinant NDV proteins. The full length ORF encoding the nucleocapsid protein (NP) of NDV LaSota was cloned into pET19b downstream of the T7 promoter as shown in figure 2.6. A hexa-histidine- and an Avi-tag were positioned in-frame and N-terminally of the ORF (Beckett et al 1999). Similarly, the sequences encoding the unique parts of the P and V (P_u, V_u) downstream of the RNA editing site of the P gene were cloned into pET19b (Figure 2.6 B). In addition, the C-terminal 99 amino acids of NDV LaSota NP (NP_{ct}), and the C-terminal 81 amino acids of the NP of APMV-8 were cloned as shown in figure 2.6 C. A heptamerization fragment of the human C4 binding protein (C4BP) was positioned between the tags and the NDV sequences (Hofmeyer et al 2013, Shinya et al 2009). SGS-linker sequences were placed between C4BP and the virus-specific sequences to ensure unrestrained folding of the latter. Plasmid pBirCam (Avidity, Aurora, U.S.A) over-expressing the bacterial biotin ligase BirA was co-transformed into strain Rosettagami to ensure co-translational mono-biotinylation of the recombinant APMV protein fragments at their Avi-tag (Zhao et al 2013). Ampicillin and chloramphenicol were used for selection. Presence of both plasmids was confirmed by plasmid-insert-specific PCRs.

Induction of expression was achieved in TYH medium supplemented with IPTG (1.5 mM) and D (+)-biotin (50 µM). After culturing for 4 hours at 37°C, cells were pelleted and lysed using ultrasonic disruption as previously described [173] to liberate inclusion bodies (ICs) into which recombinant proteins had sequestered.

Purification and reconstitution of recombinant proteins from bacterial inclusion bodies

Purified ICs were solubilized in 6 M guanidinium-HCl. A set of refolding buffers was used in a stepwise solubilisation strategy using the ProteoStat kit (Enzo, Lörrach, Germany) to determine the most appropriate buffer conditions to fold back the proteins into an antigenically authentic structure while keeping it solubilized. Antigenic properties in ELISA

were assayed using polyclonal antibodies raised in chickens. Final protein concentration in refolding buffer was determined using a coomassie protein assay kit (ThermoScientific, Rockford, IL, U.S.A.) and proteins were stored at 4°C until further use.

Production of antisera

Galline hyperimmune sera against reference isolates of APMV serotype-1 (NDV strain Ulster, pigeon paramyxovirus, class I isolate APMV-1/Mallard/Germany/R2481/2007), and other serotypes-2 (APMV-2/chicken/California/Yucaipa/56), -3a (APMV-3/turkey/England/1087/82), -3b (APMV-3/Parakeet/Netherlands/449/75), -4 (APMV-4/duck/Hong Kong/D3/75), -6 (APMV-6/Dk/HK/77), -7 (APMV-7/dove/Tennessee/4/75), -8 (APMV-8/goose/Delaware/1053/46), and -9 (APMV-9/duck/New York/22/78) had been generated in chickens by booster immunization using egg-grown virus adjuvant with incomplete Freund's adjuvant. SPF chicken sera were sampled from chickens hatched at the FLI from SPF eggs (Lohmann Tierzucht, Cuxhaven, Germany). All animal experiments had received full legal approval by the animal welfare committee of the German Federal State of Mecklenburg-Vorpommern (LALLF M-V/TSD/7221.3-2.5-004/10; LALLF M-V/TSD/7221.3-2.5-010/10).

Western blotting

Protein samples were separated by SDS-PAGE in 12.5% gels. For denaturing conditions samples were heated in Laemmli loading buffer containing dithioerythritol (DTE). Proteins were also separated under non-denaturing conditions in 10% PAGE without SDS and DTE. Proteins were transferred onto nitrocellulose membranes by semi-dry blotting at 0.8 mA/cm^2 for 1 h (PerfectBlueTM Electro Blotter, Peqlab, Erlangen, Germany). Membranes were blocked with 5% skim milk powder in Tris-buffered saline (TBS) supplemented with 0.05% (v/v) Tween (TBST) for 1h. Specific antibodies and sera were appropriately diluted in TBST and incubated on the membranes for 1 hour at room temperature. Membranes were washed three times with TBST and incubated for another hour with POD-labeled secondary antibody. Blots were washed again for three times, then incubated with substrate SuperSignal™ West Pico Chemiluminescent substrate solution (ThermoScientific, Braunschweig, Germany) before being analyzed with an imaging system (VersaDoc, Bio-Rad). Photos were edited with respect to contrast and brightness and composite blots were assembled using cut-out lanes (GIMP software).

Indirect ELISA

Recombinant proteins solubilized in the respective refolding buffers were further diluted to a concentration of 5 µg/ml using bicarbonate coating buffer (pH 9.6) of which 100 µl per

well were used to coat Maxisorb ELISA plates (NUNC, Thermofisher, Braunschweig, Germany) overnight at 4 °C. After washing, wells were blocked by 5% skim milk-TBST) for 2 hours at room temperature and then washed four times with TBST (Postel et al 2011b). Individual sera were diluted 1:200 (for the NP iELISA) or 1:500 (other antigens) in appropriate sample dilution buffer. Sera were incubated at room temperature for 1 hour. Wells were washed again four times with TBST before 100 µl of appropriately diluted goat-anti-chicken or -turkey IgY POD conjugate (Dianova, Hamburg, Germany) was added for 1 hour at room temperature. Antibody was removed and after a final washing cycle with TBST, 50 µl of chromogenic TMB substrate was added. OD450 values were measured after 10 minutes of incubation and addition of 50 µl of 1N H_2SO_4 to each well. Results were calculated and expressed in S/P units:

$$\frac{\text{ODTest} - \text{ODBackground}}{\text{ODPositive control} - \text{ODBackground}} \times 100 = S/P$$

Determination of cut-off values for indirect ELISAs

Maxisorb ELISA plates were coated with 0.5 µg of recombinant protein per well and then blocked. A predilution step of sera, as outlined above, was required for background reduction. Species-specific anti-IgY POD-conjugates were used for detection of bound antibodies. A total of 51 sera from SPF chickens were examined to determine cut-off values for the different recombinant antigens in this indirect ELISA format. None of these sera tested positive in a commercial indirect NDV ELISA (see below). Mean extinctions and standard deviations were calculated (Table 2.8). Serum S185, a hyperimmune serum raised in chicken against inactivated NDV strain Ulster, served as standard positive control for assays employing NP and P antigens. Serum S880/Hu295 was used as standard positive control for V protein-specific assays since this serum had been obtained at day 28 after immunization with live-attenuated NDV LaSota vaccine and was shown to harbor V-specific antibodies in Western blotting (Figure 2.7C). An SPF chicken serum was chosen as standard negative control. These control sera were used as standards in each of the assays to determine threshold OD values and to calculate S/P ratios. The S/P mean values of all 51 SPF sera plus 2 SD were chosen as cut-offs in the examination of chicken field sera by indirect ELISAs. Table 2.8 presents exact cut-off values for each assay.

Commercial ELISA for detection of NDV antibodies

A commercial indirect NDV-ELISA (Flock Chek NDV) based on complete ND virions was purchased from IDEXX (Ludwigsburg, Germany). Instructions of the manufacturer were followed exactly using either the version for chickens or turkeys as appropriate.

Hemagglutination inhibition assay (HI)

HI assays were performed according to O.I.E. recommendations essentially as described (Grund et al 2011). Four hemagglutinating units of egg-grown APMV-1 or APMV-8 viruses were used throughout. All sera had been heat-inactivated for 30 minutes at 56°C.

Origin of field sera

A total of 150 chicken and 147 turkey sera submitted for routine avian influenza or ND serodiagnostic investigations originated from various poultry holdings in Germany. It should be noted that, in Germany, NDV vaccination is compulsory for all gallinaceous poultry including those kept in small backyard flocks.

Origin of experimental infection sera

APMV-1

A total of 10 chickens each received immunization with a live-attenuated vaccine based on NDV strain “Clone 30” (Table 2.6, vaccinated group). An additional six SPF chickens were used as controls and did not receive vaccination. At day 21 post vaccination all chickens were challenged with the velogenic NDV strain “Herts” and serum samples were taken at days 0, 3, 7, 14, 21, and 28 post challenge.

APMV-8

Fifteen chickens were immunized with wild-type APMV-8 strain APMV-8/goose/Delaware/1053/76 (GenBank acc. no. FJ619036) one day after hatch and challenged with APMV-8 on day 21 post vaccination. as described in detail by Grund et al (Grund et al 2014). Samples used here for serological analysis were taken on days 21 post vaccination and 14 post challenge. Both animal experiments received full legal approval by the animal welfare committee of the Federal State of Mecklenburg-Vorpommern (LALLF M-V/TSD/7221.3-1.1-053/10).

2.2.3 Results

Production of recombinant proteins

Recombinant proteins were designed to represent those amino acid sequences of the APMV-1 P gene products which are unique (“u”) to P and V. Since this resulted, especially for V, in comparatively small peptides (P_u 27.6 kD; V_u 11.7 kD), the heptamerization domain of the human C4 binding protein (C4BP) was fused as a carrier module in order to facilitate handling as well as proper presentation of the specific peptide antigens (Figure

2.6). The same strategy was used to express the C-terminal 99 amino acids of the LaSota NP protein (LS-NP_{ct}) of APMV-1 which is much less conserved among APMV serotypes compared to the full-length NP protein (Table 2.8). Also, the NP_{ct} of an APMV-8 strain was expressed as an example of a non-NDV APMV serotype. In addition, full length APMV-1 NP was expressed but without the C4BP fusion. All constructs harbored hexahistidine and AVI tags (Rigaut et al 1999) to facilitate downstream processing (Figure 2.6). The AVI tag was used for co-translational mono-biotinylation in *E. coli* in which BirA, a biotin ligase, was overexpressed.

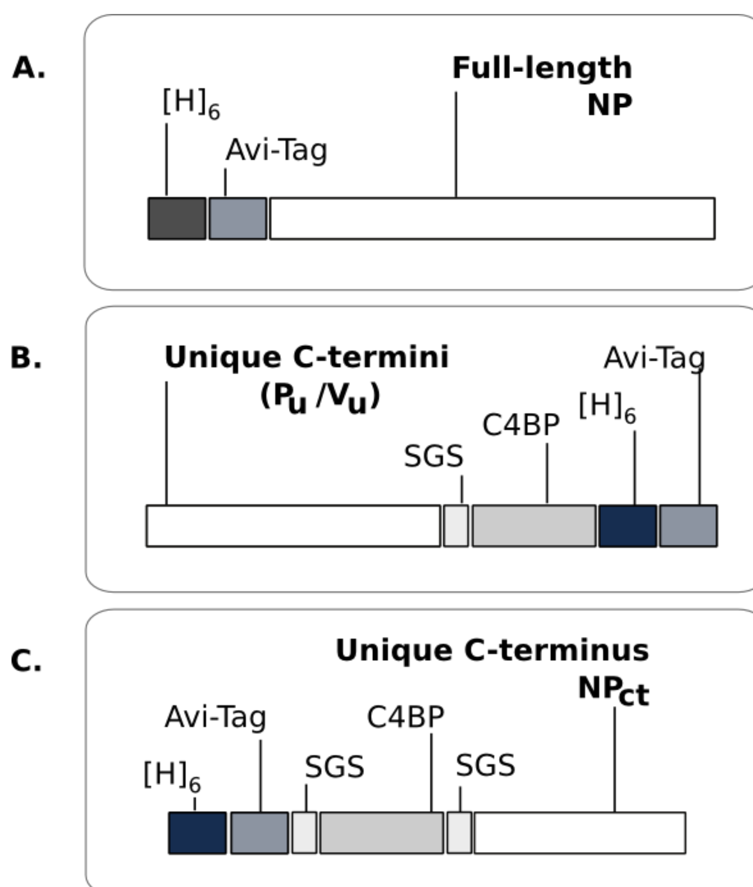


Figure 2.6 Schematic presentation of constructs used for generation of pET19b plasmids and T7-driven expression of NDV (Newcastle disease virus) proteins in Rosettagami *E. coli* cultures. All sketches show the protein C-terminus. A. Design of full-length expression of the nucleocapsid gene of NDV strain LaSota, with N-terminal hexahistidin ([H] 6) and AVI tags. B. Expression of truncated and frame-edited versions of the P gene expressing unique C-termini of the two P gene-derived proteins P and V as fusion proteins with the heptamerization domain of the human C4 binding protein (C4BP), and tags. An SGS-linker separates virus-specific from fused protein sequences. C. Expression cassette of C-terminally truncated versions of the NP protein of NDV LaSota and APMV-8, respectively.

Probing refolded recombinant proteins by Western blot analysis under reducing conditions with a monoclonal antibody against biotin yielded specific bands in the size range calculated from the amino acid sequence for NDV full-length NP as well as LS-NP_{ct}, P_u,

V_u (Figure 2.7A). The Rosettagami E.*coli* lysate and recombinant heptamerization domain of the human C4 binding protein expressed without any APMV-specific peptides were used as controls (Figure 2.7A, lanes “N and C4BP”). When separating under non-denaturing conditions, proteins that contained the C4BP domain assembled into multimeric complexes. Their molecular weight measured under native PAGE conditions were approximately sevenfold the calculated value of their corresponding monomeric protein (Figure 2.7B, lane “NP_{ct}, P_u, V_u and C4BP”). This indicated functionality of the heptamerization domain of C4BP. Intriguingly, apart from the monomers, also dimers and oligomers of the full-length NP protein were observed as well (Figure 2.7B, lane “NP”) demonstrating that the refolded full-length NP has the ability of spontaneously forming multimers without the C4BP multimerization domain helper.

Immunogenicity of the APMV-specific peptide part was confirmed, by a chicken serum (S880/Hu295) obtained at day 28 after immunization with live-attenuated NDV LaSota vaccine. Strong responses to full length NDV NP, NP_{ct} and P_u as well as weak reactivity to V_u protein, visible as faint band at the expected size, proved that the major epitopes were preserved and accessible in the recombinant proteins after the refolding procedure (Figure 2.7C). The results indicate that expressed recombinant proteins constitute suitable antigens for testing NDV specific antibodies in avian sera.

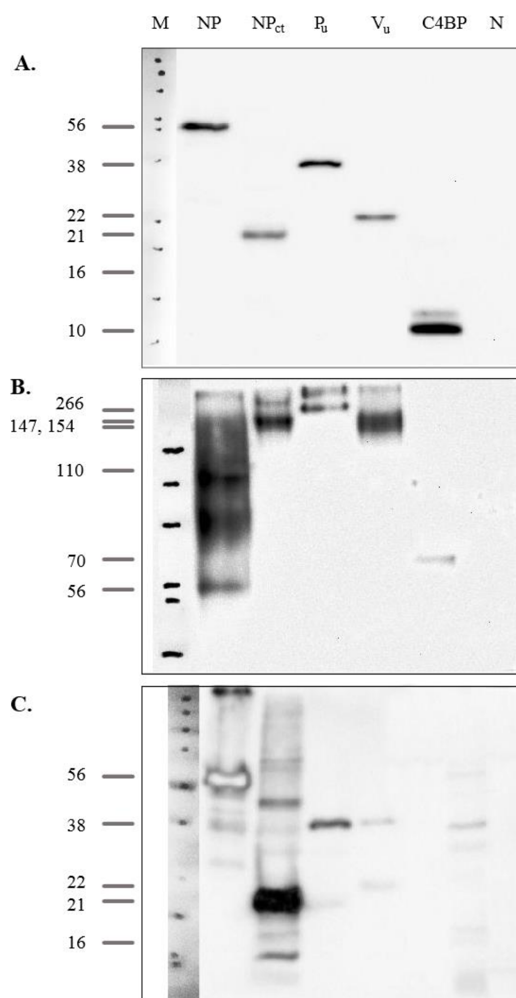


Figure 2.7 Assembled Western blot assays of monobiotinylated recombinant proteins of NDV LaSota. Panels A and B were stained using a monoclonal antibody specific for biotin. For panel C a chicken serum (S880/Hu295) was used that was obtained at day 28 after immunization with live-attenuated NDV LaSota vaccine. Panels A and C were run under denaturing conditions while panel B was obtained after protein separation in native PAGE. M – marker lane, NP – full length nucleocapsid protein; NP_{ct} -C-terminal fragment of nucleocapsid protein; P_u – unique C-terminal part of phosphoprotein; V_u – unique C-terminal part of V protein; C4BP – heptamerization domain of human C4 binding protein; N – Rosettagami *E. coli* lysate. Molecular weights of monomeric recombinant proteins (panels A, C) and multimeric conglomerates (panel B) are presented to the left of the marker lane.

Use of recombinant NDV proteins in indirect ELISAs (iELISA)

Indirect ELISA assays for all four recombinant NDV-antigens and an APMV-8 NP_{ct} antigen were established, applying a total of 51 sera from SPF chickens to determine cut-off values (Table 2.8). A defined positive serum (S880/Hu295) and a selected SPF-chicken

serum served as standards in each of the assays in order to determine validity (threshold OD values) and to calculate S/P ratios.

Analytical specificity of recombinant NDV NP- and P-gene derived proteins in iELISA with sera raised against different avian avulavirus subtypes

Recombinant iELISAs were used to test chicken hyperimmune sera raised against inactivated antigens of nine APMV serotypes; for APMV-1 and APMV-3 sera were raisted against each of 3 (APMV-1 class II/Ulster, PPMV, APMV-1 class I) and 2 (APMV-3/England; APMV-3/Netherlands) strains, respectively. Other sera were raised against APMV-2, -4, -6, -7, -8, -9. A commercial NDV ELISA based on virion-preparations as antigen was used as a control. In this ELISA, only 2 out of 3 APMV-1 sera reacted (Figure 2.8A) but a hyperimmune serum raised against APMV-1 class I was not detected. In addition, APMV-6, 7 and 9 hyperimmune sera showed clear cross reactivity (dark greyed columns in Figure 2.8), whereas the APMV-4 hyperimmune serum revealed marginal reactivity, but was considered APMV-1 positive by internal standards of the commercial ELISA. When using recombinant full length LaSota NP protein, positive reactions were obtained with all APMV-1 sera, including the APMV-1 class I serum. However, cross reactivity of APMV-6 and -9 specific sera remained (Figure 2.8A). In the NDV NP_{ct} based iELISA, in contrast, only NDV-specific sera raised against APMV-1 class II viruses (NDV LaSota, and the pigeon paramyxovirus type-1) reacted while all cross reactions were abolished (Figure 2.8B, left panel). No cross reactivity was observed with a serum specific for APMV-1 of class I. Conversely, by the APMV-8 NP_{ct} iELISA, the corresponding homologous antiserum was detected while cross reactions were limited to an APMV-7 serum only. Serotype specificity of NP_{ct} antigens to corresponding APMV-1 and APMV-8 hyperimmune sera was also confirmed in composite Western blot assays (Figure 2.9).

Similar to the recombinant full-length NP iELISA, cross reactivity of serotype-specific APMV sera was observed also for the iELISA when using the P_u fragment as the coating protein: Sera specific for APMV-3b (Netherlands), -4, -7, and -9 revealed reactivity above threshold level against the LaSota P_u protein (Figure 2.8C).

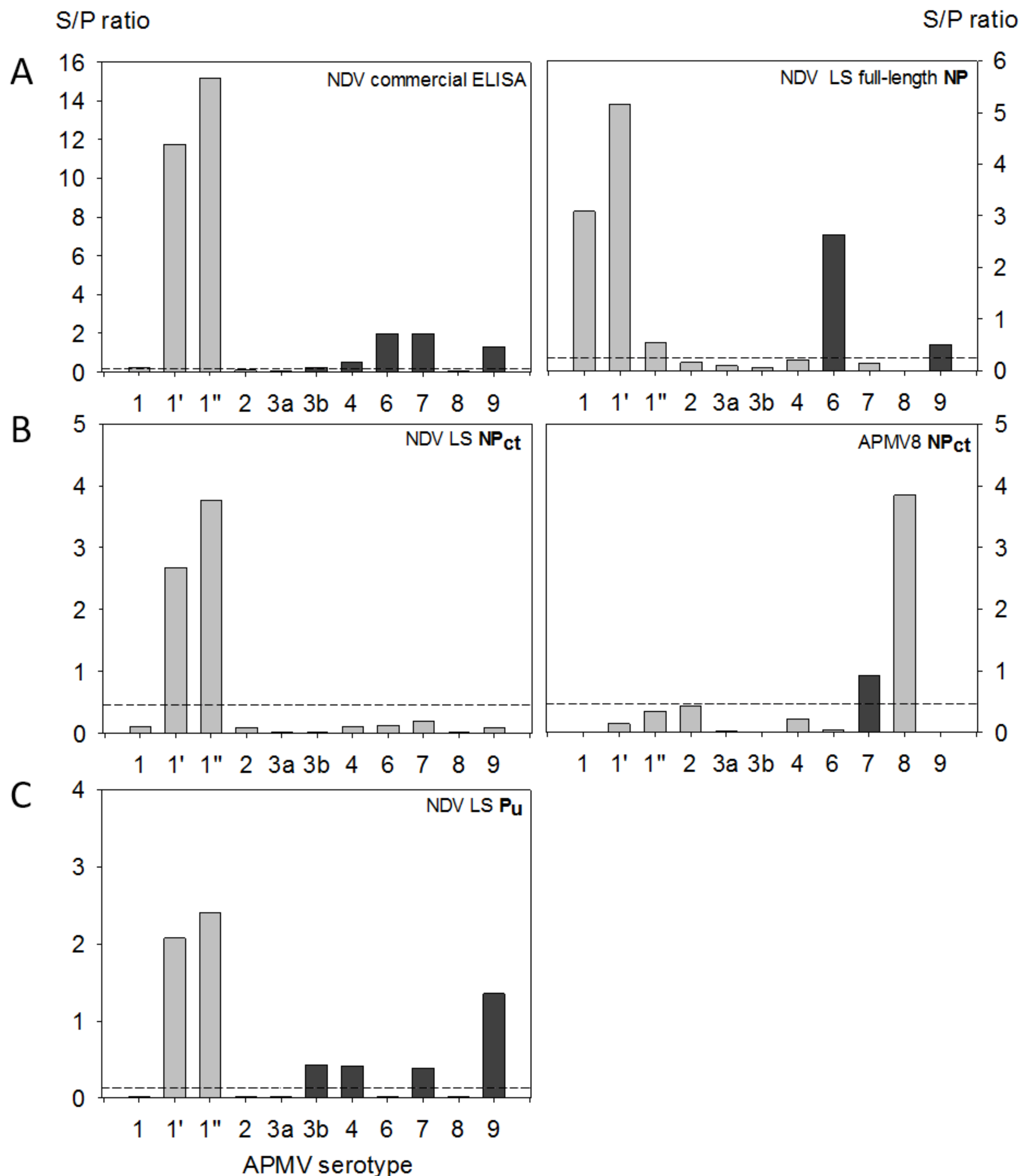


Figure 2.8 Use of NP and P gene products of APMV-1 and APMV-8 (NP) as diagnostic antigens in indirect ELISA compared to a commercial NDV ELISA. NDV commercial iELISA and Full-length NP of NDV strain LaSota (NDV LS full-length NP) iELISA were showed in panel A; C-terminal fragment of the NDV (NDV LS NP_{ct}) and the APMV-8 (APMV-8 NP_{ct}) NP protein based iELISA were presented in panel B; The truncated P (NDV LS P_u) iELISA were in the panel C. The chicken hyperimmune sera raised against avian avulavirus subtypes 1 through 9. 1 – APMV-1 class I, 1' – APMV-1 class II, LaSota, 1'' – APMV-1 class II, pigeon paramyxovirus, other serotypes as indicated. Dotted line indicates cut-off. Dark grey bars indicate unspecific reactivity.

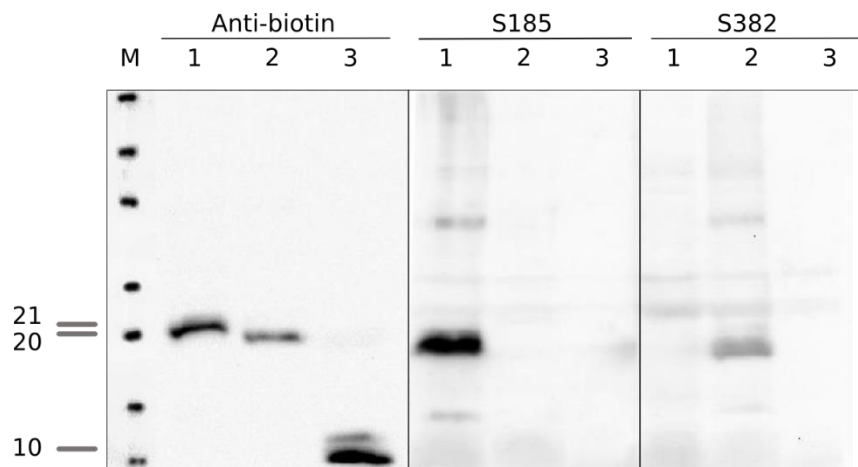


Figure 2.9 Assembled Western blot assays of monobiotinylated recombinant C-terminal fragments of NP proteins. APMV-1 in lane 1 (NDV LaSota, 21 kD), APMV-8 in lane 2 (20 kD), and the heptamerization domain of the C4 binding protein in lane 3 (10 kD). Anti-biotin – monoclonal antibody directed against biotin; S185 – hyperimmune serum raised in chicken against NDV strain Ulster; S382 –hyperimmune serum raised in chicken against APMV-8 strain/ goose/Delaware/1053/76. Molecular weights (kD) are indicated to the left.

Detection of NDV NP and P antibodies in vaccinated/challenged chickens

Ten chickens were immunized with a live-attenuated vaccine based on NDV strain "Clone 30" (Steglich et al 2013). Six SPF chickens were used as controls. At day 21 post vaccination, all chickens were challenged with the velogenic NDV strain "Herts" and serum samples of surviving chickens were taken at days 0 (= day 21 post vaccination), 3, 7, 14, 21, and 28 post challenge (p.c.). Results obtained with these sera in iELISAs using recombinant NP_{ct} and P_u proteins were compared to commercial NDV ELISA and to HI titers against the LaSota strain of NDV. HI titers higher than 1:8 was considered positive.

As shown in Table 2.6, NDV antibodies were detected in a majority of the vaccinated chickens at day 0 post challenge by HI, commercial ELISA and indirect NP_{ct} ELISA assays, whereas a lower number of birds were positive by the P_u ELISA assay. Moreover, in the unvaccinated control group, all chicken sera tested negative at day 0 p.c. and had seroconverted until 7 days p.c. in HI, commercial ELISA and indirect NP_{ct} ELISA assays, but complete seroconversion of the control group to P_u was delayed to day 14 p.c. (Table 2.6). This indicates that the iELISA based on the P_u protein is less sensitive than the other assays. The development of antibody titers as measured by the different ELISAs and

compared to HI titers of the vaccinated and control groups before and after challenge is shown in Figure 2.10.

When testing sera from fifteen chickens immunized with wild-type APMV-8 strain, 14 out of 15 serum samples were positive in the APMV-8 NP_{ct} iELISA. No cross reactivity was seen with the APMV-1 NP_{ct} based iELISA assay (Table 2.7).

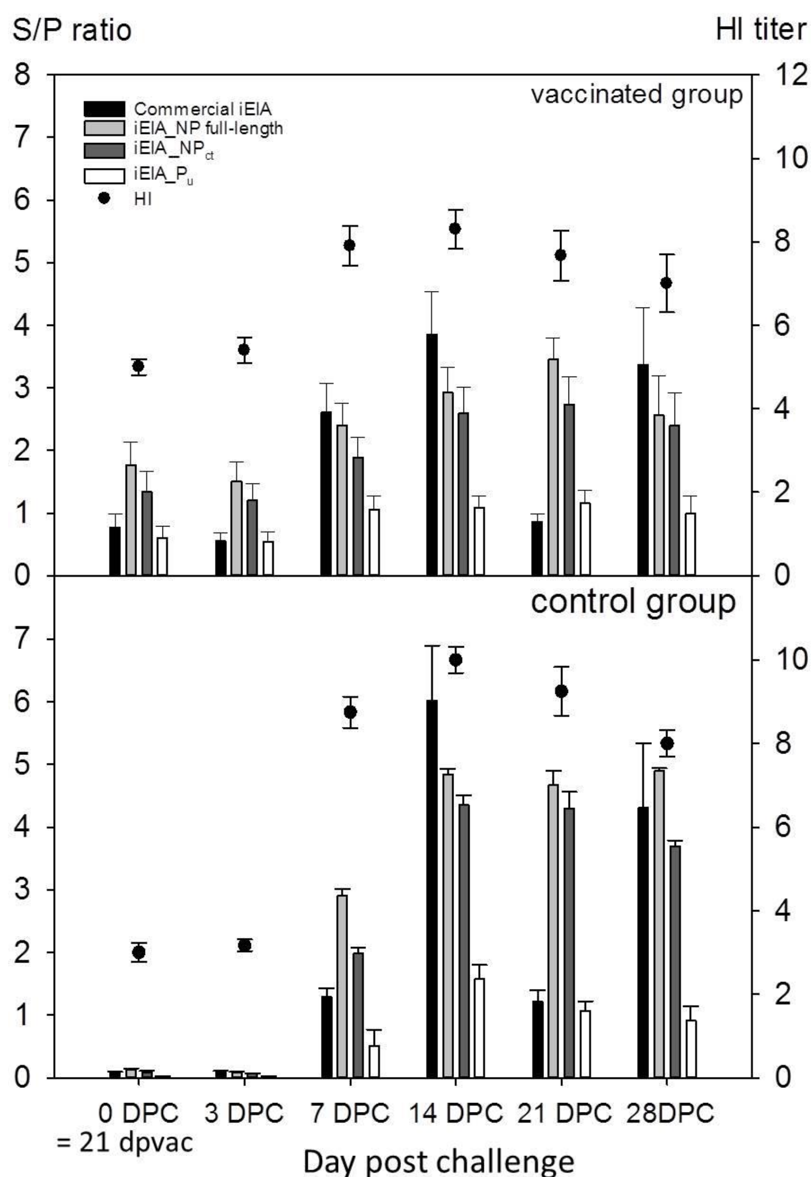


Figure 2.10 Antibody kinetics in NDV (Newcastle disease virus) vaccinated and challenged chickens against APMV-1 measured by different ELISA formats and HI (hemagglutinin inhibition) assay. Sera were obtained from ten chickens after immunization with a live attenuated vaccine based on NDV strain "Clone 30" and challenge at day 21 post vaccination (= day 0 post infection) with the velogenic NDV strain "Herts" (panel A). Panel B shows kinetics of six chickens that were not vaccinated before challenge. Antibodies were measured by indirect ELISAs as indicated in the figure legend or by HI assay against the NDV LaSota strain antigen.

Table 2.6 Serological investigation of chickens challenged with velogenic NDV strain Herts following vaccination with live-attenuated NDV vaccine.

		Serological assays				
DPC	Group	Commercial NDV iELISA	HI	iELISA-NP_{ct}	iELISA-P_u	iELISA-V_u
0 DPC	vaccinated	8/10[1]	8/10	9/10	9/10	2/10
	control	0/6	0/6	0/6	0/6	0/6
3 DPC	vaccinated	9/10	9/10	9/10	7/10	2/10
	control	0/6	0/6	0/6	0/6	0/6
7 DPC	vaccinated	10/10	10/10	10/10	8/10	7/10
	control	4/4	4/4	4/4	2/4	2/4
14 DPC	vaccinated	10/10	10/10	10/10	8/10	7/10
	control	4/4	4/4	4/4	4/4	4/4
21 DPC	vaccinated	9/9	8/9	9/9	8/9	7/9
	control	4/4	4/4	4/4	4/4	4/4
28 DPC	vaccinated	6/6	5/6	6/6	4/6	4/6
	control	4/4	4/4	4/4	4/4	2/4

Vaccinated group – Ten chickens each were vaccinated with NDV strain “Clone 30”

Control group – Six chickens did not receive vaccination before challenge

DPC – days post challenge; challenge infection with NDV strain Herts33 was carried out on day 21 post vaccination, i.e., day 0 DPC characterizes the status at day 21 post vaccination

HI – hemagglutination inhibition assay against NDV LaSota antigen

[1] N/M – numbers of seropositive/total chickens

Table 2.7 Qualitative results of serological investigations (seropositive/total samples analysed per assay).

serum panel	HI assay		Commercial ELISA	Recombinant ELISA			
	NDV LS	APMV-8		NP full-length	P_u	LS NP_{ct}	APMV-8 NP_{ct}
SPF	0/51	0/51	0/51	0/51	0/51	0/51	0/51
Experimental Infection NDV[a]	68/83	n.d.	68/83	69/83	58/83	68/83	0/83
Experimental Infection APMV-8[b]	0/15	15/15	0/15	0/15	n.d.	0/15	14/15
Field sera chicken	60/150	n.d.	103/150	91/150	90/150	79/150	0/150
Field sera turkey	67/147	n.d.	113/147	100/147	57/147	88/147	0/147

[a] Experimental NDV infection sera were obtained from chickens after immunization with a live attenuated vaccine based on NDV strain “Clone 30”; on day 21 post vaccination all chickens were challenged with the velogenic NDV strain “Herts”.

[b] Experimental APMV-8 infection sera originated from chickens that were immunized with wildtype APMV-8 strain APMV-8/goose/Delaware/1053/76 one day after hatch and challenged with APMV-8 on day 21 postvaccination. Samples used here for serological analyzes were taken on days 21 post vaccination and 14 post challenge.

Table 2.8 Determination of cut-off values of indirect ELISAs based on recombinant avian paramyxovirus NP and P gene products.

Serum samples	Values	Recombinant antigens				
		NP	P_u	V_u	NP_{ct}	APMV-8 NP_{ct}
SPF chicken sera ([S-N]/[P-N] values)	Mean	0.076	0.016	0.03	0.13	0.166
N=51	SD	0.09	0.059	0.027	0.161	0.149
Cut off	Xq + 2SD	0.257	0.134	0.083	0.451	0.465

Viruses used for production of recombinant proteins:

NP, P_u, V_u, NP_{ct} – LaSota strain of APMV-1

APMV-8 NP_{ct} – APMV-8/goose/2010

Antibody response to V_u protein in chickens vaccinated with live-attenuated NDV and challenged with a velogenic NDV strain

The same set of sera as described in the previous section was examined for V protein specific antibodies in the V_u iELISA. Before challenge with the velogenic NDV strain, the majority of vaccinated chickens had seroconverted by HI and commercial ELISA tests, but only two birds reacted in the V_u protein assay (Table 2.6). Following challenge, V_u-specific seroconversion was evident from day 7 p.c. onwards and a maximum of seven out of 10 of the challenged vaccines were positive. Additionally, V_u protein specific

antibody was detectable in all four unvaccinated control chickens from day 14 p.c. onwards. V_u-specific antibody titers declined in vaccinated and in control birds at day 28 p.c. (Table 2.6).

Detection of NDV-specific antibodies in chicken and turkey field sera

Finally, 297 field sera from NDV vaccinated chickens (n=150) and turkeys (n=147) were tested in a commercial indirect NDV ELISA, and by HI, and results were compared to data obtained with iELISAs using recombinant proteins (Table 2.7). There was high correlation between the commercial ELISA and the results obtained with recombinant full-length NP (Kappa=0.688 [95% CI 0.604-0.773]) while both assays only had moderate correlation with the HI assay using antigen of the NDV LaSota strain (commercial ELISA: Kappa=0.401; recombinant NP iELISA: Kappa=0.458). This was caused by a considerable proportion of sera which reacted positive in either of the ELISAs but remained negative by HI.

Results from APMV-1 NP_{ct} based iELISA yielded less numbers of positive sera compared to both full-length NP and commercial ELISA assays (Table 2.7). Comparison of results obtained with the commercial NDV ELISA and the two recombinant NDV NP antigens showed good agreement of the three assays (Venn diagram, Figure 2.11) although the NP_{ct} ELISA revealed reduced sensitivity. Including results obtained with the SPF sera and the experimental NDV infection sera, a sensitivity of 87.3% was calculated for the NDV NP_{ct} recombinant antigen using the HI as the standard assay. Inter-rater agreement between the NP_{ct} ELISA results and HI titers (kappa=0.53 [CI 0.438-0.622]) was slightly better compared to full length NP and the commercial ELISA. None of the field sera scored positive for APMV-8 NP_{ct} specific antibodies (Table 2.7).

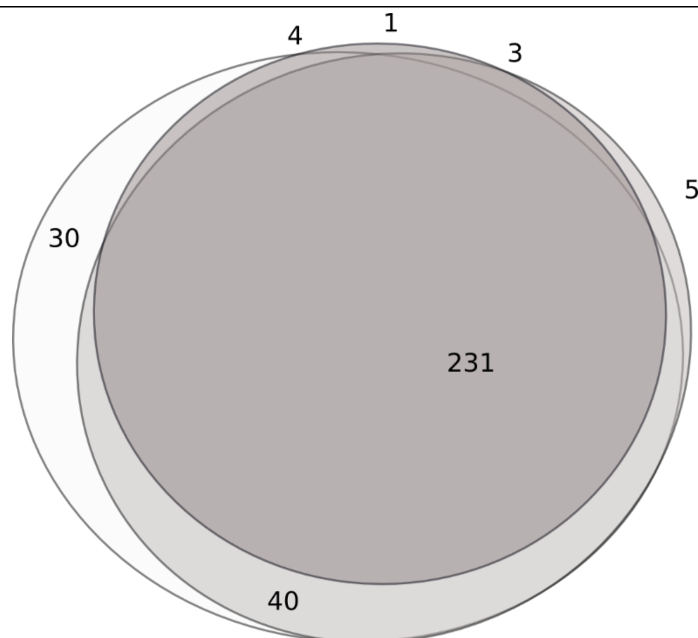


Figure 2.11 Venn diagram 223 of detection of NDV (Newcastle disease virus) NP-specific antibodies by ELISAs. Commercial indirect ELISA was shown by white ellipsis, recombinant indirect ELISA using full-length NP was shown by light grey ellipsis or the truncated C-terminal fragment of NP was shown by NDV-NP_{ct}, dark grey circle in chicken and turkey field sera. Numbers indicate positive sera in a given subset except for the centre of the circle where also congruently negative sera have been added.

2.2.4 Discussion

We successfully constructed, bacterially expressed, and purified full-length and truncated versions of the protein products of the NP and P genes of the APMV-1 strain LaSota. Truncations were chosen so as to express less conserved (C-terminal part of NP) or unique (P_u, V_u) amino acid sequence stretches (Figure 2.6). In order to improve the immunological reactivity of the rather small peptides (ranging from 11.7 to 27.6 kD) they were expressed as fusion proteins of the human C4 binding protein heptamerization domain (Shinya et al 2009). Analysis of refolded proteins revealed all of the C4BP-tagged proteins formed multimers with an approximately sevenfold molecular weight compared to monomers. This indicates that the C4BP domain is potent to promote protein multimerization. Part of full-length NP expressed without a C4BP tag was monomeric but spontaneous formation of dimers, trimers and multimers under non-denaturing PAGE conditions was observed as well (Figure 2.7). Spontaneous self-assembly/multimerization of full-length NP proteins of negative-stranded RNA viruses with helical nucleocapsids had been reported before (Bulavaite et al 2016, Errington & Emmerson 1997). Recombinant proteins were purified from bacterial inclusion bodies, solubilized, and

refolded in vitro, and their antigenic authenticity was confirmed using a chicken immune serum obtained after immunization with live-attenuated NDV vaccine (Figure 2.7). Results were encouraging to use the recombinant proteins as antigens for the detection of APMV1 specific antibodies.

Currently, serological testing for avian avulavirus antibodies, in particular of APMV-1, as employed, e.g., for surveillance purposes, is traditionally based on HI assay or indirect ELISAs using whole virus antigen (Senne et al 2004)[226] or full-length NP protein (Ahmad-Raus et al 2009, Errington et al 1995, Makkay et al 1999, Panshin et al 2000, Pinette et al 2014). Although ELISAs are widely used for screening purposes, little is known about their specificity regarding cross reacting antibodies induced after infection with other APMV serotypes. In this study, when testing hyperimmune sera raised in chickens against nine different APMV serotypes in the different ELISAs, cross reactions were observed for the commercial ELISA and also for the recombinant ELISAs based on full-length NP and the unique P proteins (Figure 2.8A and C). As hypothesized, specificity increased when using a less conserved C-terminal fragment of the NDV or the APMV-8 NP proteins (Figure 2.8B). Our results confirmed that the C-terminal NP fragment holds potential as a highly serotype-specific diagnostic antigen. However, compared to full-length NP, the number of antigenic sites is substantially reduced. This fact may be at the basis of the lower sensitivity of NP_{ct} driven indirect ELISAs (compared to commercial and full-length NP ELISAs) as observed in this study with field sera (Figure 2.11, Table 2.7). This may limit the general use of NP_{ct} fragments if a screening assay of high sensitivity is required. However, higher sensitivity, i.e., the use of complete virus or full-length NP protein as diagnostic antigens, is achieved only at the expense of a loss of specificity as our data on APMV serotype-specific hyperimmune sera showed. The NP_{ct} fragment, in contrast, provides high serotype specificity similar to that of HI assays.

To evaluate the V protein as a target antigen in the NDV DIVA strategy, sera of chickens immunized with live attenuated NDV clone 30 vaccine and subsequently challenged with virulent Herts33 were used. Notably, the complete V protein is generated from the P gene via post-transcriptional mRNA editing, and thus P and V proteins possess an identical portion at their N-terminus. To eradicate the cross reaction between P and V proteins, the distinctive C-terminal fragment of the V protein (V_u) was expressed as target antigen in the iELISA. As a result, only a few of the animals (2/10) seroconverted against the V protein after immunization with live attenuated vaccine, but when challenged with a virulent virus this number of animals rose to seven out of ten (Table 2.6). In conjunction with the observation that non-vaccinated control animals seroconverted against V_u after

challenge with the velogenic NDV Herts strain, these results indicated that V protein may be more immunogenic during infection with virulent strains. The data gave evidence that, on a flock level, (i) seroconversion to V after application of live-attenuated vaccine was incomplete and (ii) V-specific antibody titers declined within a few weeks to below detection limits. This significantly limits the use of a V protein-based DIVA strategy for NDV.

2.2.5 Conclusions

In this study, a newly configured bacterial expression system featuring co-translationally monobiotinylated fusion proteins of small protein fragments of NDV NP and P (V) gene products fused with the heptamerization domain of human C4BP yielded immune reactive products. Utility of the recombinant proteins as serodiagnostic antigens was demonstrated in indirect ELISA assays: (i) the NP_{ct} protein fragments of NDV and APMV-8 enabled a serotype specific diagnosis by ELISA and might be exploited as alternative, highly serotype-specific diagnostic tools for classical HI; (ii) the NDV V protein is able to induce some weak humoral immune response after immunization with an attenuated NDV vaccine strain. However, V-specific seroconversion within an experimentally infected flock was incomplete, and titers in individual bird transient. Therefore, a V protein-based serological DIVA strategy to distinguish infected from vaccinated (inactivated vaccines) birds is not feasible; (iii) the full-length NP protein as the ELISA antigen possessed comparable diagnostic features compared to a whole virus antigen based commercial ELISA, but cross reactivity among APMV serotypes may blur specificity.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ZN and TH conceived and designed the study. ZN had a major role in conducting the experiments. CG was responsible for obtaining avian sera. ZN and TH took the lead in drafting the manuscript. All authors critically analysed and interpreted the data. All authors read and approved the final manuscript.

Acknowledgements

N. Zhao is a recipient of a fellowship of the China Scholarship Council. We thank Dr. Y. Zheng for proof reading the manuscript. We thank all technical staff of the FLI for excellent assistance.

Funding

N. Zhao is a recipient of a fellowship of the China Scholarship Council. The Council had no influence whatsoever on any issue regarding this study.

2.3 Third Study: Tetraplex Fluorescent Microbead-Based Immunoassay for the Serodiagnosis of Newcastle Disease Virus and Avian Influenza Viruses in Poultry Sera

Na Zhao [1,2], Christian Grund [1], Martin Beer [1], Gang Wang [2] and Timm C. Harder [1,*]

[1] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Suedufer 10, 17493 Greifswald, Germany

[2] Precise Genome Engineering Center, School of Life Sciences, Guangzhou University, 510030 Guangzhou, China

*Correspondence: timm.harder@fli.de; Tel.: +49 38351 715 46

This article has been published in pathogens 2022, 11, 1059. https://doi.org/10.3390/ pathogens11091059

This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution
(CC BY) license (https://creativecommons.org/licenses/by/4.0/).

Abstract

Virulent Newcastle disease virus (NDV) as well as highly pathogenic avian influenza (HPAIV) subtypes H5 and H7 induce contagious and lethal systemic disease in poultry. In contrast, low pathogenic AIV H5 and H7 may circulate clinically unnoticed in poultry but eventually generate HPAIV. Low pathogenic NDV strains are widely used as live-attenuated vaccines against ND. Serological tools are essential to conduct active surveillance for infections with notifiable AIV-H5, - H7 and to control vaccination against NDV and HPAIV in poultry populations. Here, recombinant nucleocapsid proteins (NP) of AIV and NDV, and haemagglutinin protein fragment-1 (HA1) of AIV subtypes H5 and H7 were expressed in E. coli. Purification and refolding were required before coating fluorescent microspheres via streptavidin-biotin linkage. The tetraplexed inhibition fluorescent microsphere immunoassay (iFMIA) was then assembled for analysis on a Luminex® like platform (Bioplex®) using murine monoclonal antibodies specific for each of the four targets. The assay was evaluated by testing galliform poultry sera derived from experimental infections (n = 257) and from farms (n = 250), respectively. The tetraplex iFMIA compared favorably with commercially available ELISAs and the “gold standard” hemagglutination inhibition assay. Tetraplexed iFMIA provided a specific and sensitive tool to detect and discriminate AIV- and NDV-specific antibodies in the sera of galliform poultry.

Keywords: Newcastle disease virus; avian influenza; serosurveillance; fluorescent microspheres; immunoassay; multiplex detection

2.3.1 Introduction

Newcastle disease in poultry is caused by virulent forms of the avian orthoavulavirus-1 (AOAV-1), commonly referred to as Newcastle disease virus (NDV) (Dimitrov et al 2019). Avian influenza viruses (AIV) are influenza type A viruses which hold generic status in the Orthomyxoviridae family. So far, sixteen subtypes of the hemagglutinin (HA) and nine of the neuraminidase (NA) proteins of AIV have been distinguished in avian species (Spackman 2014). Velogenic/virulent Newcastle Disease (ND) caused by infections with NDV and highly pathogenic influenza A (HPAI) of subtypes H5 or H7, respectively, range among the most-dreaded poultry diseases worldwide. Consequently, infections in poultry with AIV subtypes H5 and H7 of both low and high pathogenicity, and with velogenic ND, are globally notifiable to the O.I.E. A tight control of these infections requires (i) prevention of virus incursions by advanced biosecurity measures, (ii) detection of outbreaks as early as possible using molecular diagnostics, (iii) suppression of further virus spread by harsh restriction measures, and (iv) eradication from affected poultry populations by, ultimately, culling affected holdings and vaccination (Sims 2013). Yet, these viruses remain enzootic in several regions of the world and continue to threaten industrial as well as backyard poultry rearing on a broad scale (Sims 2013, Spackman 2014).

Vaccination with inactivated or live virus vaccines featuring low virulent (lentogenic) NDV strains such as LaSota or B1 is a widely used preventive measure against ND (Senne et al 2004). In some countries, including Germany, vaccination of all gallinaceous poultry against NDV is mandatory. Vaccination against notifiable AI (subtypes H5 and H7) employing inactivated adjuvanted vaccines or recombinant modified-live virus vaccines, including NDV as a backbone (Steglich et al 2013) expressing the HA of AIV, is used more restrictedly. Currently, AI vaccination is restricted for use in poultry populations in which AIV has gained endemic status (Sims 2013).

Serological monitoring of poultry populations for NDV and AIV subtypes H5/H7 is an important control tool to estimate infection prevalence or to evaluate efficacy of vaccination campaigns (Wood et al 2012, Zhao et al 2018, Zhao et al 2013). A number of serological assay formats such as agar gel immunodiffusion (AGID), hemagglutination inhibition (HI), and enzyme-linked immunosorbent assays (ELISA) are available for this purpose (Chappell et al 2014, Grund et al 2011, Jenson 2014). The AGID test detects precipitating antibodies and although it is a robust test format it suffers from limited sensitivity and cannot be used with sera of waterfowl (Jenson 2014). The HI assay detects antibodies that specifically inhibit hemagglutination of NDV and AIV H5/H7 due to

interference with receptor-binding of the NDV hemagglutinin-neuraminidase (HN) or the AIV HA proteins, respectively; this assay remains the “gold standard” for detecting NDV or subtype influenza virus-specific antibodies (Charlton et al 2009, Miller & Torchetti 2014). Unfortunately, HI assays are labor intensive, require the production of antigens from potentially hazardous viruses, and, due to the high specificity of the assay, sensitivity may suffer if the antigenic profiles of the test antigen and the field viruses in circulation are not sufficiently matching (Katz et al 2011, Wood et al 2012). ELISA-based assays for detection antibodies may overcome many of these limitations: They are easy and safe to use and can be tailored more flexibly to the diagnostic needs. Several ELISA kits specific for the NDV nucleocapsid protein (NP) or the AIV NP, H5 and H7 HA proteins are commercially available (Grund et al 2011, Häuslaigner et al 2009, Zhao et al 2018). However, such solid phase ELISAs are limited to detecting antibodies against a single target and demand substantial costs (and time) when antibodies against all four targets mentioned above are to be measured.

The Luminex® suspension array system utilizes xMAP® technology and permits the multiplex probing of up to five hundred different analytes within a single reaction. Luminex technology enables the fast, accurate, and flexible measurement of multiple biomarkers such as RNA or protein targets simultaneously in a single sample (Bohm et al 2017, Chen et al 2015, Dunbar et al 2015, Jimenez-Munguia et al 2017, Kamminga et al 2018, Li et al 2017a, Mechaly et al 2018).This technique is based on distinct fluorescent color-coded beads that can be conjugated with analyte-specific targets. Multiplexing is achieved by mixing different distinctly coded analyte-specific beads that are probed in a liquid suspension assay. This assay format combines the advantages of solid phase ELISAs with the benefits of a lower sample volume, reduced costs and turn-around time if multiplexing is applied.

Here we explored a tetraplex inhibition fluorescent microsphere immunoassay (4plex iFMIA) based on Luminex® bead technology to simultaneously detect NP-specific antibodies of NDV and AIV as well as AIV H5- and H7-specific antibodies using recombinantly expressed proteins as targets in a blocking liquid array format.

2.3.2 Materials and methods

Animal Experimentation Permits

All animal experiments for the production of reference sera received full legal ap-proval by the animal welfare committee of the German Federal State of Mecklen-burg-Western Pomerania (LALLF M-V/TSD/7221.3-2.5-004/10; LALLF M-V/TSD/7221.3-2.5-010/10).

Virus propagation

New castle disease vaccine strain LaSota (LS) and AI viruses were grown in the allantoic cavity of embryonated chicken eggs as detailed elsewhere (Häuslaigner et al 2009, Starick et al 2011b). Recombinant influenza A virus nucleoprotein was produced from A/swine/Germany/R1738/2010 (H1N1, [EPI426141]) and recombinant NDV nucleoprotein was derived from NDV vaccine LaSota. Truncated AIV recombinant hemagglutinin proteins originated from A/turkey/Germany/R1612/08 (H5N3 LP) and A/chicken/Germany/R28/03 (H7N7 HP, [AJ620350]).

Cloning and bacterial expression of recombinant proteins

Recombinant pET19b vector was used to express ND and AI proteins in Rosettagami *E. coli* cells (Novagen, Darmstadt, Germany). Full length ORFs encoding the NP of NDV LaSota (489 amino acids) and AIV (498 amino acids) were cloned each into pET19b downstream of the T7 promoter using primer-directed techniques. An octa-histidine and an Avi-tag, the latter used as a biotin acceptor site (Rigaut et al 1999) were positioned N-terminally of the NP (Figure 2.12). Similarly, fragments of the hemagglutinin HA1 coding part representing amino acids (aa) 17-342 (H5) and 19-337 (H7), respectively, of the AIV isolates were cloned into pET19b (Figure 2.12). Plasmid pBirCam (Avidity, Aurora, U.S.A) over-expressing the bacterial biotin ligase BirA was co-transformed with either of the four constructs into *E. coli* strain Rosettagami to ensure co-translational mono-biotinylation of the recombinant NP and HA1 proteins at the lysine residue of their respective Avi-tag (Zhao et al 2018, Zhao et al 2013). Ampicillin and chloramphenicol were used for selection, and plasmid-insert-specific PCRs were used for identification of double transformants.

The induction of expression was achieved in tryptone-yeast-hepes (TYH) medium (20 g of tryptone; 10 g of yeast extract; 5 g of NaCl; 1 g of $MgSO4$; 11 g of HEPES in 1 L aq. bidest.) supplemented with IPTG (1.5 mM) and D (+)-biotin (50 µM). After culturing for 4 h at 37 °C, the cells were pelleted and lysated using ultrasonic disruption, as previously

described (Khurana et al 2011), in order to liberate the inclusion bodies (ICs) into which the recombinant proteins had sequestered.

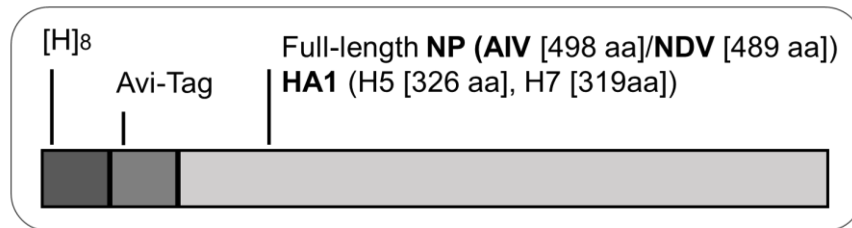


Figure 2.12 Schematic presentation of constructs used for the generation of pET19b plasmids and T7-driven expression of NDV (Newcastle disease virus) and AIV (avian influenza virus) proteins in Rosettagami *E. coli* cultures. Protein N-terminus is shown on left with N-terminal octahistidin ($[H]_8$)- and Avi-tags. The Avi-tag is used for mono-biotinylation in vivo. “aa” —amino acid.

Purification and reconstitution of recombinant proteins from bacterial inclusion bodies

Purified inclusion bodies (ICs) were solubilized in 6 M of guanidinium-HCl. A set of refolding buffers was used in a stepwise solubilization strategy (ProteoStat kit, Enzo, Lörrach, Germany). Finally, an optimized refolding buffer was identified consisting of 50 mM of Tris, a pH of 8.3, 20 mM of NaCl, 0.8 mM of KCl, 0.8 M of L-Arginine, and 0.12 M of sucrose. Antigenic properties in solid phase ELISA were assayed using polyclonal antibodies raised in chickens or with specific monoclonal antibodies. The final protein concentration in refolding buffer was determined using a Coomassie protein assay kit (ThermoScientific, Rockford, IL, USA) and proteins were stored at 4 °C until further use.

Production of positive control sera

Serum S185, a hyperimmune serum raised in chicken against inactivated NDV strain Ulster, served as standard positive control for assays employing NDV rNP. Likewise, hyperimmune serum S304, produced against the AIV isolate A/duck/Italy/636/2003 (H7N3) and serum S82, raised against the HA of A/Vietnam/1194/2004 H5N1 [expressed by recombinant A/Puerto Rico clone NIBRG-14; kindly provided by Dr. J. McCauley, Mill Hill, UK, in the frame of the WHO PIP program] were used for assays employing AIV rNP, H5-rHA1 or H7-rHA1. An SPF chicken serum was chosen as negative control. The immune status of these control sera was assessed by three different methods (Table 2.12). These control sera were used as standards in each of the assays to determine validity (threshold FI [fluorescence intensity]) and to calculate S/N ratios.

Production of experimental infection or vaccination sera in chickens

Table 2.9 gives an overview of the status of different sera series used for evaluation of the 4plex iFMIA assay. Sera were produced in chickens in the frame of several animal experiments conducted at FLI.

Origin of Field Sera Not Used for Reference Purposes

A total of 150 chicken and 130 turkey sera submitted for routine AI or ND serodi-agnosis originated from various poultry holdings in Germany and were investigated by 4plex iFMIA assay and other routine assays in this study.

Monoclonal antibodies (mAbs)

A mAb-horse raddish peroxidase (HRPO) conjugate specific for biotin was purchased from NEB (#7075). MAb NP-36, specific for the NP protein of NDV, had been generated against NDV strain LaSota (Werner et al 1999) and was kindly provided by Dr. B. Koellner, Friedrich-Loeffler-Institute, Riems, Germany. The NP protein of influenza A viruses is recognized by mAb HB-65 which was purchased from ATCC (H16-L10-4R5). The AIV HA H5 specific monoclonal antibody 8292, generated against recA/Vietnam/1194/2004 (NIBRG-14), was provided by Dr. R. Ehricht (Alere, Jena, Germany) while Mab 15C7 raised against an unknown low pathogenic AIV isolate of subtype H7 was kindly supplied by Dr. P.Pourquier (ID-Vet, Montpellier, France) (Postel et al 2011a). A phycoerythrin (PE)-labeled mAb directed against hexahistidine was purchased from Abcam (Ad1.1.10).

Western blotting

Protein samples were separated by denaturing SDS-PAGE in 12.5% gels and transferred onto nitrocellulose membranes by semi-dry blotting at 0.8 mA/cm^2 for 1 h (PerfectBlue™ Electro Blotter, Peqlab, Erlangen, Germany). Membranes were blocked with 5% skim milk powder in Tris-buffered saline (TBS) supplemented with 0.05% (v/v) Tween 20 (TBST) for 1h. Specific mAbs or sera were appropriately diluted in TBST and incubated on the membranes for one hour at room temperature. Membranes were washed three times with TBST and incubated for another hour with anti-murine IgG or -gallid IgY HRPO-labeled secondary antibody conjugate (Santa Cruz Biotechnology). Blots were washed three times, incubated with SuperSignal™ West Pico Chemiluminescent substrate solution (ThermoScientific, Braunschweig, Germany) before being analyzed on an imaging system (VersaDoc, Bio-Rad). Photos were edited with respect to contrast and brightness and composite blots were assembled using cut-out lanes (GIMP software).

Coupling of recombinant antigens to strepavidin precoated Luminex beads

Refolded, biotinylated recombinant proteins were coupled to individual strepavidin precoated Lumilite® beads according to the manufacturer's instructions (Progen Biotechnik Corporation, Heidelberg, Germany). Briefly, the recombinant proteins NDV-rNP, AIV-rNP, AIV subtype H5-rHA1 and AIV subtype H7-rHA1 were coupled with $2×10^6$ fluorescent beads (Lumilite® MMLL03-10, -23, -50, -56) by 100 µg, 200 µg, 25 µg and 20 µg of each antigen respectively, shaking for more than 2 hours at the room temperature in the dark, then the beads were washed three times in assay buffer (PBS [pH 7.4] supplemented with 1% [w/v] biotin-free bovine serum albumin, and 0.05%[v/v] Tween 20). After coupling, the antigen- coated beads were stored in the dark at 4℃. Microspheres were protected from the light throughout the whole procedure.

Multiplex bead-based immunoassay

Multiscreen HTS IP 0,45µm 96-well filter plates (Millipore, Bedford, Mass.) were prewashed once with 150 µl of assay buffer and washing fluids were aspirated using a vacuum manifold (Millipore, Bedford, Mass.). Recombinant antigens coated beads were sonicated and vortexed for 20s to disrupt the aggregation, then mixed and diluted to a final concentration of $4×10^4$/mL beads each in assay buffer. Finally, 50 µL of this suspension containing 2000 beads/antigen was used per well of the filter plate in this assay. Next, serum samples diluted 1:20 in 50 µL assay buffer were applied to the plates and incubated for 40 min, then removing the solution and washing the beads. Meanwhile, a set of four previously determined serum (S185, S82, S304 and SPF, [Table 2.12]) were run on each plate as inter-assay and controls. After that, the selected monoclonal antibodies against four antigens diluted properly individually were mixed together, taken 50 µL into each well for incubation for 30 min. After washing, 50 µL of diluted 1:4000 PE-labelled polyclonal antibodies raised in goats against murine IgG (Fab')2 (Santa Cruz Biotechnology, Heidelberg, Germany) were added to each well and incubated for 30 min. After final washing, the beads were resuspended in 125 µL of assay buffer, and plates were placed on the shaker for 15 min to counteract beads aggregation. In this assay, washing step performed three times with 200 µL assay buffer and solution was aspirated from the plate by the vacuum manifold. All incubation steps were carried on a rocking platform in the dark at room temperature. The samples were measured in a BioPlex 100 instrument running the Bioplex Manager 6.1 software (Bio-Rad, California, USA). The instrument was calibrated using the Bio-Rad calibration sets CL1/CL2 before each use, and 50-100 beads per species and samples were measured in a sample volume of 50 µL. Throughout, the results were calculated and presented as S/N ratios, in which S value

arises from median fluorescence intensity (MFI) of samples and N is from MFI of negative control.

Commercial ELISAs for detection of AIV and NDV antibodies

A commercial indirect NDV-ELISA (Flock Chek NDV) based on complete ND virions was purchased from IDEXX (Ludwigsburg, Germany). Instructions of the manufacturer were followed exactly using either the version for chickens or turkeys as appropriate. Commercial AIV competitive ELISA kits (ID.Vet, Montpellier, France) were used for AIV NP, H5 or H7 specific antibody detection in poultry sera. Assays were performed according to the manufacturer's protocols. For NDV assay, sera were considered as positive if the S/P ([sample- negative control]/[positive control-negative control] OD450) ratio was >0.2. For AIV assay, inhibition values were calculated by [Sample OD450/Negative control OD450] *100, then compared with the recommended cut-offs by the manufacturer to determine the sample sera as “positive”, “questionable” or “negative”.

Hemagglutination inhibition assay (HI)

HI assays were performed according to O.I.E. recommendations as described by Grund (Grund et al 2011). Briefly, four hemagglutinating units of inactivated antigens of NDV strain LaSota or AIV strains A/common teal/England/7894/2006 (H5N3) and A/turkey/England/647/1977 (H7N7) were used. The AIV antigens were produced at the Community Reference Laboratory for Avian Influenza at APHA, Weybrigde, UK, and are used in the European sero-monitoring program for poultry. All sera had been heat-inactivated for 30 minutes at 56°C. Serum HI titers >1:8 was considered positive.

Statistical analysis

For the four serological assay systems held here as a gold standard (HI NDV, HI H5, HI H7, ELISA NDV) prevalence were estimated separately for the groups of sera from experimental infections (EXP) or from the field. Cut-off values for the 4plex iFMIA were selected according to receiver-operating-curve analyses (ROC) with regard to the criterion “minimum ROC distance” whereby combining groups EXP and Field. Sensitivity and specificity were calculated on basis of the gold standard assays, and for each system all assays were compared pairwise using kappa statistics; EXP and F groups were treated separately. Calculations were performed using the program “R” (version 2.13.0 (2011-04-13) with the package Diagnosis Med, Version 0.2.3.) according to the R Development Core Team (Fawcett 2006).

2.3.3 Results

Bacterial expression of recombinant proteins

Recombinant proteins were purified from bacterial inclusion bodies, denatured and refolded in vitro. Biotinylated protein products matching the predicted molecular weights of 56.1 kDa (NDV-rNP), and 59.2 kDa, 39.7 kDa, and 37.9 kDa for IAV-rNP, rH5-HA1, and rH7-HA1, respectively, were detected by Western blotting using a biotin-specific mAb (Figure 2.13). The proteins also reacted specifically with positive polyclonal chicken control sera against NDV and AIV H5/H7 viruses (Figure 2.13 B-D) although these were raised against heterologous antigens. The influenza rNP antigen, although derived from a porcine H1N1 virus, was recognized by sera raised against avian-origin influenza A viruses of subtypes H5N3 and H7N7 (lanes 1 of Figure 2.13C, D). The NDV-rNP antigen originating from the LaSota vaccine strain reacted with sera produced against inactivated NDV strain Ulster (lane 4, Figure 2.13B). Similarly, a chicken serum raised against H5N1 was specific for the rHA1 protein of H5N3 (lane 2, Figure 2.13D) and serum S304 raised against H7N3 was specific for the rHA1 proteins of H7N7 (lane 3, Figure 2.13). Results indicate that recombinant proteins of the expected sizes and broadly specific antigenicity were successfully expressed, purified and refolded.

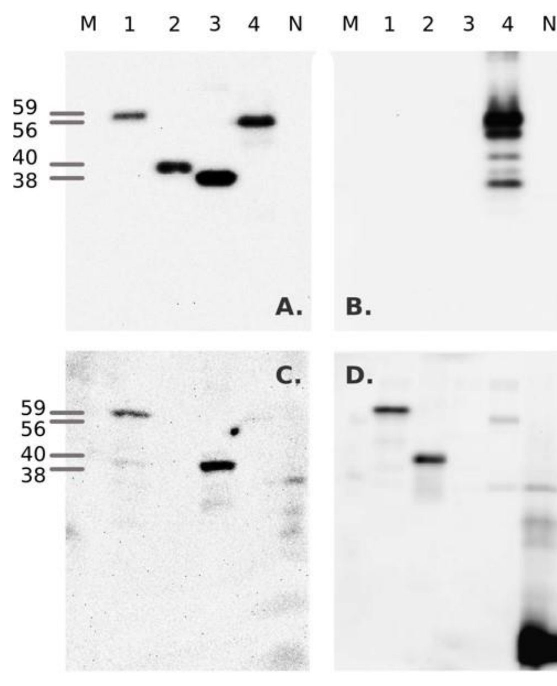


Figure 2.13 Assembled Western blots of recombinant, in vivo biotinylated HA1 and NP proteins of avian influenza and Newcastle Disease viruses. M – protein size standards (the size of relevant proteins is indicated to the left), 1 – rNP_R1738/10; 2 – rHA1_H5_R1612/11; 3 – rHA1_H7_R28/01; 4 – rNP_NDV_LS; N – lysate of *E. coli* Rosettagami cells transfected with an empty pET19b vector and induced with IPTG. Blots were stained as follows: A – anti-biotin monoclonal antibody, B – chicken serum S185 raised against NDV strain Ulster, C – chicken serum S304 produced against A/duck/Italy/636/2003 H7N3, D – chicken serum S82 against rHA A/Vietnam/1194/2004 H5N1 [NIBRG-14]).

To establish the inhibition fluorescent microsphere immunoassay, the specific epitopes within these four antigens which can be recognized by monoclonal antibodies should be preserved properly after refolding. A solid phase indirect ELISA format was used to verify authentically refolded epitopes. Briefly, the four recombinant proteins were coated in a 96 well plate each, then dilution series of the four mAbs were added, finally a HRPO-conjugated antibody against mAbs was detected to evaluate interactions between recombinant proteins and their corresponding mAb; TMB substrate was used. As a result, all four recombinant antigens were recognized at high dilutions by the specific mAbs (data not shown).

Development of the 4plex iFMIA assay

The principle of our 4plex iFMIA assay used in this study is depicted in Figure 2.14. The biotin labeled recombinant proteins were coupled to strepavidin precoated luminex beads

for setting up the 4plex iFMA assay. To optimize the quantity of antigens coupled, a series of different concentrations of recombinant proteins coated with beads were assayed by a PE-labeled mAb against HIS tag, which were measured in the BioPlex 100 instrument. The quantity of protein was considered optimal if the MFI values were between 2000-6000 (data not shown). As a result, the optimal quantity of NDV-rNP, IAV-rNP, AIV H5-rHA1 and AIV H5-rHA1 was 50 µg, 100 µg, 12.5 µg and 10 µg of protein/10^6 beads, respectively. These protein quantities were used for all subsequent experiments. Next, the checker-board titration test was applied to optimize further assay conditions, i.e., dilutions of serum sample, each mAb (against four antigens) and PE-labelled polyclonal anti-murine antibodies. Finally, sample sera were diluted 1:20 and the 2nd PE-labeled antibody 1:4000; the four mAbs against NDV-rNP, IAV-rNP, AIV H5-rHA1, AIV H5-rHA1 were used at dilutions of 1:40, 1:320, 1:3000, and 1:4000, respectively. Lastly, the Luminex beads were resuspended in the assay buffer and analyzed in the Bioplex 100 instrument, which enabled to identify the unique fluorophore-encoded beads and the PE fluorescence signal of each bead. Due to the mAb-directed blocking principle described above, this assay is species independent.

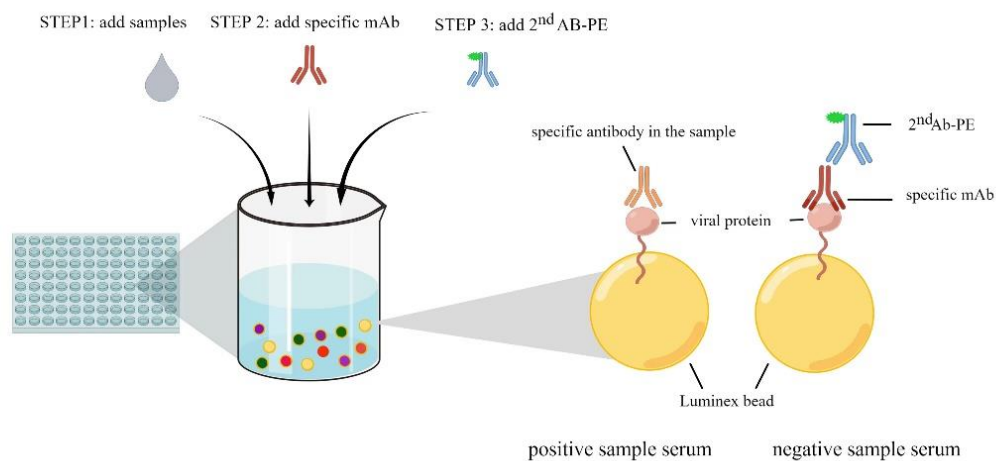


Figure 2.14 Schematic principle of the Luminex bead-based assay utilized in this study. Appropriate amounts of four recombinant antigens were coated onto each of our Luminex microbead species distinguished by the emitted fluorescence; aliquots of 2000 antigen-coated microbeads/protein were used in assays. Samples were incubated with mixtures of the four microbead species for 30 min; beads were then washed to remove unbound components (washing steps also followed all further incubation steps) (step 1). Mixtures of four monoclonal antibodies specific for each of the recombinant target proteins were added into each well and incubated for 30 min (step 2). PE-labelled polyclonal antibodies raised in goats against murine IgG were incubated with beads for 30 min. After the final washing, the microbeads were resuspended in assay buffer and left shaking for 15 min (step3). The samples were measured and data analyzed in the Luminex-BioPlex 100 instrument.

4plex iFMIA cut off determination and performances compared with ELISAs and HI

To set the cut off values for the 4plex iFMIA, a series of sera derived from experimental NDV/AIV infected and/or vaccinated animals with different immune status were tested (Table 2.9). Receiver operating characteristic (ROC) curve analysis was used to analyze the data and calculate cut off values for each of the four antigens, in which the HI assay was used as “golden standard”. As a result, the cut off of NDV-rNP, AIV-rNP, H5-rHA1, H7-rHA1 were 91.8, 75.4, 61.3 and 39,4 respectively (Figure 2.16).

According to the cut off values above, the sensitivity and specificity for the 4plex iFMIA assay for recombinant proteins NDV-rNP, AIV-rNP, H5-rHA1, H7-rHA1 were calculated individually under 95% C-Index (CI) condition by ROC curve analyses. Results are presented in Table 2.10. The AUCs (area under the ROC curve; values equal to 1 represent perfect performance) measured 0.937 for NDV-rNP, 0.987 for AIV-rNP, 0.996 for H5-rHA1 and 0.986 for H7-rHA1. These data indicate that the 4plex iFMIA assay exhibited promising sensitivity and specificity using sera derived from experimentally infected poultry. Meanwhile, commercial ELISAs for each pathogen were also used to detect specific antibodies of these experimental sera. Thereafter, data were also analyzed by ROC similar to those of iFMIA for comparison purposes (Table 2.10). The results revealed that for all four targets the performance characteristics of the 4plex iFMIA compared favourably with the commercial ELISAs when using HI titers as a gold standard. It should be noted that for detection of H5 and H7 specific antibodies the 4plex iFMIA showed a slightly increased sensitivity compared to commercial ELISA. According to these cut off values, we re-determined the number of positive samples among the sera obtained from experimental infections/vaccinations. Results from this analysis are summarized in supplementary Table 2.13. Here, in seven groups of sera with different immune status, rates of seropositives as analysed by HI, ELISA and iFMIA assay revealed a high level of congruence.

Furthermore, sera from thirty SPF chickens which received immunization with a live-attenuated vaccine based on NDV strain “Clone 30” were examined for the kinetics of antibody development (Figure 2.15). Results indicate that the NDV NP specific seroresponse can be accurately detected by the 4plex iFMIA format, similar to commercial ELISA and homologous HI assay. Simultaneously, chickens remained seronegative for AIV by 4plex iFMIA and the other assays indicating a high level of congruence among the three assay formats (data not shown)

Table 2.9 Origin and characteristics of 177 NDV- and AIV-specific sera and 80 SPF sera obtained from chicken and turkeys.

Panel	Serum source	No. of samples	Species	Flock immune status			
				ND-NP	AI-NP	H5	H7
A	ND vaccination, EXP	70	chicken	+	-	-	-
B	AI H5/ND vaccination, EXP	40	chicken	+	+	+	-
C	AI H7 vaccination, EXP	10	chicken	-	+	-	+
D	AI H7 infection in ND vaccinated birds, EXP, Field	11	turkey	+	+	-	+
E	recND-H7 vaccination, EXP	26	chicken	+	-	-	+
F	AI H9N2 infection, EXP	10	chicken	-	+	-	-
G	AI H9N2 infection in ND vaccinated birds, EXP, Field	10	turkey	+	+	-	-
H	SPF	50/30	chicken/ turkey	-	-	-	-

EXP – Sera derived from experimental infection/vaccination

A – sera collected from specific pathogen free (SPF) chickens vaccinated with NDV strain "clone 30"

B – sera collected from NDV vaccinated SPF chickens experimentally infected with AIV-H5N2

C – sera collected from SPF chickens vaccinated against AIV H7N7

D – sera collected from NDV vaccinated turkeys (field origin) experimentally infected with AIV-H7N7

E – sera collected from SPF chickens vaccinated with recombinant H7-NDV

F – sera collected from turkeys experimentally infected with AIV H9N2

G – sera collected from NDV vaccinated SPF chickens experimentally infected with AIV H9N2

H – sera collected from SPF chickens and turkeys before experimental infections

(+)—seropositive and (−)—negative on pretests against the indicated antigen.

Table 2.10 Comparison of performance characteristics (sensitivity, specificity with 95% confidence intervals) of commercial ELISAs and the 4plex iFMIA assay using the hemagglutination inhibition (HI) assay as gold standard.

Target	Assay format[a]	Sensitivity	95% CI	Specificity	95% CI	AUC[b]
NDV Full virion	iELISA (IDEXX)	0.93	0.87-0.96	0.95	0.86-0.99	
NDV-rNP	4plex iFMIA	0.93	0.87-0.96	0.91	0.81-0.97	0.937
AIV Full virion	c-ELISA (iD.Vet)	0.98	0.92-1	0.96	0.92-0.99	
AIV-rNP	4plex iFMIA	0.97	0.91-1	0.96	0.91-0.99	0.987
Subtype H5 virion	c-ELISA (iD.Vet)	0.85	0.69-0.94	0.99	0.97-1	
H5-rHA1	4plex iFMIA	1	0.91-1	0.98	0.95- 1	0.996
Subtype H7 virion	c-ELISA (iD.Vet)	0.9	0.76-0.97	0.99	0.97-1	
H7-rHA1	4plex iFMIA	0.95	0.83-1	0.98	0.94-1	0.986

[a]- Sera of groups A-H (Table 2.9) were used to run the assays.

[b]- AUC - values of area under (ROC) curves approaching 1 signal very good performance characteristics based on the ROC curve prediction model [278]. See also Figure 2.16.

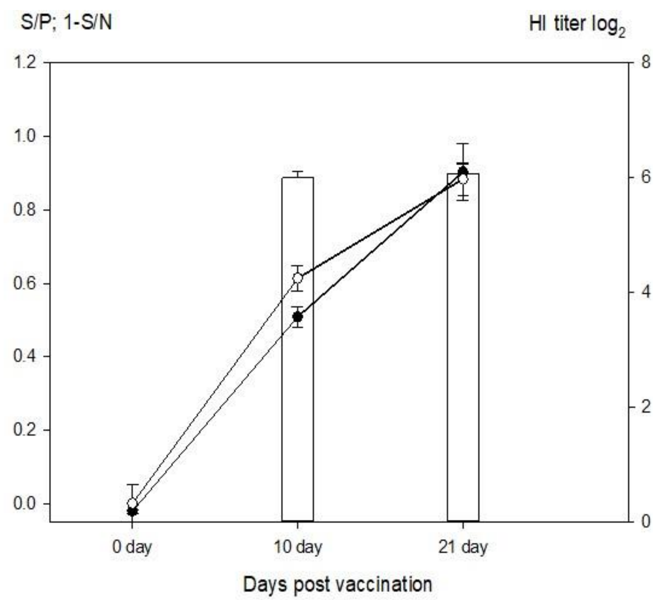


Figure 2.15 Antibody kinetics in SPF chickens vaccinated against NDV (Newcastle disease virus) as measured by HI (hemagglutinin inhibition), commercial ELISA and 4plex iFMIA assay. Sera were obtained from thirty chickens after immunization with a live-attenuated vaccine based on NDV strain "clone 30" at days 0, 10 and 14 post vaccination Columns – HI titer against the homologous antigen (NDV clone 30); open circle – commercial ELISA (IDEXX); black dot – 4plex iFMIA format.

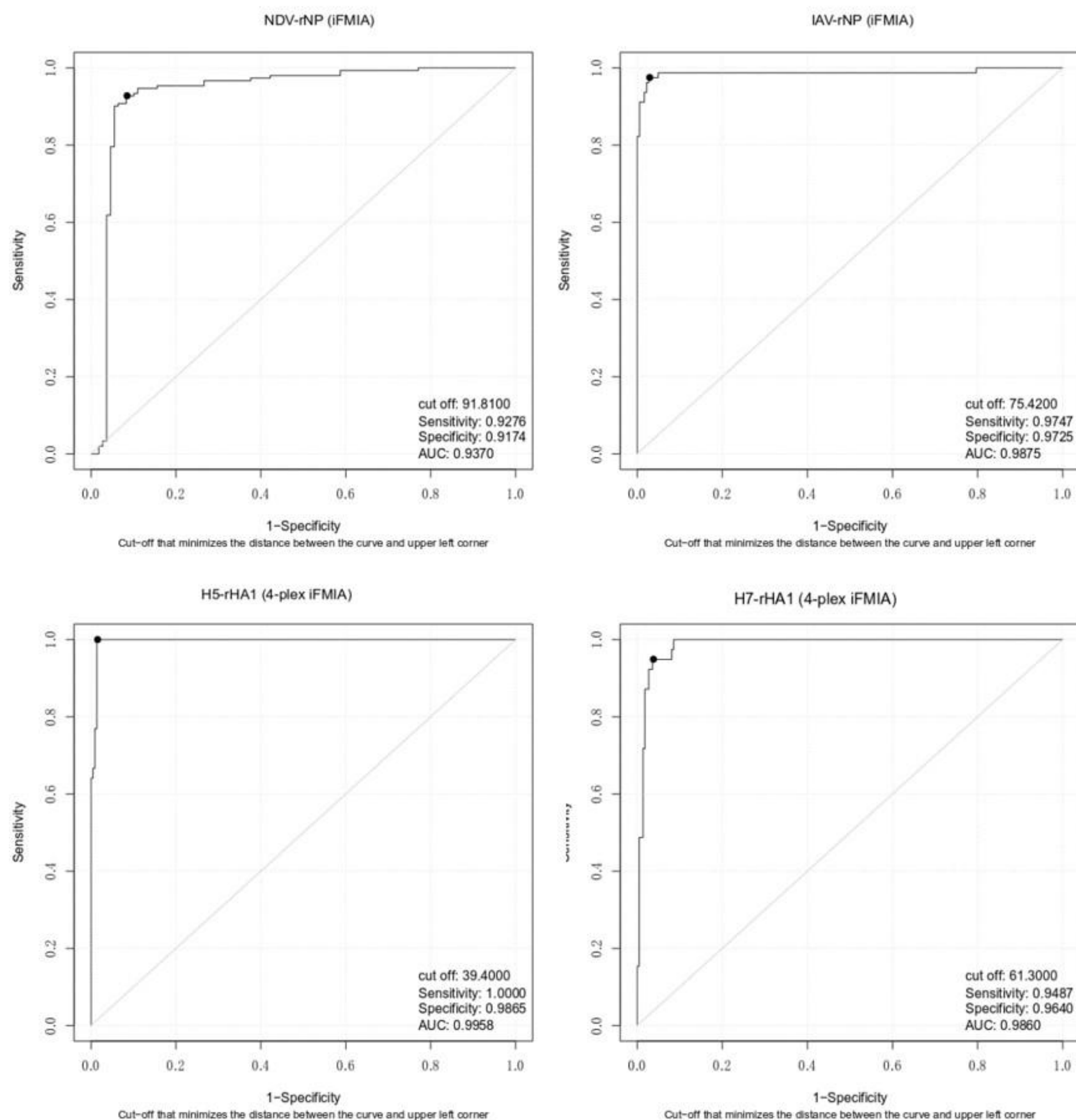


Figure 2.16 ROC analyses of the 4plex iFMIA for performance characteristics in sera from experimentally infected and/or vaccinated galliform poultry. ROC curves of the 4plex iFMIA reactivity to 4 recombinant proteins of NDV and AIV were compared with those of HI assay (n = 257).

Performance characteristics of the 4plex iFMIA assay with field sera

A total of 250 field sera collected from commercial chickens and turkeys in various regions of Germany was tested by HI, commercial ELISAs, and 4plex iFMIA assays. The results are presented in Table 2.11. Antibody detection by iFMIA for AIV H5 and H7 in field serum samples exhibited a high sensitivity and specificity (0.99 and 1) similar to results obtained with sera of experimental infections. For AIV-rNP, sensitivity of the 4plex iFMIA format declined slightly from 0.97 when testing experimental sera to 0.87 when testing field sera. For the NDV-rNP specific iFMIA the number of field sera scoring positive was excessively

higher compared to HI, resulting in a decrease of specificity from 0.91 to 0.56. A similar situation was observed when using the commercial ND ELISA assay and comparing results to the HI assay (Table 2.11).

Table 2.11 Performance characteristics of the 4plex iFMIA assay compared to commercial ELISA and HI (hemagglutinin inhibition) assay in 250 sera of field origin.

Target	Assay	No. of positives	Sensitivity[a]	95% CI	Specificity[a]	95% CI
NDV	HI	129				
	iELISA (IDEXX)	179	0.83	0.76-0.89	0.82	0.74-0.89
	4plex iFMIA	177	0.96	0.91-0.99	0.56	0.47-0.65
AIV	c-ELISA (iD.Vet)	88	n.a.[b]		n.a.	
	4plex iFMIA	91	0.87	0.79-0.93	0.95	0.91-0.98
Subtype-H5	HI	0				
	c-ELISA (iD.Vet)	0	No HI-positives[c]		1	0.98-1
	4plex iFMIA	2	No HI-positives		0.99	0.97-1
Subtype-H7	HI	40				
	c-ELISA (iD.Vet)	40	1	0.91-1	1	0.97-1
	4plex iFMIA	42	1	0.91-1	0.99	0.97-1

[a] - Calculated using the HI results as gold standard.

[b] - Not assessed; no gold standard assay available to control the reactivity of the generic NP (nucleocapsid protein) ELISA since positive reactions could have been due to infections with any of the16 HA subtypes of AIV.

[c] - no H5-specific sera available from the field.

Table 2.12 Positive and negative control sera for assay validation

	HI			Commercial ELISA				Western Blotting			
Serum	NDV clone 30	AIV H5N3	AIV H7N7	IDEXX NDV NP	iD.Vet AIV NP	iD.Vet H5	iD.Vet H7	NDV rNP	IAV rNP	H5N3 rHA1	H7N7 rHA1
S82 (H5N1[a])	4	>512	4	neg.	pos.	pos.	neg.	-	+	+	-
S304 (H7N3[a])	2	<2	>512	neg.	pos.	neg.	pos.	-	+	-	+
S185 (ND-Ulster[1])	>512[b]	<2	<2	pos.	neg.	neg.	neg.	+	-	-	-
SPF serum	4	<2	4	neg.	neg.	neg.	neg.	-	-	-	-

[a] - Antigen used to raise the sera

[b] – Bold-face values indicate specifically positive reactions. Western blotting reactions are also depicted in Figure 2.13

Table 2.13 Qualitative results of serological investigations (seropositive/total samples analysed per assay)

	Detection Antigens										
	NDV NP			AIV NP		HA-H5			HA-H7		
Serum panel[a]	HI	IDEXX	4plex iFMIA	iD.Vet	4plex iFMIA	HI	iD.Vet	4plex iFMIA	HI	iD.Vet	4plex iFMIA
ND vaccination chicken	10/10	9/10	9/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
AI-H5 vaccination in ND vaccinated chicken	39/40	39/40	33/40	32 (1)/40[b]	36/40	40/40	31 (2)/40	40/40	0/40	0/40	0/40
AI H7 vaccination chicken	0/10	0/10	0/10	10/10	10/10	0/10	0/10	0/10	8/10	5 (4)/10	6/10
recombinant ND-H7 vaccination chicken	26/26	25/26	26/26	0/26	0/26	0/26	0/26	0/26	21/26	14 (5)/26	19/26
experimental AI-H7 infection in ND vaccinated turkey	11/11	6/11	3/11	11/11	11/11	0/11	0/11	0/11	10/11	7 (2)/11	9/11
AI H9N2 infection, EXP	0/10	0/10	0/10	10/10	10/10	0/10	0/10	0/10	0/10	0/10	0/10
AI H9N2 infection in ND vaccinated birds, Field	9/10	8/10	9/10	10/10	10/10	0/10	0/10	0/10	0/10	0/10	0/10

[a]-serum details were provided in Table 2.9.

[b]- brackets indicates doubtful result from commercial ELISA assay.

2.3.4 Discussion

In this report, we demonstrate the feasibility of developing a Luminex microbead-based liquid immunoassay for simultaneously detecting specific antibodies against avian pathogens NDV and AIV of subtypes H5 and H7 in a single serum sample. The 4plex iFMIA represents an interesting alternative to the aforementioned assays. The time required to measure 96-well plates and the dependency on Luminex®/Bioplex® machinery are the current constraints of the 4plex iFMIA. In general, the economic efficacy of multiplexed iFMIA will increase with the number of sera to be examined at the same time, as a calibration of the Bioplex® instrument is an essential time-consuming prerequisite for each run. An optimization of the number of beads and flow of beads to be measured is pivotal in economizing time for scanning. Moreover, care must be taken to avoid aggregating the beads. As such, work is still required to render 4plex iFMIA feasible for commercial high-throughput surveillance testing.

In contrast to the results obtained with ND positive sera from experimentally vaccinated birds, there was less congruence of the ND-specific results for avian field sera with the results of the HI assay, resulting in lower specificity. It should be noted that, in Germany, NDV vaccination is compulsory for all gallinaceous poultry, including those kept in small backyard herds; all field sera originated from flocks in Germany. A similar effect was observed using the commercial ND ELISA when compared with HI. This discrepancy has also been observed by previous studies (Häuslaigner et al 2009, Phan et al 2013). Although the HI assay is considered a “golden standard”, it measures antibodies that inhibit hemagglutination by the HN ND viral protein, whereas both the 4plex iFMA format and the commercial ELISA measured NP-specific antibodies. It may be speculated that ND vaccination in the field may induce a higher level of NP- versus HN-specific antibodies, and/or that NP-specific antibodies have slower declining kinetics post-vaccination (Häuslaigner et al 2009, Matsubara et al 2012, Phan et al 2013). In any case, the HI assay did not prove an ideal control in the ROC analysis in the field sera measurements.

To our knowledge, only a few further reports on the use of fluorescent microsphere technology for the detection of ND- and AI-specific antibodies in avian sera have been published (Deregt et al 2006, Lupiani et al 2010, Pinette et al 2014, Watson et al 2009). These assays were limited to the duplex detection of antibodies directed against the ND and AI NP (Deregt et al 2006, Pinette et al 2014), or AI NP and H5 (Lupiani et al 2010), or the triplex detection of AI NP, M1, and NS proteins (Watson et al 2009). Recombinant antigens expressed by either the baculovirus or an alphavirus replicon system were used

in those studies. Performance characteristics comparable to the values obtained in this study were reported. The currently developed 4plex iFMIA system provides advantages over the published systems: (i) The use of bacterially expressed recombinant antigens that are mono-biotinylated in vivo allows the production of larger amounts of target proteins in less demanding bacterial culture systems. (ii) An inhibition format is employed that is based on the use of specific mAbs for each of the targets. This, in turn, will allow the testing of avian sera independent of the poultry species. Previous assays depended on species-specific conjugates. (iii) The simultaneous detection of generic and subtype-specific antibodies against notifiable AI is facilitated.

Demands to examine an ever-growing number of infection-related serological analytes in small sample volumes have constantly increased. With respect to the notifiable avian viral diseases AI and ND, at least four serological parameters need to be measured in surveillance programs to study disease prevalence or to control vaccination efficacy at the population level. Attempts to multiplex such assays are a logical consequence. Fluorescent microsphere systems offer vast opportunities in this respect and should be more intensely explored in the future. The current work could be extended to include the possible quantification of viral/antigen loads, and the detection of antibodies against other AIV subtypes (e.g., the H9), further NDV proteins (e.g., the HN), and other significant avian virus diseases (e.g., the infectious bronchitis or infectious bursal disease viruses).

Author Contributions: N.Z., C.G., and T.C.H. conceived the study and drafted the manuscript. N.Z. carried out the molecular and serological work and analyzed the samples. G.W. conceived the study and analyzed the samples. M.B. conceived the study, provided funds, and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by of the China Scholarship Council (CSC), grant number 2010325035 to N.Z.

Institutional Review Board Statement: The animal study protocol was approved by the animal welfare committee of the German Federal State of Mecklenburg-Western Pomerania (LALLF M-V/TSD/7221.3-2.5-004/10; LALLF M-V/TSD/7221.3-2.5-010/10).

Informed Consent Statement: Not applicable.

Acknowledgments: We thank Mario Ziller, Institute of Epidemiology, FLI, for kindly analyzing the data in this study. We gratefully acknowledge excellent technical support by Sven Sander, Katja Hartwig, and Cornelia Illing.

Conflicts of Interest: The authors declare no conflict of interest.

3

General Discussion

3.1 New approaches to serodiagnostic investigations for IAV and NDV infections in avian and porcine hosts

Avian influenza virus and Newcastle disease virus are two of the most important, and hence globally notifiable, contagious diseases of poultry. Due to their high rates of morbidity and mortality the induced diseases cause significant economic losses to poultry production systems (Tanner et al 2015). Additionally, swine influenza is an economically important disease of pigs caused by swine influenza virus. Swine influenza virus is highly variable and the swine as a host of influenza A viruses is capable to generate reassortants of influenza A viruses derived from human, avian and porcine hosts. This function as a “mixing vessel” of influenza A viruses has been implicated to contribute to the generation of new human pandemic influenza viruses (Thacker & Janke 2008). Therefore, rapid, simple and reliable diagnostic methods are essential for early detection, surveillance of these infections and vaccine effectiveness evaluation including a One Health perspective.

The serodiagnostic approach plays an important role in this aspect. The hemagglutination inhibition (HI) test as a standard method is widely used for the diagnosis of infection caused by influenza virus or paramyxoviruses. However, this test does not only suffer from moderate sensitivity but requires intensive laboratory support if performed in a standardized mode (Pedersen 2014). Consequently, there is evidence for poor reproducibility between laboratories (Stephenson et al 2007, Zacour et al 2016). ELISA formats now serve as the most commonly used serological assays which can overcome

some of the limitations of the HI assay. To date, several commercial ELISA kits are available for serodiagnosis of AIV, SIV and NDV infections, however there are still some drawbacks of these assays, related for instance with high costs, and lack of sufficient subtype-specific applications (Barr et al 2006, Brookes et al 2010, He et al 2013, Luo et al 2009, Moreno et al 2009, Velumani et al 2008). Work collated in this PhD study focused on the broadening of the selection of serodiagnostic tools for detection of IAV- and NDV-specific antibodies. It was attempted to use recombinantly expressed viral antigens as target antigens in ELISA applications whereby exploiting bacterial expression systems and adapting easy-to-handle purification methods. Along these lines, we succeeded in setting up an indirect ELISAs format based on truncated influenza HA proteins (HA1) for detection of subtype-specific antibodies in porcine sera (experimental study 2.1). Second, we demonstrated that Avian orthoavulavirus 1 full-length NP protein as the coating antigen in the indirect ELISA format, compared favorably with the whole virus antigen in the commercial NDV ELISA, although some cross reactivity was found with different AOaV subtypes. Such cross reactivity can be eliminated when using the less conserved C-terminal fragment of AOaV-1 NP as the antigen. Additionally, we also demonstrated that a serological DIVA strategy built on a unique part of AOaV-1 V protein was not feasible (experimental study 2.2). Finally, we developed a reliable, sensitive and high throughput inhibition-fluorescent microsphere immunoassay based on Luminex® bead technology to detect seroresponses to AIV and NDV simultaneously in sera of galliform poultry (experimental study 2.3). The findings of experimental studies will be discussed in the following sections.

3.2 Recombinant viral proteins as serological diagnostic reagents: Tailoring for purpose

The use of recombinantly expressed virus proteins has become a routine measure for many target antigens (Cuzzubbo et al 2001, Das & Kumar 2015, Katz et al 2012, Perelygina et al 2005, Saijo et al 2006, Yu et al 2006). However, the quality of antigens as defined by conformation, stability, accessible epitopes and purity, is essential to ensure high analytical and diagnostic sensitivity and specificity. Currently, there is a plethora of systems available for recombinant proteins expression in, e.g., bacteria, yeast, plant, insect or mammalian cells. Although expressing recombinant protein in insect and mammalian cells confers many advantages related to biological activities, post-translational modifications including glycosilation, and bona fide protein folding of the target proteins, the main drawbacks are high costs and typically lower yields of

recombinant proteins (Khan 2013, Unger & Peleg 2012). Plants as vectors of viral antigens to be in diagnostic approaches but more so as edible vaccines have been studied extensively but so far, no commercialized products have become available (Jain et al 2013, Lee et al 2015, Song et al 2021). The yeast and bacterial expression systems have similar characteristics, such as rapid expression, low cost, simple scaling-up, well characterized genetics and easy purification of recombinant proteins (Baneyx 1999, Chen 2012, Swartz 2001). According to our study aim, eventually a bacterial expression system has been selected, despite the risk that expressed proteins may form improper conformational structures and tend to aggregate into highly insoluble inclusion bodies. However, we show such limitations can be overcome by careful sequence and plasmid selection and post-expression treatments (experimental study 2.2). We benefitted from a number of reports documenting that inclusion bodies can be solubilized and refolded in vitro to restore high recovery rates of bioactive proteins (Mayer & Buchner 2004, Singh & Panda 2005, Yamaguchi & Miyazaki 2014). All our recombinant viral proteins were expressed as inclusion bodies, but we found this even an advantage as it facilitated protein isolation and purification processes. Restoring bioactive function, requiring proper folding to represent conformation-dependent epitopes, was then a function of inclusion body solubilization and in vitro-refolding in appropriate refolding buffers to acquire native conformation. The recombinant proteins and their proper conformation were identified by SDS-PAGE, native PAGE and/or indirect ELISA utilizing monoclonal antibody recognizing specific conformational epitopes. Overall, the bacterially expressed recombinant viral proteins showed high recovery rates of bioactive protein from inclusion bodies and were deemed to be fit for using as serological diagnostic reagents.

3.3 Recombinant viral hemagglutinin facilitates subtype-specific detection of antibodies against influenza A viruses in swine

As mentioned earlier (page 16), swine are capable of infection with both avian and human influenza viruses. When more than one subtype influenza viruses infect an individual pig simultaneously, genetic reassortment between these viruses can occur, resulting in creating novel influenza A virus reassortants with new gene constellations that may be associated with new phenotypic properties. For example, the new IAV strain may exert increased spreading potential in swine and could be transmitted to other mammalian populations including humans starting eventually a new pandemic (Hilleman 2002). Prevention and management of future pandemics with an animal origin remain among the most prominent and difficult tasks as the most recent example of SARS-Cov2 has

demonstrated again (Holmes et al 2021). In the influenza field it has been recommended to tackle potential pandemic viruses already at their root, in animal populations, before they have a chance to form and spread (Yamaji et al 2020). Vaccination of swine as a pre-emptive intervention strategy combined with an effective surveillance and diagnosis is able to reduce the risk of generating new pandemic viruses. According to these aims, serodiagnosis is an important measure. The detection of antibodies against viral proteins, can be used to confirm recent infection (retrospective diagnosis) and evaluate vaccine effectiveness.

The key challenge of serodiagnosis of swine influenza virus infection worldwide is anchored in the cocirculation of several viral subtypes. In addition, the use of multivalent vaccines complicates this situation further. Currently, the HI assay remains the routine method for subtype-specific antibody differentiation. To overcome limitations of the HI assay (page 16), ELISA formats suitable for high throughput investigation of samples are considered as a promising alternative strategy.

In this study, the well conserved influenza A virus NP protein expressed in bacteria was used as a capture antigen - instead of the whole virion preparation - in an indirect ELISA to detect generic IAV-specific antibodies. A positive reaction in this assay would state that the animal has been infected at some time by an influenza A virus. The full-length recombinant NP protein was recognized by specific porcine immune sera from experimentally IAV-infected and field animals in the indirect ELISA. The diagnostic results correlated well with the HI assay and a commercial blocking NP ELISA assay (experimental study 2.1). Using the highly variable HA1 section of the HA glycoprotein as diagnostic reagents in the indirect ELISA, subtype H1 and H3 specific antibodies were discernable in Western blotting and indirect ELISA (experimental study 2.1). On average, the HA1 protein of subtypes H1 and H3 only share approximately 25% homology (Janke 2000). A number of conserved epitopes between the different subtypes should probably also exist in the HA1, thus the observed weak cross reactivity is not unexpected but, in all cases the homologous HA1 subtype antigens gave the highest signal intensity. Testing results from indirect HA1 ELISAs agreed with the HI assay for the majority of swine field sera. However, due to the fact that at least four lineages of the H1 subtype are cocirculating globally in swine, the application of recombinant H1 HA1 protein can result in strong cross reactivity among these H1-subtype lineages when using an indirect ELISA format, while a distinction may still be possible when using the HI test (Starick et al 2012). The examination of swine field sera yielded a variable agreement for the different antigens, especially for the H1av and H1huN2 lineages. Some samples scored negative

in the indirect ELISAs when these sera showed low HI titers. This situation may be due to limitations of the indirect ELISA, e.g., loss of some epitopes, or caused by lack of specificity of the HI assays. A true gold standard that would allow objective assessment of the assay's performance characteristics is unfortunately not available.

As noted above, in this study we successfully expressed the recombinant NP and HA1 proteins by a bacterial expression system and refolded these proteins in vitro to re-acquire native antigenic conformation. These refolded and purified proteins served as antigens in indirect ELISAs and were found suitable as an alternative to commercial blocking NP ELISA and HI assays for HA subtype-specific serodiagnosis on a herd basis. In order to improve HA lineage-specific distinction of antibodies a competitive ELISA format using monoclonal lineage-specific antibodies for competition should be considered in future research and development.

3.4 Trimming recombinant avian orthoavulavirus nucleoproteins increases specificity of NDV serodiagnosis

Newcastle Disease is one of the most dreaded viral infections of gallinaceous poultry worldwide and vaccination of poultry is the most important strategy to prevent ND. In many countries, including Germany, vaccination of all gallinaceous poultry is mandatory. Currently, vaccine strains LaSota or B1 of low virulence (sy. Lentogenic) are broadly used as live virus as well as in adjuvanted inactivated vaccines. The induction of a sufficient humoral immune response vaccination is the essential factor for ND control (Kapczynski et al 2013), and serological control of the vaccine-induced humoral immunity is crucial to assessing the protection status of a flock. Several further avian AOAV serotypes are in circulation which do not cause significant disease but induce an avian orthoavulavirus-1 cross reactive, whereas not cross-protective humoral immune response (Nayak et al 2012).

This study mainly focused on detection of AOAV-1 specific antibodies using bacterially expressed recombinant proteins as antigens in indirect ELISA, aiming at an improved differentiation among the various AOAV serotypes. In addition, we were seeking to establish a serological DIVA diagnosis whereby distinguishing poultry that was seropositive following vaccination versus field virus infection. We assumed that the non-structural viral V protein might induce humoral immunity. The V protein is only expressed in cells infected by replication-competent viruses. Therefore, the antibody specifically

against V protein might be used to distinguish infected birds from those that had received inactivated virus vaccines.

Traditionally, HI assays and commercial ELISAs with whole virus antigen are routinely used in serodiagnosis of NDV surveillance programs, but little is known about the cross reactivity with other serotypes. To overcome this limitation, the less conserved C-terminal parts of the NP protein (NP_{ct}) as well as the variable P protein were used as antigens, expecting to obtain a more serotype-specific indirect ELISA assay. To evaluate the specificity, chicken hyperimmune serum against nine different serotypes of AOAV were investigated by these ELISA formats. Extensive cross reactivity was observed in commercial ELISA and by use of the recombinant full-length NP-based indirect ELISA (experimental studies 2.2). Interestingly, specificity increased significantly when using NP_{ct} fragments of the NDV and APMV-8 as antigens. These data indicated that the NP_{ct} holds potential as a highly serotype-specific diagnostic antigen in the indirect ELISA format. On the other hand, due to loss of numerous antigenic epitopes compared to full length NP protein, the sensitivity might be reduced in an indirect ELISA. Probably this has contributed to detecting a lower number of NP-positive sera by the NP_{ct} ELISA compared with other corresponding tests in the field sera. The recombinant full-length NP of NDV as the antigen in the indirect ELISA resulted in similar sensitivity and specificity compared with commercial ELISA. It cannot be excluded that both these assays in fact scored a number of non-NDV infected birds as AOAV-1 seropositive due to lower specificity.

The nonstructural V protein was considered as the ideal target antigen to differentiate between viral infection and vaccination. This protein is generated from the P gene via post-transcriptional mRNA editing, and thus shares the N-terminal amino acid sequences with the P protein but possesses its own unique C-terminal domain. To eradicate cross reactivity between P and V proteins, the distinctive C-terminal portion of V individually was expressed as a target antigen, termed V_u. The feasibility of this strategy was tested with sera from chickens infected either with vaccine strain LaSota or with virulent Herts33 NDV. A few of chickens (2/10) showed positive results post immunization with live attenuated vaccine, but more chickens converted after challenge with the velogenic Herts strain (7/10). Since no clear-cut V-specific seroconversion patterns were obtained and interference from modified-live vaccination was recognized as a problem, a V protein-based DIVA strategy relevant to AOAV-1 virus is not feasible (experimental studies 2.2).

Taken together, this study demonstrated that (i) NP_{ct} protein fragments enabled a serotype-specific diagnosis by indirect ELISA and might be exploited as alternative diagnostic tools to classical HI assay; (ii) the full-length NP protein as the ELISA antigen

possessed comparable diagnostic features compared to a whole virus antigen-based commercial ELISA, but cross reactivity among AOAV serotypes may blur specificity; (iii) V protein-based serological DIVA strategy is not recommended, since V-specific seroconversion within an experimentally infected flock was incomplete as well as interference with attenuated vaccination was observed.

3.5 Liquid array technology widens the scope for simultaneous detection and discrimination of generic and subtype-specific antibodies against AIV and NDV

Both AI and ND are contagious and often fatal diseases in poultry. Because of the similar clinical symptoms caused by the different viruses, it is difficult to distinguish between AI and ND on basis of clinical signs. In addition, H5 and H7 are two of most important subtypes among all subtype AIVs, as both viruses are able to mutate into a highly pathogenic form after infection of poultry. Therefore, early detection and intervention is essential for controlling disease spreading. Active serosurveillance plays an important role in this line (EU directive 437/2006/EC). ELISA and HI are routine diagnostic methods used in surveillance programs However, there are obvious limitations of these techniques: ELISA and HI lack multiplexing capabilities, and the HI assays are labor-intensive and time-consuming.

To address these issues, the technology of fluorescent microsphere immunoassay (FMIA) was explored to detect specific antibodies against the nucleocapsid proteins (NP) of NDV and AIV (as a generic serodiagnostic assay), as well as against the hemagglutinin protein (HA) of H5 and H7 subtypes, in a single poultry serum simultaneously in the same sample.

The Luminex® 100/200™ system is a flexible analyzer based on the principles of flow cytometry that enables to simultaneously measure up to 100 analytes in a single microplate consuming very small volume samples. The platform consists of microspheres, a detection instrument and analysis software. Microspheres consist micron-sized polystyrene beads that are intrinsically labeled with fluorescent dyes. The dye acts as the identifier of the bead and the solid surface of the sphere is employed in fixing antigens or other types of reactants. Bead-based technology is becoming a key component for many biological applications currently (Cook et al 2019, Coppock & Stratis-Cullum 2019, Gary et al 2019, Germeraad et al 2019, Gonsalves et al 2019, Graham et al 2019, Hwang et al 2019, Kadam et al 2019, Kamminga et al 2018, Manglani et al 2019, McFarlin & Curtis

2019, Zhao et al 2022). Microspheres are suited to carry a variety of target types (proteins, nucleic acids, and other molecular structures) to provide them in biomarker interactions (Cook et al 2019, Coppock & Stratis-Cullum 2019, Manglani et al 2019, McFarlin et al 2016). Besides, bead-based assays also have the capacity of combined detection of nucleic acid targets and proteins in a single assay (Graham et al 2019, Kamminga et al 2018, Zhao et al 2022). These advantages are widely expanding the capacities of older methodologies such as single analyte immuno- and enzymatic assays. Beyond multiplexing, this technology bears capacity to extend the lower limit of detection of analytes , which allows exploration of more complex biological pathways (Gary et al 2019, Hwang et al 2019) or finding early stages of pathogenic infections (Beckmann & Hirsch 2016, Brenner et al 2019, Germeraad et al 2019). The bead-based method today has been further developed to focus on clinical diagnosis that offers rapid, high-throughput detection of a variety of viral and bacterial pathogens or the host-specific responses to such infections (Brenner et al 2019, Germeraad et al 2019, Gonsalves et al 2019, Kadam et al 2019, Zhao et al 2022).

Although several studies have used the FMIA technology for the detection of ND and AI specific antibodies in avian sera (Bao et al 2017, Deregt et al 2006, Germeraad et al 2019, Li et al 2017b, Lupiani et al 2010, Pinette et al 2014, Watson et al 2009), until to date no 4plex iFMIA system to detect NDV, AIV and AIV subtype H5, H7 antibodies simultaneously has been reported. In our study, distinct fluorescent color-coded beads coated with four kinds of target antigens (AIV-NP, NDV-NP, H5-HA and H7-HA) were used. Moreover, corresponding monoclonal antibodies against the four antigens were used in a competition format of the liquid array FMIA format, the obvious benefit of which is to ensure a highly specific and species-independent detection of antibodies.

Series of chicken and turkey sera derived from experimental infection or vaccination trials were used for evaluation of the performance characteristics. The obtained results indicated the feasibility of this approach (experimental study 2.3). Further efforts in evaluating sensitivity and specificity involved numerous field sera. The results of 4plex-iFMIA, commercial ELISA and the gold standard HI assay were compared using ROC analyses. Based on this, all parameters of the bead-based assay correlated favorably with the other two classical assays (experimental study 2.3).

Taken together, the 4plex iFMIA proved to be a reliable and efficient method to detect antibodies against NDV- and AIV-specific antigens. The multiplexed FMIA platform holds

potential for a sensitive, specific, fast and high throughput detection of AIV and NDV antibodies.

3.6 Conclusion and outlook

In this thesis, we successfully expressed in easy-to-handle bacterial systems recombinant NP and HA proteins or fragments thereof of influenza and paramyxoviruses to serve as antigens in indirect ELISAs. Liquid arrays proved to be a promising technology to combine and straighten serodiagnostic assays in a meaningful way which is especially well situated for high-throughput screening. Along these lines future efforts could focus on widening the scope of target antigens to further HA but also NA subtypes of influenza viruses. In addition, NDV proteins F and HN which represent the main targets of protective humoral immunity against NDV infection are rewarding targets when aiming at an improved evaluation of vaccination efficacy in flocks. Moreover, non-structural proteins as target antigens might be useful to distinguish vaccinated and infected animals as soon as subunit vaccines become available. In an even wider scope, the fluorescent microsphere system can be expanded to target other avian diseases for instance infectious bronchitis or infectious bursal disease.

References

A. Ahad MR, T. Yaqub, A. Mahmood1, Z. H. Kuthu2, M. Z. Shabbir, A. A. Sheikh and H. Gohar. 2013. Detection of antibody to newcastle disease virus in human sera in Pakistan. *The Journal of Animal & Plant Sciences* 23: 5

Abdallah F, Hassanin O. 2015. Positive regulation of humoral and innate immune responses induced by inactivated Avian Influenza Virus vaccine in broiler chickens. *Veterinary research communications* 39: 211-6

Abdelwhab el SM, Erfan AM, Grund C, Ziller M, Arafa AS, et al. 2010. Simultaneous detection and differentiation by multiplex real time RT-PCR of highly pathogenic avian influenza subtype H5N1 classic (clade 2.2.1 proper) and escape mutant (clade 2.2.1 variant) lineages in Egypt. *Virology journal* 7: 260

Ahmad-Raus R, Ali AM, Tan WS, Salleh HM, Eshaghi M, Yusoff K. 2009. Localization of the antigenic sites of newcastle disease virus nucleocapsid using a panel of monoclonal antibodies. *Research in veterinary science* 86: 174-82

Al-Garib SO, Gielkens AL, Gruys DE, Hartog L, Koch G. 2003. Immunoglobulin class distribution of systemic and mucosal antibody responses to Newcastle disease in chickens. *Avian diseases* 47: 32-40

Alamares JG, Elankumaran S, Samal SK, Iorio RM. 2010. The interferon antagonistic activities of the V proteins from two strains of Newcastle disease virus correlate with their known virulence properties. *Virus research* 147: 153-7

Alexander DJ. 2000. Newcastle disease and other avian paramyxoviruses. *Revue scientifique et technique (International Office of Epizootics)* 19: 443-62

Alexander DJ. 2007. An overview of the epidemiology of avian influenza. *Vaccine* 25: 5637-44

Alexander DJ, Aldous EW, Fuller CM. 2012. The long view: a selective review of 40 years of Newcastle disease research. *Avian pathology : journal of the W.V.P.A* 41: 329-35

Alexander DJ, Brown IH. 2009. History of highly pathogenic avian influenza. *Revue scientifique et technique (International Office of Epizootics)* 28: 19-38

Arafa AS, Selim AA, Hassan MK, Aly MM. 2010. Genetic characterization of variant strains of highly pathogenic avian influenza H5N1 that escaped detection by real-time reverse transcriptase-PCR diagnostic tests. *Avian diseases* 54: 673-6

Baigent SJ, McCauley JW. 2003. Influenza type A in humans, mammals and birds: determinants of virus virulence, host-range and interspecies transmission. *BioEssays : news and reviews in molecular, cellular and developmental biology* 25: 657-71

Baneyx F. 1999. Recombinant protein expression in Escherichia coli. *Current opinion in biotechnology* 10: 411-21

Bano S, Naeem K, Malik SA. 2003. Evaluation of pathogenic potential of avian influenza virus serotype H9N2 in chickens. *Avian diseases* 47: 817-22

Bao DT, Kim DTH, Park H, Cuc BT, Ngoc NM, et al. 2017. Rapid Detection of Avian Influenza Virus by Fluorescent Diagnostic Assay using an Epitope-Derived Peptide. *Theranostics* 7: 1835-46

Barbe F, Labarque G, Pensaert M, Van Reeth K. 2009. Performance of a commercial Swine influenza virus H1N1 and H3N2 antibody enzyme-linked immunosorbent assay in pigs experimentally infected with European influenza viruses. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* 21: 88-96

Barr IG, McCaig M, Durrant C, Shaw R. 2006. The rapid identification of human influenza neuraminidase N1 and N2 subtypes by ELISA. *Vaccine* 24: 6675-8

Baudon E, Peyre M, Peiris M, Cowling BJ. 2017. Epidemiological features of influenza circulation in swine populations: A systematic review and meta-analysis. *PloS one* 12: e0179044

Beckett D, Kovaleva E, Schatz PJ. 1999. A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation. *Protein science : a publication of the Protein Society* 8: 921-9

Beckmann C, Hirsch HH. 2016. Comparing Luminex NxTAG-Respiratory Pathogen Panel and RespiFinder-22 for multiplex detection of respiratory pathogens. *Journal of medical virology* 88: 1319-24

Bergfeld J, Meers J, Bingham J, Harper J, Payne J, et al. 2017. An Australian Newcastle Disease Virus With a Virulent Fusion Protein Cleavage Site Produces Minimal Pathogenicity in Chickens. *Veterinary pathology* 54: 649-60

Berinstein A, Seal BS, Zanetti F, Kaloghlian A, Segade G, Carrillo E. 1999. Newcastle disease virus surveillance in Argentina: use of reverse transcription-polymerase chain reaction and sequencing for molecular typification. *Avian diseases* 43: 792-7

Berinstein A, Sellers HS, King DJ, Seal BS. 2001. Use of a heteroduplex mobility assay to detect differences in the fusion protein cleavage site coding sequence among Newcastle disease virus isolates. *Journal of clinical microbiology* 39: 3171-8

Bissonnette ML, Donald JE, DeGrado WF, Jardetzky TS, Lamb RA. 2009. Functional analysis of the transmembrane domain in paramyxovirus F protein-mediated membrane fusion. *J Mol Biol* 386: 14-36

Bohm K, Filomena A, Schneiderhan-Marra N, Krause G, Sievers C. 2017. Validation of HAV biomarker 2A for differential diagnostic of hepatitis A infected and vaccinated individuals using multiplex serology. *Vaccine* 35: 5883-89

Boulo S, Akarsu H, Ruigrok RW, Baudin F. 2007. Nuclear traffic of influenza virus proteins and ribonucleoprotein complexes. *Virus research* 124: 12-21

Bouvier NM, Palese P. 2008. The biology of influenza viruses. *Vaccine* 26 Suppl 4: D49-53

Brandenburg B, Koudstaal W, Goudsmit J, Klaren V, Tang C, et al. 2013. Mechanisms of hemagglutinin targeted influenza virus neutralization. *PloS one* 8: e80034

Brenner N, Butt J, Bomfim IL, Tabatabai J, Pawlita M, et al. 2019. Validation of monoplex assays detecting antibodies against Corynebacterium diphtheriae and Clostridium tetani toxins, rubella virus and parvovirus B19 for incorporation into Multiplex Serology. *Methods* 158: 44-53

Briand FX, Henry A, Massin P, Jestin V. 2012. Complete genome sequence of a novel avian paramyxovirus. *Journal of virology* 86: 7710

Brookes SM, Nunez A, Choudhury B, Matrosovich M, Essen SC, et al. 2010. Replication, pathogenesis and transmission of pandemic (H1N1) 2009 virus in non-immune pigs. *PloS one* 5: e9068

Brown IH. 2013. History and epidemiology of Swine influenza in Europe. *Current topics in microbiology and immunology* 370: 133-46

Brown IH, Harris PA, McCauley JW, Alexander DJ. 1998. Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype. *The Journal of general virology* 79 (Pt 12): 2947-55

Brown IH, Hill ML, Harris PA, Alexander DJ, McCauley JW. 1997. Genetic characterisation of an influenza A virus of unusual subtype (H1N7) isolated from pigs in England. *Archives of virology* 142: 1045-50

Bulavaite A, Lasickiene R, Vaitiekaite A, Sasnauskas K, Zvirbliene A. 2016. Synthesis of human parainfluenza virus 2 nucleocapsid protein in yeast as nucleocapsid-like particles and investigation of its antigenic structure. *Applied microbiology and biotechnology* 100: 4523-34

Cao X, Yang F, Wu H, Xu L. 2017. Genetic characterization of novel reassortant H5N6-subtype influenza viruses isolated from cats in eastern China. *Archives of virology*

Capua I, Marangon S. 2007. Control and prevention of avian influenza in an evolving scenario. *Vaccine* 25: 5645-52

Carrat F, Flahault A. 2007. Influenza vaccine: the challenge of antigenic drift. *Vaccine* 25: 6852-62

Chaharaein B, Omar AR, Aini I, Yusoff K, Hassan SS. 2009. Detection of H5, H7 and H9 subtypes of avian influenza viruses by multiplex reverse transcription-polymerase chain reaction. *Microbiological research* 164: 174-9

Chaka H, Goutard F, Gil P, Abolnik C, Servan de Almeida R, et al. 2013. Serological and molecular investigation of Newcastle disease in household chicken flocks and associated markets in Eastern Shewa zone, Ethiopia. *Tropical animal health and production* 45: 705-14

Chambers P, Samson AC. 1982. Non-structural proteins in Newcastle disease virus-infected cells. *The Journal of general virology* 58 Pt 1: 1-12

Chappell L, Killian ML, Spackman E. 2014. Detection of influenza A antibodies in avian serum samples by ELISA. *Methods in molecular biology (Clifton, N.J.)* 1161: 151-67

Charlton B, Crossley B, Hietala S. 2009. Conventional and future diagnostics for avian influenza. *Comparative immunology, microbiology and infectious diseases* 32: 341-50

Chen Q, Madson D, Miller CL, Harris DL. 2012. Vaccine development for protecting swine against influenza virus. *Animal health research reviews* 13: 181-95

Chen R. 2012. Bacterial expression systems for recombinant protein production: E. coli and beyond. *Biotechnol Adv* 30: 1102-7

Chen R, Yu XL, Gao XB, Xue CY, Song CX, et al. 2015. Bead-based suspension array for simultaneous differential detection of five major swine viruses. *Applied microbiology and biotechnology* 99: 919-28

Cheng JH, Sun YJ, Zhang FQ, Zhang XR, Qiu XS, et al. 2016. Newcastle disease virus NP and P proteins induce autophagy via the endoplasmic reticulum stress-related unfolded protein response. *Sci Rep* 6: 24721

Cheung TK, Poon LL. 2007. Biology of influenza a virus. *Ann N Y Acad Sci* 1102: 1-25

Chiapponi C, Faccini S, De Mattia A, Baioni L, Barbieri I, et al. 2016. Detection of Influenza D Virus among Swine and Cattle, Italy. *Emerging infectious diseases* 22: 352-4

Chiu YC, Chu WY, Tsao Z, Wang CH. 2012. Antigen-capture enzyme-linked immunosorbent assays for detection of different H5 avian Influenza A virus. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* 24: 716-8

Choi JH, Kim MS, Lee JY, Lee NJ, Kwon D, et al. 2013. Development and evaluation of multiplex real-time RT-PCR assays for seasonal, pandemic A/H1pdm09 and avian A/H5 influenza viruses detection. *Journal of microbiology (Seoul, Korea)* 51: 252-7

Choi YK, Lee JH, Erickson G, Goyal SM, Joo HS, et al. 2004. H3N2 influenza virus transmission from swine to turkeys, United States. *Emerging infectious diseases* 10: 2156-60

Chumbe A, Izquierdo-Lara R, Calderón K, Fernández-Díaz M, Vakharia VN. 2017. Development of a novel Newcastle disease virus (NDV) neutralization test based on recombinant NDV expressing enhanced green fluorescent protein. *Virology journal* 14: 232-32

Ciacci-Zanella JR, Vincent AL, Prickett JR, Zimmerman SM, Zimmerman JJ. 2010. Detection of anti-influenza A nucleoprotein antibodies in pigs using a commercial influenza epitope-blocking enzyme-linked immunosorbent assay developed for avian species. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* 22: 3-9

Collins PL, Hightower LE. 1982. Newcastle disease virus stimulates the cellular accumulation of stress (heat shock) mRNAs and proteins. *Journal of virology* 44: 703-7

Collins PL, Wertz GW, Ball LA, Hightower LE. 1982. Coding assignments of the five smaller mRNAs of Newcastle disease virus. *Journal of virology* 43: 1024-31

Cook DB, McLucas BC, Montoya LA, Brotski CM, Das S, et al. 2019. Multiplexing protein and gene level measurements on a single Luminex platform. *Methods* 158: 27-32

Coppock MB, Stratis-Cullum DN. 2019. A universal method for the functionalization of dyed magnetic microspheres with peptides. *Methods (San Diego, Calif.)* 158: 12-16

Cros JF, Palese P. 2003. Trafficking of viral genomic RNA into and out of the nucleus: influenza, Thogoto and Borna disease viruses. *Virus research* 95: 3-12

Cuzzubbo AJ, Endy TP, Nisalak A, Kalayanarooj S, Vaughn DW, et al. 2001. Use of recombinant envelope proteins for serological diagnosis of Dengue virus infection in an immunochromatographic assay. *Clinical and diagnostic laboratory immunology* 8: 1150-5

Czifra G, Nilsson M, Alexander DJ, Manvell R, Kecskemeti S, Engstrom BE. 1996. Detection of PMV-1 specific antibodies with a monoclonal antibody blocking enzyme-linked immunosorbent assay. *Avian pathology : journal of the W.V.P.A* 25: 691-703

Das M, Kumar S. 2015. Recombinant phosphoprotein based single serum dilution ELISA for rapid serological detection of Newcastle disease virus. *Journal of virological methods* 225: 64-9

de Oliveira ES, Silva KR, Fernando FS, Goncalves MC, Fernandes CC, et al. 2013. A liquid-phase-blocking concanavalin A enzyme-linked immunosorbent assay for the detection of antibodies against Newcastle disease virus in serum of free-ranging pigeons. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* 25: 720-6

de Sousa RLM, Montassier HJ, Pinto AA. 2000. Detection and Quantification of Antibodies to Newcastle Disease Virus in Ostrich and Rhea Sera Using a Liquid Phase Blocking Enzyme-Linked Immunosorbent Assay. *Clinical and diagnostic laboratory immunology* 7: 940-4

Deng R, Wang Z, Mahon PJ, Marinello M, Mirza A, Iorio RM. 1999. Mutations in the Newcastle disease virus hemagglutinin-neuraminidase protein that interfere with its ability to interact with the homologous F protein in the promotion of fusion. *Virology* 253: 43-54

Deregt D, Furukawa-Stoffer TL, Tokaryk KL, Pasick J, Hughes KM, et al. 2006. A microsphere immunoassay for detection of antibodies to avian influenza virus. *Journal of virological methods* 137: 88-94

Detmer S, Gramer M, Goyal S, Torremorell M, Torrison J. 2013. Diagnostics and surveillance for Swine influenza. *Current topics in microbiology and immunology* 370: 85-112

Di Trani L, Bedini B, Donatelli I, Campitelli L, Chiappini B, et al. 2006. A sensitive one-step real-time PCR for detection of avian influenza viruses using a MGB probe and an internal positive control. *BMC infectious diseases* 6: 87

Diel DG, da Silva LH, Liu H, Wang Z, Miller PJ, Afonso CL. 2012. Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* 12: 1770-9

Digard P, Elton D, Bishop K, Medcalf E, Weeds A, Pope B. 1999. Modulation of nuclear localization of the influenza virus nucleoprotein through interaction with actin filaments. *Journal of virology* 73: 2222-31

DiLillo DJ, Tan GS, Palese P, Ravetch JV. 2014. Broadly neutralizing hemagglutinin stalk-specific antibodies require FcgammaR interactions for protection against influenza virus in vivo. *Nature medicine* 20: 143-51

Dimitrov KM, Abolnik C, Afonso CL, Albina E, Bahl J, et al. 2019. Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* 74: 103917

Ducatez MF, Pelletier C, Meyer G. 2015. Influenza D virus in cattle, France, 2011-2014. *Emerging infectious diseases* 21: 368-71

Dunbar SA, Ritchie VB, Hoffmeyer MR, Rana GS, Zhang H. 2015. Luminex((R)) multiplex bead suspension arrays for the detection and serotyping of Salmonella spp. *Methods in molecular biology (Clifton, N.J.)* 1225: 1-27

Elton D, Medcalf L, Bishop K, Harrison D, Digard P. 1999. Identification of amino acid residues of influenza virus nucleoprotein essential for RNA binding. *Journal of virology* 73: 7357-67

Erf GF. 2004. Cell-mediated immunity in poultry. *Poultry science* 83: 580-90

Errington W, Emmerson PT. 1997. Assembly of recombinant Newcastle disease virus nucleocapsid protein into nucleocapsid-like structures is inhibited by the phosphoprotein. *The Journal of general virology* 78 (Pt 9): 2335-9

Errington W, Steward M, Emmerson PT. 1995. A diagnostic immunoassay for Newcastle disease virus based on the nucleocapsid protein expressed by a recombinant baculovirus. *Journal of virological methods* 55: 357-65

Fawcett T. 2006. An introduction to ROC analysis. *Pattern Recognition Letters* 27: 861-74

Ferguson L, Eckard L, Epperson WB, Long LP, Smith D, et al. 2015. Influenza D virus infection in Mississippi beef cattle. *Virology* 486: 28-34

Ferguson L, Olivier AK, Genova S, Epperson WB, Smith DR, et al. 2016. Pathogenesis of Influenza D Virus in Cattle. *Journal of virology* 90: 5636-42

Fleiss JL CJ, Everitt BS. 1969. Large sample standard errors of kappa and weighted kappa. *Psychological Bull.* 10: 5

Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, et al. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *Journal of virology* 79: 2814-22

Gary MA, Tanner EA, Davis AA, McFarlin BK. 2019. Combined bead-based multiplex detection of RNA and protein biomarkers: Implications for understanding the time course of skeletal muscle injury and repair. *Methods* 158: 92-96

Gerhard W, Yewdell J, Frankel ME, Webster R. 1981. Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies. *Nature* 290: 713-7

Germeraad E, Achterberg R, Venema S, Post J, de Leeuw O, et al. 2019. The development of a multiplex serological assay for avian influenza based on Luminex technology. *Methods* 158: 54-60

Gogoi P, Ganar K, Kumar S. 2017. Avian Paramyxovirus: A Brief Review. *Transbound Emerg Dis* 64: 53-67

Gonsalves S, Mahony J, Rao A, Dunbar S, Juretschko S. 2019. Multiplexed detection and identification of respiratory pathogens using the NxTAG® respiratory pathogen panel. *Methods* 158: 61-68

Goraichuk I, Sharma P, Stegniy B, Muzyka D, Pantin-Jackwood MJ, et al. 2016. Complete Genome Sequence of an Avian Paramyxovirus Representative of Putative New Serotype 13. *Genome Announcements* 4

Graham H, Chandler DJ, Dunbar SA. 2019. The genesis and evolution of bead-based multiplexing. *Methods* 158: 2-11

Grund C, Steglich C, Huthmann E, Beer M, Mettenleiter TC, Romer-Oberdorfer A. 2014. Avian paramyoxvirus-8 immunization reduces viral shedding after homologous APMV-8 challenge but fails to protect against Newcastle disease. *Virology journal* 11: 179

Grund S, Adams O, Wahlisch S, Schweiger B. 2011. Comparison of hemagglutination inhibition assay, an ELISA-based micro-neutralization assay and colorimetric microneutralization assay to detect antibody responses to vaccination against influenza A H1N1 2009 virus. *Journal of virological methods* 171: 369-73

Guan R, Ma LC, Leonard PG, Amer BR, Sridharan H, et al. 2011. Structural basis for the sequence-specific recognition of human ISG15 by the NS1 protein of influenza B virus.

Proceedings of the National Academy of Sciences of the United States of America 108: 13468-73
Guo YJ, Jin FG, Wang P, Wang M, Zhu JM. 1983. Isolation of influenza C virus from pigs and experimental infection of pigs with influenza C virus. *The Journal of general virology* 64 (Pt 1): 177-82
Häuslaigner R, Sonnenburg J, Kothlow S, Kaspers B, Staubach C, Grund C. 2009. Evaluation of an indirect enzyme-linked immunosorbent assay to study the specific humoral immune response of Muscovy ducks (Cairina moschata) and domestic geese (Anser anser var. domestica) after vaccination against Newcastle disease virus. *Avian pathology : journal of the W.V.P.A* 38: 89-95
Hamaguchi M, Yoshida T, Nishikawa K, Naruse H, Nagai Y. 1983. Transcriptive complex of Newcastle disease virus. I. Both L and P proteins are required to constitute an active complex. *Virology* 128: 105-17
Hamilton BS, Whittaker GR, Daniel S. 2012. Influenza virus-mediated membrane fusion: determinants of hemagglutinin fusogenic activity and experimental approaches for assessing virus fusion. *Viruses* 4: 1144-68
Harder TC RA, Buda S. 2011. Zoonotic influenza [ger. Influenza als Zoonose]. *In Epidemiological report on influenza in Germany. 12th edition. Berlin, Germany: Robert-Koch-Institut;* : 7
Harder TC, Vahlenkamp TW. 2010. Influenza virus infections in dogs and cats. *Veterinary immunology and immunopathology* 134: 54-60
Hass J, Matuszewski S, Cieslik D, Haase M. 2011. The role of swine as "mixing vessel" for interspecies transmission of the influenza A subtype H1N1: a simultaneous Bayesian inference of phylogeny and ancestral hosts. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* 11: 437-41
Hauslaigner R, Sonnenburg J, Kothlow S, Kaspers B, Staubach C, Grund C. 2009. Evaluation of an indirect enzyme-linked immunosorbent assay to study the specific humoral immune response of Muscovy ducks (Cairina moschata) and domestic geese (Anser anser var. domestica) after vaccination against Newcastle disease virus. *Avian pathology : journal of the W.V.P.A* 38: 89-95
Hausmann S, Garcin D, Delenda C, Kolakofsky D. 1999. The Versatility of Paramyxovirus RNA Polymerase Stuttering. *Journal of virology* 73: 5568-76
He F, Prabakaran M, Tan Y, Indira K, Kumar SR, Kwang J. 2013. Development of dual-function ELISA for effective antigen and antibody detection against H7 avian influenza virus. *BMC microbiology* 13: 219
Hefferon KL. 2014. Broadly neutralizing antibodies and the promise of universal vaccines: where are we now? *Immunotherapy* 6: 51-7
Hemmatzadeh F, Kazemimanesh M. 2017. Detection of specific antigens of Newcastle disease virus using an absorbed Western blotting method. *Iranian journal of veterinary research* 18: 92-96
Hill SC, Manvell RJ, Schulenburg B, Shell W, Wikramaratna PS, et al. 2016. Antibody responses to avian influenza viruses in wild birds broaden with age. *Proceedings. Biological sciences* 283
Hilleman MR. 2002. Realities and enigmas of human viral influenza: pathogenesis, epidemiology and control. *Vaccine* 20: 3068-87
Hines NL, Miller CL. 2012. Avian paramyxovirus serotype-1: a review of disease distribution, clinical symptoms, and laboratory diagnostics. *Vet Med Int* 2012: 708216
Hoffmann B, Harder T, Starick E, Depner K, Werner O, Beer M. 2007. Rapid and highly sensitive pathotyping of avian influenza A H5N1 virus by using real-time reverse transcription-PCR. *Journal of clinical microbiology* 45: 600-3

Hofmeyer T, Schmelz S, Degiacomi MT, Dal Peraro M, Daneschdar M, et al. 2013. Arranged sevenfold: structural insights into the C-terminal oligomerization domain of human C4b-binding protein. *J Mol Biol* 425: 1302-17

Holmes EC, Goldstein SA, Rasmussen AL, Robertson DL, Crits-Christoph A, et al. 2021. The origins of SARS-CoV-2: A critical review. *Cell* 184: 4848-56

Horimoto T, Hiono T, Mekata H, Odagiri T, Lei Z, et al. 2016. Nationwide Distribution of Bovine Influenza D Virus Infection in Japan. *PloS one* 11: e0163828

Horimoto T, Kawaoka Y. 2005. Influenza: lessons from past pandemics, warnings from current incidents. *Nature reviews. Microbiology* 3: 591-600

Hsieh YC, Wu TZ, Liu DP, Shao PL, Chang LY, et al. 2006. Influenza pandemics: past, present and future. *Journal of the Formosan Medical Association = Taiwan yi zhi* 105: 1-6

Huang Z, Krishnamurthy S, Panda A, Samal SK. 2003. Newcastle disease virus V protein is associated with viral pathogenesis and functions as an alpha interferon antagonist. *Journal of virology* 77: 8676-85

Hwang J, Banerjee M, Venable AS, Walden Z, Jolly J, et al. 2019. Quantitation of low abundant soluble biomarkers using high sensitivity Single Molecule Counting technology. *Methods* 158: 69-76

Iwasaki A, Pillai PS. 2014. Innate immunity to influenza virus infection. *Nature reviews. Immunology* 14: 315-28

Jain A, Saini V, Kohli DV. 2013. Edible transgenic plant vaccines for different diseases. *Current pharmaceutical biotechnology* 14: 594-614

Janke BH. 2000. Diagnosis of swine influenza. *Swine Health Prod* 8: 6

Jeeninga RE, Jan B, van den Berg H, Berkhout B. 2006. Construction of doxycyline-dependent mini-HIV-I variants for the development of a virotherapy against leukemias. *Retrovirology* 3

Jennelle CS, Carstensen M, Hildebrand EC, Wolf PC, Grear DA, et al. 2017. Surveillance for Highly Pathogenic Avian Influenza in Wild Turkeys (Meleagris gallopavo) of Minnesota, USA during 2015 Outbreaks in Domestic Poultry. *Journal of wildlife diseases* 53: 616-20

Jenson TA. 2014. Agar gel immunodiffusion assay to detect antibodies to Type A influenza virus. *Methods in molecular biology (Clifton, N.J.)* 1161: 141-50

Ji Y, Liu T, Jia Y, Liu B, Yu Q, et al. 2017. Two single mutations in the fusion protein of Newcastle disease virus confer hemagglutinin-neuraminidase independent fusion promotion and attenuate the pathogenicity in chickens. *Virology* 509: 146-51

Jimenez-Munguia I, van Wamel WJB, Rodriguez-Ortega MJ, Obando I. 2017. Detection of Natural Antibodies and Serological Diagnosis of Pneumococcal Pneumonia Using a Bead-Based High-Throughput Assay. *Methods in molecular biology (Clifton, N.J.)* 1643: 169-77

Jin M, Wang G, Zhang R, Zhao S, Li H, et al. 2004. Development of enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection of antibodies to avian influenza virus. *Avian diseases* 48: 870-8

Jordan IK, Sutter BAt, McClure MA. 2000. Molecular evolution of the Paramyxoviridae and Rhabdoviridae multiple-protein-encoding P gene. *Molecular biology and evolution* 17: 75-86

Kadam L, Patel K, Gautam M, Thorat S, Kale P, et al. 2019. Development and validation of magnetic bead pentaplex immunoassay for simultaneous quantification of murine serum IgG antibodies to acellular pertussis, diphtheria and tetanus antigens used in combination vaccines. *Methods* 158: 33-43

Kai Y, Hu Z, Xu H, Hu S, Zhu J, et al. 2015. The M, F and HN genes of genotype VIId Newcastle disease virus are associated with the severe pathological changes in the spleen of chickens. *Virology journal* 12: 133

Kamminga S, van der Meijden E, Wunderink HF, Touze A, Zaaijer HL, Feltkamp MCW. 2018. Development and Evaluation of a Broad Bead-Based Multiplex Immunoassay To Measure IgG Seroreactivity against Human Polyomaviruses. *Journal of clinical microbiology* 56

Kapczynski DR, Afonso CL, Miller PJ. 2013. Immune responses of poultry to Newcastle disease virus. *Developmental and comparative immunology* 41: 447-53

Karamendin K, Kydyrmanov A, Seidalina A, Asanova S, Sayatov M, et al. 2016. Complete Genome Sequence of a Novel Avian Paramyxovirus (APMV-13) Isolated from a Wild Bird in Kazakhstan. *Genome Announcements* 4

Karasin AI, Landgraf J, Swenson S, Erickson G, Goyal S, et al. 2002. Genetic characterization of H1N2 influenza A viruses isolated from pigs throughout the United States. *Journal of clinical microbiology* 40: 1073-9

Kash JC, Goodman AG, Korth MJ, Katze MG. 2006. Hijacking of the host-cell response and translational control during influenza virus infection. *Virus research* 119: 111-20

Katz D, Shi W, Patrusheva I, Perelygina L, Gowda MS, et al. 2012. An automated ELISA using recombinant antigens for serologic diagnosis of B virus infections in macaques. *Comparative medicine* 62: 527-34

Katz JM, Hancock K, Xu X. 2011. Serologic assays for influenza surveillance, diagnosis and vaccine evaluation. *Expert review of anti-infective therapy* 9: 669-83

Kawaoka Y, Nestorowicz A, Alexander DJ, Webster RG. 1987. Molecular analyses of the hemagglutinin genes of H5 influenza viruses: origin of a virulent turkey strain. *Virology* 158: 218-27

Khan KH. 2013. Gene expression in Mammalian cells and its applications. *Advanced pharmaceutical bulletin* 3: 257-63

Kho CL, Mohd-Azmi ML, Arshad SS, Yusoff K. 2000. Performance of an RT-nested PCR ELISA for detection of Newcastle disease virus. *Journal of virological methods* 86: 71-83

Kho CL, Tan WS, Tey BT, Yusoff K. 2004. Regions on nucleocapsid protein of Newcastle disease virus that interact with its phosphoprotein. *Archives of virology* 149: 997-1005

Khurana S, Larkin C, Verma S, Joshi MB, Fontana J, et al. 2011. Recombinant HA1 produced in E. coli forms functional oligomers and generates strain-specific SRID potency antibodies for pandemic influenza vaccines. *Vaccine* 29: 5657-65

Kim SH, Chen Z, Yoshida A, Paldurai A, Xiao S, Samal SK. 2017. Evaluation of fusion protein cleavage site sequences of Newcastle disease virus in genotype matched vaccines. *PloS one* 12: e0173965

Kim WI, Wu WH, Janke B, Yoon KJ. 2006. Characterization of the humoral immune response of experimentally infected and vaccinated pigs to swine influenza viral proteins. *Archives of virology* 151: 23-36

Klenk HD, Rott R, Orlich M. 1977. Further studies on the activation of influenza virus by proteolytic cleavage of the haemagglutinin. *The Journal of general virology* 36: 151-61

Kodihalli S, Sivanandan V, Halvorson DA, Nagaraja KV, Kumar MC. 1993. Antigen-capture ELISA for rapid diagnosis of avian influenza virus in commercial turkey flocks. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* 5: 438-40

Kothalawala H, Toussaint MJ, Gruys E. 2006. An overview of swine influenza. *The Veterinary quarterly* 28: 46-53

Krammer F. 2019. The human antibody response to influenza A virus infection and vaccination. *Nature reviews. Immunology*

Krammer F, Smith GJD, Fouchier RAM, Peiris M, Kedzierska K, et al. 2018. Influenza. *Nature Reviews Disease Primers* 4: 3

Kreijtz JH, Fouchier RA, Rimmelzwaan GF. 2011. Immune responses to influenza virus infection. *Virus research* 162: 19-30

Krishnamurthy S, Samal SK. 1998. Nucleotide sequences of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beaudette C and completion of the entire genome sequence. *The Journal of general virology* 79 (Pt 10): 2419-24

Kumar S, Nayak B, Collins PL, Samal SK. 2011. Evaluation of the Newcastle disease virus F and HN proteins in protective immunity by using a recombinant avian paramyxovirus type 3 vector in chickens. *Journal of virology* 85: 6521-34

Kyriakis CS, Rose N, Foni E, Maldonado J, Loeffen WL, et al. 2013. Influenza A virus infection dynamics in swine farms in Belgium, France, Italy and Spain, 2006-2008. *Veterinary microbiology* 162: 543-50

Lamb RA, Choppin PW. 1983. The gene structure and replication of influenza virus. *Annual review of biochemistry* 52: 467-506

Lamb RA, Paterson RG, Jardetzky TS. 2006. Paramyxovirus membrane fusion: lessons from the F and HN atomic structures. *Virology* 344: 30-7

Lamb RP, G. 2007. *Paramyxoviridae: the viruses and their replication*. pp. 1449-96.

Lang C S, Dürrwald R, Herwig V, Selbitz HJ, Sommerfeld-Stur I, Schmoll F. 2007. Hämagglutinationsinhibitiontest und kommerzielle ELISA-Kits zum Nachweis von Antikörpern gegen Schweineinfluenzavirus TypA-H1N1- und –H3N2 differieren in ihrer diagnostischen Aussagekraft [ger. Hemagglutination inhibition assay and commercial ELISA for detection of antibodies against porcine influenza virus subtype H1N1 and H3N2 differ in their diagnostic significance.]. pp. 8. Wien Tierärztl Mschr

Lange E, Kalthoff D, Blohm U, Teifke JP, Breithaupt A, et al. 2009. Pathogenesis and transmission of the novel swine-origin influenza virus A/H1N1 after experimental infection of pigs. *The Journal of general virology* 90: 2119-23

Lee CW, Saif YM. 2009. Avian influenza virus. *Comparative immunology, microbiology and infectious diseases* 32: 301-10

Lee CW, Swayne DE, Linares JA, Senne DA, Suarez DL. 2005. H5N2 avian influenza outbreak in Texas in 2004: the first highly pathogenic strain in the United States in 20 years? *Journal of virology* 79: 11412-21

Lee EK, Jeon WJ, Kwon JH, Yang CB, Choi KS. 2009. Molecular epidemiological investigation of Newcastle disease virus from domestic ducks in Korea. *Veterinary microbiology* 134: 241-8

Lee G, Na YJ, Yang BG, Choi JP, Seo YB, et al. 2015. Oral immunization of haemaggulutinin H5 expressed in plant endoplasmic reticulum with adjuvant saponin protects mice against highly pathogenic avian influenza A virus infection. *Plant biotechnology journal* 13: 62-72

Lee HJ, Kim JY, Lee YJ, Lee EK, Song BM, et al. 2017. A Novel Avian Paramyxovirus (Putative Serotype 15) Isolated from Wild Birds. *Frontiers in microbiology* 8: 786

Lee MS, Chang PC, Shien JH, Cheng MC, Shieh HK. 2001. Identification and subtyping of avian influenza viruses by reverse transcription-PCR. *Journal of virological methods* 97: 13-22

Li L, Wagner B, Freer H, Schilling M, Bannantine JP, et al. 2017a. Early detection of Mycobacterium avium subsp. paratuberculosis infection in cattle with multiplex-bead based immunoassays. *PloS one* 12: e0189783

Li PQ, Zhang J, Muller CP, Chen JX, Yang ZF, et al. 2008. Development of a multiplex real-time polymerase chain reaction for the detection of influenza virus type A including H5 and H9 subtypes. *Diagnostic microbiology and infectious disease* 61: 192-7

Li ZN, Trost JF, Weber KM, LeMasters EH, Nasreen S, et al. 2017b. Novel multiplex assay platforms to detect influenza A hemagglutinin subtype-specific antibody responses for high-throughput and in-field applications. *Influenza and other respiratory viruses* 11: 289-97

Liu Q, Zhou B, Ma W, Bawa B, Ma J, et al. 2014. Analysis of recombinant H7N9 wild-type and mutant viruses in pigs shows that the Q226L mutation in HA is important for transmission. *Journal of virology* 88: 8153-65

Loeffen WL, Nodelijk G, Heinen PP, van Leengoed LA, Hunneman WA, Verheijden JH. 2003. Estimating the incidence of influenza-virus infections in Dutch weaned piglets using blood samples from a cross-sectional study. *Veterinary microbiology* 91: 295-308

Loving CL, Vincent AL, Pena L, Perez DR. 2012. Heightened adaptive immune responses following vaccination with a temperature-sensitive, live-attenuated influenza virus compared to adjuvanted, whole-inactivated virus in pigs. *Vaccine* 30: 5830-8

Luo J, Ferguson L, Smith DR, Woolums AR, Epperson WB, Wan XF. 2017. Serological evidence for high prevalence of Influenza D Viruses in Cattle, Nebraska, United States, 2003-2004. *Virology* 501: 88-91

Luo Q, Huang H, Zou W, Dan H, Guo X, et al. 2009. An indirect sandwich ELISA for the detection of avian influenza H5 subtype viruses using anti-hemagglutinin protein monoclonal antibody. *Veterinary microbiology* 137: 24-30

Lupiani B, Mozisek B, Mason PW, Lamichhane C, Reddy SM. 2010. Simultaneous detection of avian influenza virus NP and H5 antibodies in chicken sera using a fluorescence microsphere immunoassay. *Avian diseases* 54: 668-72

Ma W, Kahn RE, Richt JA. 2008. The pig as a mixing vessel for influenza viruses: Human and veterinary implications. *Journal of molecular and genetic medicine : an international journal of biomedical research* 3: 158-66

Ma W, Lager KM, Vincent AL, Janke BH, Gramer MR, Richt JA. 2009. The role of swine in the generation of novel influenza viruses. *Zoonoses and public health* 56: 326-37

Mahon PJ, Mirza AM, Iorio RM. 2011. Role of the two sialic acid binding sites on the newcastle disease virus HN protein in triggering the interaction with the F protein required for the promotion of fusion. *Journal of virology* 85: 12079-82

Makkay AM, Krell PJ, Nagy E. 1999. Antibody detection-based differential ELISA for NDV-infected or vaccinated chickens versus NDV HN-subunit vaccinated chickens. *Veterinary microbiology* 66: 209-22

Manglani M, Rua R, Hendricksen A, Braunschweig D, Gao Q, et al. 2019. Method to quantify cytokines and chemokines in mouse brain tissue using Bio-Plex multiplex immunoassays. *Methods* 158: 22-26

Manual OT. 2012. *NEWCASTLE DISEASE*. pp. 555-568.

Manuguerra JC, Hannoun C. 1992. Natural infection of dogs by influenza C virus. *Research in virology* 143: 199-204

Marks FS, Rodenbusch CR, Okino CH, Hein HE, Costa EF, et al. 2014. Targeted survey of Newcastle disease virus in backyard poultry flocks located in wintering site for migratory birds from Southern Brazil. *Preventive veterinary medicine* 116: 197-202

Marozin S, Gregory V, Cameron K, Bennett M, Valette M, et al. 2002. Antigenic and genetic diversity among swine influenza A H1N1 and H1N2 viruses in Europe. *The Journal of general virology* 83: 735-45

Martin K, Helenius A. 1991. Transport of incoming influenza virus nucleocapsids into the nucleus. *Journal of virology* 65: 232-44

Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD. 2004. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *Proceedings of the National Academy of Sciences of the United States of America* 101: 4620-4

Matsubara K, Iwata S, Nakayama T. 2012. Antibodies against mumps virus component proteins. *Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy* 18: 466-71

Matsubara K, Sakano T, Takao S, Daikoku K. 2004. [Influenza C virus isolated in Hiroshima Prefecture during the 1999/2000 winter season--a clinical and epidemiological study]. *Kansenshogaku zasshi. The Journal of the Japanese Association for Infectious Diseases* 78: 470-5

Mayer M, Buchner J. 2004. Refolding of inclusion body proteins. *Methods in molecular medicine* 94: 239-54

McFarlin BK, Curtis JH. 2019. Advances in Bead-Based Biomarker Detection. *Methods* 158: 1

McFarlin BK, Venable AS, Henning AL, Sampson JNB, Pennel K, et al. 2016. Reduced inflammatory and muscle damage biomarkers following oral supplementation with bioavailable curcumin. *BBA Clinical* 5: 72-78

McGinnes L, McQuain C, Morrison T. 1988. The P protein and the nonstructural 38K and 29K proteins of Newcastle disease virus are derived from the same open reading frame. *Virology* 164: 256-64

Mechaly A, Vitner E, Levy H, Weiss S, Bar-David E, et al. 2018. Simultaneous Immunodetection of Anthrax, Plague, and Tularemia from Blood Cultures by Use of Multiplexed Suspension Arrays. *Journal of clinical microbiology* 56

Medcalf L, Poole E, Elton D, Digard P. 1999. Temperature-sensitive lesions in two influenza A viruses defective for replicative transcription disrupt RNA binding by the nucleoprotein. *Journal of virology* 73: 7349-56

Mehle A. 2014. Unusual influenza A viruses in bats. *Viruses* 6: 3438-49

Miers LA, Bankowski RA, Zee YC. 1983. Optimizing the enzyme-linked immunosorbent assay for evaluating immunity of chickens to Newcastle disease. *Avian diseases* 27: 1112-25

Miller PJ, Afonso CL, Spackman E, Scott MA, Pedersen JC, et al. 2010. Evidence for a new avian paramyxovirus serotype 10 detected in rockhopper penguins from the Falkland Islands. *Journal of virology* 84: 11496-504

Miller PJ, Torchetti MK. 2014. Newcastle disease virus detection and differentiation from avian influenza. *Methods in molecular biology (Clifton, N.J.)* 1161: 235-9

Mitnaul LJ, Castrucci MR, Murti KG, Kawaoka Y. 1996. The cytoplasmic tail of influenza A virus neuraminidase (NA) affects NA incorporation into virions, virion morphology, and virulence in mice but is not essential for virus replication. *Journal of virology* 70: 873-9

Mohan CM, Dey S, Rai A, Kataria JM. 2006. Recombinant haemagglutinin neuraminidase antigen-based single serum dilution ELISA for rapid serological profiling of Newcastle disease virus. *Journal of virological methods* 138: 117-22

Moreno A, Brocchi E, Lelli D, Gamba D, Tranquillo M, Cordioli P. 2009. Monoclonal antibody based ELISA tests to detect antibodies against neuraminidase subtypes 1, 2 and 3 of avian influenza viruses in avian sera. *Vaccine* 27: 4967-74

Morrison TG. 2003. Structure and function of a paramyxovirus fusion protein. *Biochimica et biophysica acta* 1614: 73-84

Moser M, Leo O. 2010. Key concepts in immunology. *Vaccine* 28 Suppl 3: C2-13

Mozdzanowska K, Maiese K, Furchner M, Gerhard W. 1999. Treatment of influenza virus-infected SCID mice with nonneutralizing antibodies specific for the transmembrane proteins matrix 2 and neuraminidase reduces the pulmonary virus titer but fails to clear the infection. *Virology* 254: 138-46

Munch M, Nielsen LP, Handberg KJ, Jorgensen PH. 2001. Detection and subtyping (H5 and H7) of avian type A influenza virus by reverse transcription-PCR and PCR-ELISA. *Archives of virology* 146: 87-97

Munster VJ, Baas C, Lexmond P, Waldenstrom J, Wallensten A, et al. 2007. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. *PLoS pathogens* 3: e61

Naffakh N, Massin P, Escriou N, Crescenzo-Chaigne B, van der Werf S. 2000. Genetic analysis of the compatibility between polymerase proteins from human and avian strains of influenza A viruses. *The Journal of general virology* 81: 1283-91

Nagai Y, Klenk HD, Rott R. 1976. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. *Virology* 72: 494-508

Nayak B, Dias FM, Kumar S, Paldurai A, Collins PL, Samal SK. 2012. Avian paramyxovirus serotypes 2-9 (APMV-2-9) vary in the ability to induce protective immunity in chickens against challenge with virulent Newcastle disease virus (APMV-1). *Vaccine* 30: 2220-7

Neumann G, Noda T, Kawaoka Y. 2009. Emergence and pandemic potential of swine-origin H1N1 influenza virus. *Nature* 459: 931-9

Nili H, Asasi K. 2002. Natural cases and an experimental study of H9N2 avian influenza in commercial broiler chickens of Iran. *Avian pathology : journal of the W.V.P.A* 31: 247-52

Noda T, Kawaoka Y. 2010. Structure of influenza virus ribonucleoprotein complexes and their packaging into virions. *Reviews in medical virology* 20: 380-91

Oh HL, Akerstrom S, Shen S, Bereczky S, Karlberg H, et al. 2010. An antibody against a novel and conserved epitope in the hemagglutinin 1 subunit neutralizes numerous H5N1 influenza viruses. *Journal of virology* 84: 8275-86

OIE. 2012. *AVIAN INFLUENZA*. pp. 2.3.4. 436 pp.

Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA. 2006. Global patterns of influenza a virus in wild birds. *Science* 312: 384-8

Olsen CW, Brammer L, Easterday BC, Arden N, Belay E, et al. 2002. Serologic evidence of H1 swine Influenza virus infection in swine farm residents and employees. *Emerging infectious diseases* 8: 814-9

Opriessnig T, Gimenez-Lirola LG, Halbur PG. 2011. Polymicrobial respiratory disease in pigs. *Animal health research reviews* 12: 133-48

Osterhaus AD, Rimmelzwaan GF, Martina BE, Bestebroer TM, Fouchier RA. 2000. Influenza B virus in seals. *Science* 288: 1051-3

Panshin A, Shihmanter E, Weisman Y, Orvell C, Kydyrmanov A, et al. 2000. Antigenic characterization of the nucleocapsid protein of Newcastle disease virus by means of a new panel of monoclonal antibodies. *Comparative immunology, microbiology and infectious diseases* 23: 209-20

Park MS, Garcia-Sastre A, Cros JF, Basler CF, Palese P. 2003. Newcastle disease virus V protein is a determinant of host range restriction. *Journal of virology* 77: 9522-32

Pasick J, Handel K, Robinson J, Copps J, Ridd D, et al. 2005. Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia. *The Journal of general virology* 86: 727-31

Patterson KD, Pyle GF. 1991. The geography and mortality of the 1918 influenza pandemic. *Bulletin of the history of medicine* 65: 4-21

Pedersen JC. 2014. Hemagglutination-inhibition assay for influenza virus subtype identification and the detection and quantitation of serum antibodies to influenza virus. *Methods in molecular biology (Clifton, N.J.)* 1161: 11-25

Peeters BP, Gruijthuijsen YK, de Leeuw OS, Gielkens AL. 2000. Genome replication of Newcastle disease virus: involvement of the rule-of-six. *Archives of virology* 145: 1829-45

Perelygina L, Patrusheva I, Hombaiah S, Zurkuhlen H, Wildes MJ, et al. 2005. Production of herpes B virus recombinant glycoproteins and evaluation of their diagnostic potential. *Journal of clinical microbiology* 43: 620-8

Phan LV, Park MJ, Kye SJ, Kim JY, Lee HS, Choi KS. 2013. Development and field application of a competitive enzyme-linked immunosorbent assay for detection of Newcastle disease virus antibodies in chickens and ducks. *Poultry science* 92: 2034-43

Pinette MM, Rodriguez-Lecompte JC, Pasick J, Ojkic D, Leith M, et al. 2014. Development of a duplex Fluorescent Microsphere Immunoassay (FMIA) for the detection of antibody responses to influenza A and newcastle disease viruses. *Journal of immunological methods* 405: 167-77

Poland GA, Jacobson RM, Targonski PV. 2007. Avian and pandemic influenza: an overview. *Vaccine* 25: 3057-61

Porotto M, Salah Z, DeVito I, Talekar A, Palmer SG, et al. 2012. The second receptor binding site of the globular head of the Newcastle disease virus hemagglutinin-neuraminidase activates the stalk of multiple paramyxovirus receptor binding proteins to trigger fusion. *Journal of virology* 86: 5730-41

Portela A, Digard P. 2002. The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. *The Journal of general virology* 83: 723-34

Postel A, Letzel T, Muller F, Ehricht R, Pourquier P, et al. 2011a. In vivo biotinylated recombinant influenza A virus hemagglutinin for use in subtype-specific serodiagnostic assays. *Analytical biochemistry* 411: 22-31

Postel A, Ziller M, Rudolf M, Letzel T, Ehricht R, et al. 2011b. Broad spectrum reactivity versus subtype specificity-trade-offs in serodiagnosis of influenza A virus infections by competitive ELISA. *Journal of virological methods* 173: 49-59

Qiu X, Zhan Y, Meng C, Wang J, Dong L, et al. 2016. Identification and functional analysis of phosphorylation in Newcastle disease virus phosphoprotein. *Archives of virology* 161: 2103-16

Quast M, Sreenivasan C, Sexton G, Nedland H, Singrey A, et al. 2015. Serological evidence for the presence of influenza D virus in small ruminants. *Veterinary microbiology* 180: 281-85

Rabu A, Tan WS, Kho CL, Omar AR, Yusoff K. 2002. Chimeric Newcastle disease virus nucleocapsid with parts of viral hemagglutinin-neuraminidase and fusion proteins. *Acta virologica* 46: 211-7

Rashid S, Naeem K, Ahmed Z, Saddique N, Abbas MA, Malik SA. 2009. Multiplex polymerase chain reaction for the detection and differentiation of avian influenza viruses and other poultry respiratory pathogens. *Poultry science* 88: 2526-31

Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Seraphin B. 1999. A generic protein purification method for protein complex characterization and proteome exploration. *Nature biotechnology* 17: 1030-2

Rivetz B, Weisman Y, Ritterband M, Fish F, Herzberg M. 1985. Evaluation of a novel rapid kit for the visual detection of Newcastle disease virus antibodies. *Avian diseases* 29: 929-42

Rodgers P FJ, Stapleton G, Howse J.*, Portland, OR, USA: Diagrammatic Representation and Inference, 6th International Conference, Diagrams 2010;, 2010*.

Rossman JS, Lamb RA. 2011. Influenza virus assembly and budding. *Virology* 411: 229-36

Rott R. 1979. Molecular basis of infectivity and pathogenicity of myxovirus. Brief review. *Archives of virology* 59: 285-98

Rott R. 1992. The pathogenic determinant of influenza virus. *Veterinary microbiology* 33: 303-10

Rout SN, Samal SK. 2008. The large polymerase protein is associated with the virulence of Newcastle disease virus. *Journal of virology* 82: 7828-36

Saberfar E, Forghani-Fard MM, Mosavi M. 2007. Multiplex reverse transcriptase-PCR assay for typing and subtyping of influenza A (H5 & H9) virus in Iran. *Iranian biomedical journal* 11: 69-74

Saijo M, Niikura M, Ikegami T, Kurane I, Kurata T, Morikawa S. 2006. Laboratory diagnostic systems for Ebola and Marburg hemorrhagic fevers developed with recombinant proteins. *Clinical and vaccine immunology : CVI* 13: 444-51

Samson AC, Levesley I, Russell PH. 1991. The 36K polypeptide synthesized in Newcastle disease virus-infected cells possesses properties predicted for the hypothesized 'V' protein. *The Journal of general virology* 72 (Pt 7): 1709-13

Scholtissek C, Ludwig S, Fitch WM. 1993. Analysis of influenza A virus nucleoproteins for the assessment of molecular genetic mechanisms leading to new phylogenetic virus lineages. *Archives of virology* 131: 237-50

Schultz-Cherry S, Olsen CW, Easterday BC. 2013. History of Swine influenza. *Current topics in microbiology and immunology* 370: 21-8

Senne DA, King DJ, Kapczynski DR. 2004. Control of Newcastle disease by vaccination. *Developments in biologicals* 119: 165-70

Senne DA, Panigrahy B, Kawaoka Y, Pearson JE, Suss J, et al. 1996. Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. *Avian diseases* 40: 425-37

Sheerar MG, Easterday BC, Hinshaw VS. 1989. Antigenic conservation of H1N1 swine influenza viruses. *The Journal of general virology* 70 (Pt 12): 3297-303

Shinya E, Owaki A, Norose Y, Sato S, Takahashi H. 2009. Quick method of multimeric protein production for biologically active substances such as human GM-CSF (hGM-CSF). *Biochem Biophys Res Commun* 386: 40-4

Shope RE. 1931. SWINE INFLUENZA : III. FILTRATION EXPERIMENTS AND ETIOLOGY. *The Journal of experimental medicine* 54: 373-85

Shtyrya YA, Mochalova LV, Bovin NV. 2009. Influenza virus neuraminidase: structure and function. *Acta naturae* 1: 26-32

Shu LL, Bean WJ, Webster RG. 1993. Analysis of the evolution and variation of the human influenza A virus nucleoprotein gene from 1933 to 1990. *Journal of virology* 67: 2723-9

Sidoti F, Rizzo F, Costa C, Astegiano S, Curtoni A, et al. 2010. Development of real time RT-PCR assays for detection of type A influenza virus and for subtyping of avian H5 and H7 hemagglutinin subtypes. *Molecular biotechnology* 44: 41-50

Sims LD. 2013. Intervention strategies to reduce the risk of zoonotic infection with avian influenza viruses: scientific basis, challenges and knowledge gaps. *Influenza and other respiratory viruses* 7 Suppl 2: 15-25

Singh SM, Panda AK. 2005. Solubilization and refolding of bacterial inclusion body proteins. *Journal of bioscience and bioengineering* 99: 303-10

Slemons RD, Swayne DE. 1995. Tissue tropism and replicative properties of waterfowl-origin influenza viruses in chickens. *Avian diseases* 39: 521-7

Slomka MJ, Coward VJ, Banks J, Londt BZ, Brown IH, et al. 2007a. Identification of sensitive and specific avian influenza polymerase chain reaction methods through blind ring trials organized in the European Union. *Avian diseases* 51: 227-34

Slomka MJ, Pavlidis T, Banks J, Shell W, McNally A, et al. 2007b. Validated H5 Eurasian real-time reverse transcriptase-polymerase chain reaction and its application in H5N1 outbreaks in 2005-2006. *Avian diseases* 51: 373-7

Smith EC, Popa A, Chang A, Masante C, Dutch RE. 2009. Viral entry mechanisms: the increasing diversity of paramyxovirus entry. *Febs j* 276: 7217-27

Song SJ, Shin GI, Noh J, Lee J, Kim DH, et al. 2021. Plant-based, adjuvant-free, potent multivalent vaccines for avian influenza virus via Lactococcus surface display. *Journal of integrative plant biology* 63: 1505-20

Spackman E. 2014. A brief introduction to avian influenza virus. *Methods in molecular biology (Clifton, N.J.)* 1161: 61-8

Spackman E, Ip HS, Suarez DL, Slemons RD, Stallknecht DE. 2008. Analytical validation of a real-time reverse transcription polymerase chain reaction test for Pan-American lineage H7 subtype Avian influenza viruses. *Journal of veterinary diagnostic investigation : official*

publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 20: 612-6

Spackman E, Senne DA, Bulaga LL, Myers TJ, Perdue ML, et al. 2003. Development of real-time RT-PCR for the detection of avian influenza virus. *Avian diseases* 47: 1079-82

Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, et al. 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *Journal of clinical microbiology* 40: 3256-60

Spackman E, Suarez, D. L. and Senne, D. A. 2008. Avian Influenza Diagnostics and Surveillance Methods, in Avian Influenza. pp. 305. Book, Avian Influenza

Sreta D, Jittimanee S, Charoenvisal N, Amonsin A, Kitikoon P, Thanawongnuwech R. 2013. Retrospective swine influenza serological surveillance in the four highest pig density provinces of Thailand before the introduction of the 2009 pandemic Influenza A virus subtype H1N1 using various antibody detection assays. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* 25: 45-53

Sriwilaijaroen N, Suzuki Y. 2012. Molecular basis of the structure and function of H1 hemagglutinin of influenza virus. *Proceedings of the Japan Academy. Series B, Physical and biological sciences* 88: 226-49

Starick E, Fereidouni SR, Lange E, Grund C, Vahlenkamp T, et al. 2011a. Analysis of influenza A viruses of subtype H1 from wild birds, turkeys and pigs in Germany reveals interspecies transmission events. *Influenza and other respiratory viruses* 5: 276-84

Starick E, Lange E, Fereidouni S, Bunzenthal C, Hoveler R, et al. 2011b. Reassorted pandemic (H1N1) 2009 influenza A virus discovered from pigs in Germany. *The Journal of general virology* 92: 1184-8

Starick E, Lange E, Grund C, Grosse Beilage E, Dohring S, et al. 2012. Reassortants of pandemic influenza A virus H1N1/2009 and endemic porcine HxN2 viruses emerge in swine populations in Germany. *The Journal of general virology* 93: 1658-63

Starick E, Romer-Oberdorfer A, Werner O. 2000. Type- and subtype-specific RT-PCR assays for avian influenza A viruses (AIV). *Journal of veterinary medicine. B, Infectious diseases and veterinary public health* 47: 295-301

Stech J, Stech O, Herwig A, Altmeppen H, Hundt J, et al. 2008. Rapid and reliable universal cloning of influenza A virus genes by target-primed plasmid amplification. *Nucleic acids research* 36: e139

Steglich C, Grund C, Ramp K, Breithaupt A, Hoper D, et al. 2013. Chimeric newcastle disease virus protects chickens against avian influenza in the presence of maternally derived NDV immunity. *PloS one* 8: e72530

Steinhauer DA. 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. *Virology* 258: 1-20

Stephenson I, Das RG, Wood JM, Katz JM. 2007. Comparison of neutralising antibody assays for detection of antibody to influenza A/H3N2 viruses: an international collaborative study. *Vaccine* 25: 4056-63

Steward M, Samson AC, Errington W, Emmerson PT. 1995. The Newcastle disease virus V protein binds zinc. *Archives of virology* 140: 1321-8

Steward M, Vipond IB, Millar NS, Emmerson PT. 1993. RNA editing in Newcastle disease virus. *The Journal of general virology* 74 (Pt 12): 2539-47

Stockton J, Ellis JS, Saville M, Clewley JP, Zambon MC. 1998. Multiplex PCR for typing and subtyping influenza and respiratory syncytial viruses. *Journal of clinical microbiology* 36: 2990-5

Stone-Hulslander J, Morrison TG. 1997. Detection of an interaction between the HN and F proteins in Newcastle disease virus-infected cells. *Journal of virology* 71: 6287-95

Sturm-Ramirez KM, Ellis T, Bousfield B, Bissett L, Dyrting K, et al. 2004. Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. *Journal of virology* 78: 4892-901

Suarez DL, Senne DA, Banks J, Brown IH, Essen SC, et al. 2004. Recombination resulting in virulence shift in avian influenza outbreak, Chile. *Emerging infectious diseases* 10: 693-9

Subbarao K. 2018. Avian influenza H7N9 viruses: a rare second warning. *Cell research* 28: 1-2

Sutton TC. 2018. The Pandemic Threat of Emerging H5 and H7 Avian Influenza Viruses. *Viruses* 10

Swartz JR. 2001. Advances in Escherichia coli production of therapeutic proteins. *Current opinion in biotechnology* 12: 195-201

Swayne DE, Kapczynski D. 2008. Strategies and challenges for eliciting immunity against avian influenza virus in birds. *Immunological reviews* 225: 314-31

Sylte MJ, Suarez DL. 2009. Influenza neuraminidase as a vaccine antigen. *Current topics in microbiology and immunology* 333: 227-41

Takahashi T, Suzuki T. 2015. Role of sulfatide in influenza A virus replication. *Biological & pharmaceutical bulletin* 38: 809-16

Tanner WD, Toth DJ, Gundlapalli AV. 2015. The pandemic potential of avian influenza A(H7N9) virus: a review. *Epidemiology and infection* 143: 3359-74

Taubenberger JK, Morens DM. 2006. 1918 Influenza: the mother of all pandemics. *Emerging infectious diseases* 12: 15-22

Taubenberger JK, Morens DM. 2010. Influenza: the once and future pandemic. *Public health reports (Washington, D.C. : 1974)* 125 Suppl 3: 16-26

Terregino C, Aldous EW, Heidari A, Fuller CM, De Nardi R, et al. 2013. Antigenic and genetic analyses of isolate APMV/wigeon/Italy/3920-1/2005 indicate that it represents a new avian paramyxovirus (APMV-12). *Archives of virology* 158: 2233-43

Thacker E, Janke B. 2008. Swine influenza virus: zoonotic potential and vaccination strategies for the control of avian and swine influenzas. *The Journal of infectious diseases* 197 Suppl 1: S19-24

Thampaisarn R, Bui VN, Trinh DQ, Nagai M, Mizutani T, et al. 2017. Characterization of avian paramyxovirus serotype 14, a novel serotype, isolated from a duck fecal sample in Japan. *Virus research* 228: 46-57

Thomazelli LM, de Araujo J, Fabrizio T, Walker D, Reischak D, et al. 2017. Novel avian paramyxovirus (APMV-15) isolated from a migratory bird in South America. *PloS one* 12: e0177214

Times UY. 2007. A diagram of influenza viral cell invasion and replication. https://commons.wikimedia.org/wiki/File:Virus_Replication_large.svg

Tong S, Compans RW. 1999a. Alternative mechanisms of interaction between homotypic and heterotypic parainfluenza virus HN and F proteins. *The Journal of general virology* 80 (Pt 1): 107-15

Tong S, Compans RW. 1999b. Alternative mechanisms of interaction between homotypic and heterotypic parainfluenza virus HN and F proteins. *The Journal of general virology* 80 (Pt 1): 107-15

Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, et al. 2012. A distinct lineage of influenza A virus from bats. *Proceedings of the National Academy of Sciences of the United States of America* 109: 4269-74

Tong S, Zhu X, Li Y, Shi M, Zhang J, et al. 2013. New world bats harbor diverse influenza A viruses. *PLoS pathogens* 9: e1003657

Torremorell M, Allerson M, Corzo C, Diaz A, Gramer M. 2012. Transmission of influenza A virus in pigs. *Transbound Emerg Dis* 59 Suppl 1: 68-84

Treanor J. 2004. Influenza vaccine--outmaneuvering antigenic shift and drift. *N Engl J Med* 350: 218-20

Trebbien R, Larsen LE, Viuff BM. 2011. Distribution of sialic acid receptors and influenza A virus of avian and swine origin in experimentally infected pigs. *Virology journal* 8: 434

Umali DV, Ito H, Katoh H, Ito T. 2014. Surveillance of avian paramyxovirus in migratory waterfowls in the San-in region of western Japan from 2006 to 2012. *The Journal of veterinary medical science* 76: 423-30

Unger T, Peleg Y. 2012. Recombinant protein expression in the baculovirus-infected insect cell system. *Methods in molecular biology (Clifton, N.J.)* 800: 187-99

Velumani S, Du Q, Fenner BJ, Prabakaran M, Wee LC, et al. 2008. Development of an antigen-capture ELISA for detection of H7 subtype avian influenza from experimentally infected chickens. *Journal of virological methods* 147: 219-25

Vemula SV, Zhao J, Liu J, Wang X, Biswas S, Hewlett I. 2016. Current Approaches for Diagnosis of Influenza Virus Infections in Humans. *Viruses* 8: 96

Vey M, Orlich M, Adler S, Klenk HD, Rott R, Garten W. 1992. Hemagglutinin activation of pathogenic avian influenza viruses of serotype H7 requires the protease recognition motif R-X-K/R-R. *Virology* 188: 408-13

Watson DS, Reddy SM, Brahmakshatriya V, Lupiani B. 2009. A multiplexed immunoassay for detection of antibodies against avian influenza virus. *Journal of immunological methods* 340: 123-31

Webby RJ, Swenson SL, Krauss SL, Gerrish PJ, Goyal SM, Webster RG. 2000. Evolution of swine H3N2 influenza viruses in the United States. *Journal of virology* 74: 8243-51

Weber S, Harder T, Starick E, Beer M, Werner O, et al. 2007. Molecular analysis of highly pathogenic avian influenza virus of subtype H5N1 isolated from wild birds and mammals in northern Germany. *The Journal of general virology* 88: 554-8

Webster RG. 2002. The importance of animal influenza for human disease. *Vaccine* 20 Suppl 2: S16-20

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. 1992. Evolution and ecology of influenza A viruses. *Microbiological reviews* 56: 152-79

Werner O, Romer-Oberdorfer A, Kollner B, Manvell RJ, Alexander DJ. 1999. Characterization of avian paramyxovirus type 1 strains isolated in Germany during 1992 to 1996. *Avian pathology : journal of the W.V.P.A* 28: 79-88

Wiley DC, Skehel JJ. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annual review of biochemistry* 56: 365-94

Wood JM, Major D, Heath A, Newman RW, Hoschler K, et al. 2012. Reproducibility of serology assays for pandemic influenza H1N1: collaborative study to evaluate a candidate WHO International Standard. *Vaccine* 30: 210-7

Wood JM, Montomoli E, Newman RW, Daas A, Buchheit KH, Terao E. 2011. Collaborative study on influenza vaccine clinical trial serology - part 2: reproducibility study. *Pharmeuropa bio & scientific notes* 2011: 36-54

Woolcock PR, Cardona CJ. 2005. Commercial immunoassay kits for the detection of influenza virus type A: evaluation of their use with poultry. *Avian diseases* 49: 477-81

Woolcock PR, Suarez DL, Kuney D. 2003. Low-pathogenicity avian influenza virus (H6N2) in chickens in California, 2000-02. *Avian diseases* 47: 872-81

Xie Z, Pang YS, Liu J, Deng X, Tang X, et al. 2006. A multiplex RT-PCR for detection of type A influenza virus and differentiation of avian H5, H7, and H9 hemagglutinin subtypes. *Molecular and cellular probes* 20: 245-9

Xu H, Lohr J, Greiner M. 1997. The selection of ELISA cut-off points for testing antibody to Newcastle disease by two-graph receiver operating characteristic (TG-ROC) analysis. *Journal of immunological methods* 208: 61-4

Yamaguchi H, Miyazaki M. 2014. Refolding techniques for recovering biologically active recombinant proteins from inclusion bodies. *Biomolecules* 4: 235-51

Yamaji R, Saad MD, Davis CT, Swayne DE, Wang D, et al. 2020. Pandemic potential of highly pathogenic avian influenza clade 2.3.4.4 A(H5) viruses. *Reviews in medical virology* 30: e2099

Ye Z, Liu T, Offringa DP, McInnis J, Levandowski RA. 1999. Association of influenza virus matrix protein with ribonucleoproteins. *Journal of virology* 73: 7467-73

Youzbashi E, Marschall M, Chaloupka I, Meier-Ewert H. 1996. [Distribution of influenza C virus infection in dogs and pigs in Bavaria]. *Tierarztliche Praxis* 24: 337-42

Yu F, Khairullah NS, Inoue S, Balasubramaniam V, Berendam SJ, et al. 2006. Serodiagnosis using recombinant nipah virus nucleocapsid protein expressed in Escherichia coli. *Journal of clinical microbiology* 44: 3134-8

Yu XH, Cheng JL, Xue J, Jin JH, Song Y, et al. 2017. Roles of the Polymerase-Associated Protein Genes in Newcastle Disease Virus Virulence. *Frontiers in microbiology* 8: 161

Zacour M, Ward BJ, Brewer A, Tang P, Boivin G, et al. 2016. Standardization of Hemagglutination Inhibition Assay for Influenza Serology Allows for High Reproducibility between Laboratories. *Clinical and vaccine immunology : CVI* 23: 236-42

Zeynalova S, Guliyev F, Vatani M, Abbasov B. 2015. Biosurveillance of avian influenza and Newcastle disease viruses in the Barda region of Azerbaijan using real time RT-PCR and hemagglutination inhibition. *Frontiers in microbiology* 6: 1128

Zhai SL, Zhang H, Chen SN, Zhou X, Lin T, et al. 2017. Influenza D Virus in Animal Species in Guangdong Province, Southern China. *Emerging infectious diseases* 23: 1392-96

Zhang A, Jin M, Liu F, Guo X, Hu Q, et al. 2006. Development and evaluation of a DAS-ELISA for rapid detection of avian influenza viruses. *Avian diseases* 50: 325-30

Zhao N, Grund C, Beer M, Harder TC. 2018. Engineered recombinant protein products of the avian paramyxovirus type-1 nucleocapsid and phosphoprotein genes for serological diagnosis. *Virology journal* 15: 8

Zhao N, Grund C, Beer M, Wang G, Harder TC. 2022. Tetraplex Fluorescent Microbead-Based Immunoassay for the Serodiagnosis of Newcastle Disease Virus and Avian Influenza Viruses in Poultry Sera. *Pathogens* 11: 1059

Zhao N, Lange E, Kubald S, Grund C, Beer M, Harder TC. 2013. Distinction of subtype-specific antibodies against European porcine influenza viruses by indirect ELISA based on recombinant hemagglutinin protein fragment-1. *Virology journal* 10: 246

Zhou EM, Chan M, Heckert RA, Riva J, Cantin MF. 1998. Evaluation of a competitive ELISA for detection of antibodies against avian influenza virus nucleoprotein. *Avian diseases* 42: 517-22

Summary

We have expressed several recombinant proteins of influenza A virus and APMV-1 virus via bacterial expression system and promoted these proteins folded properly in vitro. Bacterially expressed recombinant proteins from IAV and NDV were applied for diagnostic reagents with mainly benefits including rapid expression, low cost and easy purification. The serological assays based on these recombined proteins are potential alternative method to the HI assay.

We demonstrated that the well conserved influenza NP protein linked with biotin tag can be used for serological detection to discriminate between influenza-specific and other avian respiratory viral infection, which presented a good agreement when compared to HI and commercial blocking ELISA assays in both experimental and field sera. Therefore, it can be considered as an alternative method for routine serodiagnosis. In contrast, the highly varied portion of IAV HA protein (HA1) as diagnostic antigens received some cross reactivity between the different subtypes. Although these truncated HA1 proteins exhibited an interesting alternative to HI assays in indirect ELISAs, it is not feasible to use them as the targets for porcine influenza viral subtype or lineage-specific discrimination.

Bacterially expressed recombinant proteins NP and P of NDV as the antigens were sensitive to detect seroconversion after NDV infection, but revealed some degree of cross reactivity between serotypes. however, the cross reactivity was abolished when employing a C-terminal fragment of the NP of NDV and APMV-8 as targets in APMV subtype-specific ELISA assays. Unfortunately, NDV V protein cannot be used for serological DIVA strategy to discriminate APMV-1 virus infection from vaccination.

Finally, a competitive liquid array based on Luminex® bead technology was established for simultanously measuring seroresponses to AIV and NDV in poultry sera. Good performance characteristics were obtained with both experimental and field sera. This application provided some obvious benefits related to high throughput, overall time savings and being species independent. Additionally, this fluorescent microsphere approach has the potential to be expanded to up to 500 targets simultaneously and their offers potential to include further pathogens of interest, immunological or biochemical parameters.

Zusammenfassung

In dieser Arbeit wurden mehrere rekombinante Proteine des Influenza A- Virus (IAV) und des aviären Paramyxovirus-1 (Newcastle Disease virus, NDV) in einem bakteriellen Expressionssystem exprimiert und diese in vitro in authentisch gefaltete Proteine konvertiert. Bakteriell exprimierte rekombinante Proteine von IAV und NDV wurden als diagnostische Reagenzien in indirekten ELISA Verfahren eingesetzt; das Expressionssystem erwies sich als schnell und kostengünstig, da auch eine technisch einfache Reinigungsmethode angewendet werden konnte. Die mit diesen rekombinanten Proteinen hier entwickelten serologischen Tests stellen eine mögliche alternative Methode zum Hämagglutinationshemmtest (HAH) dar, der als Goldstandard in der serologischen Diagnostik dieser Virusinfektionen gilt, jedoch zeitaufwändig und schwer zu standardisieren ist.

Wir haben gezeigt, dass das gut konservierte Influenza-Nukleokapsid (NP)-Protein, das mit dem Biotin-Tag verknüpft wurde, zum serologischen Nachweis verwendet werden kann, der zwischen einer Influenza-spezifischen und einer Infektion mit anderen Erregern von Atemwegserkrankungen unterscheidet. Im Vergleich zum HAH und zu kommerziellen kompetitiven ELISA-Tests ergab sich eine gute Übereinstimmung mit Seren aus experimentellen Infektionen als auch mit Feldseren. Daher kann die entwickelte Methode als Alternative zur routinemäßigen Serodiagnostik in Betracht gezogen werden. Im Gegensatz dazu zeigte der stark variable Teil des IAV-HA-Proteins (HA1) als diagnostisches Antigen eine gewisse Kreuzreaktivität zwischen den verschiedenen Subtypen. Obwohl trunkierte HA1-Proteine in indirekten ELISAs eine interessante Alternative zum HAH darstellen, war es nicht möglich, sie zur serologischen Subtypisierung oder zu einer linienspezifischen Diskriminierung porciner Influenzaviren zu verwenden.

Die bakteriell exprimierten rekombinanten NDV Volllängenproteine NP und P von NDV zeigten als Antigene in indirekten ELISA Verfahren ebenfalls einen gewissen Grad an Kreuzreaktivität zwischen den verschiedenen Serotypen aviärer Paramyxoviren. Die Kreuzreaktivität wurde jedoch aufgehoben, wenn lediglich ein gering konserviertes C-terminales Fragment des NP von NDV in APMV-Subtyp-spezifischen ELISA-Assays verwendet wurde. In reziproker Weise funktionierte ein C-terminales NP Fragment des

APMV Subtyps 8. Zur serologischen Unterscheidung geimpfter und infizierter Tiere werden sogenannte DIVA-Tests eingesetzt. Der von uns entwickelte DIVA-Test für NDV basierte auf bakteriell exprimierten viralem V Protein, das im Wesentlichen in infizierten Zellen exprimiert, jedoch nicht oder nur in Spuren in Virionen eingebaut wird. Allerdings erwies sich dieser Test als nicht ausreichend diskriminativ und kann daher nicht für die serologische DIVA-Strategie für das APMV-1-Virus verwendet werden.

Im letzten Teil der Studie wurde ein kompetitiver Suspensiontest basierend auf der Luminex ® Bead - Technologie entwickelt. In diesem Test sollten Antikörper gegen Influenza A Viren und NDV gleichzeitig in Geflügelseren gemessen werden. Dieses Testdesign bietet Vorteile in Bezug auf einen hohen Durchsatz, Zeitersparnis und Speziesunabhängigkeit. In unserer Pilotstudie mit Seren aus experimentellen Infektionen und aus dem Feld ergaben sich gute Leistungsdaten hinsichtlich Sensitivität und Spezifität. Da das Luminex Bead System die Kombination von bis zu 500 unterschiedliche Testantigene erlaubt, hat diese Testplattform erhebliches Erweiterungspotenzial zum Nachweis von Antikörpern auch gegen weitere relevante Krankheitserreger.

Acknowledgements

First of all, I would like to give my sincerest thanks to my supervisor Prof. Dr. Timm Harder for giving me the wonderful opportunity to work on such an interesting, challenging and promising project in his lab in Friedrich Loeffler Institute. Dear Timm, it is my great pleasure to be your student and to work with you. Your door is always open for me no matter how occupied you are. Thank you so much for your continuous support on my career, especially for the understanding and fighting for the best for me all the time. I also appreciate very much for your help on my living in Greifswald and treating me like a family member, making me spend an unforgettable time in Germany. Thank you for everything you did for me.

I would also like to acknowledge Prof. Dr. Mohr and Prof. Dr. Helen Louton for accepting me as a Ph.D. student in Rostock University. Thank you very much for your support in the defense!

I would also like to thank our diagnostic institute head Dr. Martin Beer. Thank you for the help in all aspects during my PhD.

Many thanks to Dr. Christian Grund for his help in my lab work and the well organization of team work which guarantees everything is running well during my Ph.D. study. Many thanks are also given to Sybille Kubald for her kind help and spend an memorable time with me. I will never forget time that you came to see me in the Netherlands with your two sons. Thanks for Cony Illing, Sven Sander, Katja for their contribution to the lab and kind help. I feel cozy during the stay in this lab and I learnt a lot from you guys. Thank you all for the company during my PhD study and I wish our group continue to flourish and all the best of you.

I will also thank my friend Xiaocui, the only Chinese in FLI. It is reassuring to meet people who speak the same language and share the common culture. I will never forget the frequent dinners we had and the time we travel together. Thank you for your company on the way to and from work every day.

My thanks also go to the Chinese friends I met in AMC, the Netherlands. Xiaoxia, Zhongliang, Zheng Yi, Zhao Jing, Chen Mei, Xudong, I really enjoyed the time with you and thank you all. I sincerely wish you success in your future.

Any words would never be too exaggerated to express my gratefulness to my family, my beloved parents and for always being so supportive and loving me without any reserve. Wish you both enjoy every day in the future. I thank my husband Gang Wang for the constructive discussion for my work, and providing continuous support in my life and believing in me no matter what hard circumstance I have encountered. I love you. The last word to my two little daughters Tiantian (甜甜) and Tangtang (糖糖), may you both grow up healthily and Mum love you forever!

For all, thank you Na Zhao 赵娜

Scientific Curriculum Vitae

Education and Research Experience

03/2018 – current		Precise Genome Engineering Center, School of Life Sciences, Guangzhou University, China
07/2015 – 02/2018	Post doctor	Laboratory of Experimental Virology, Department of Medical Microbiology, Academic Medical Center, University of Amsterdam, The Netherlands.
	Project	Utilizing Crispr Cas9 system to eradicate HIV genome from infected cells
10/2010 – 02/2014	PhD student	Institute of Diagnostic Virology, Friedrich-Loeffler-Institute, Germany
	Project	Serodiagnosis of influenza and Newcastle disease viruses – new molecular approaches
09/2007 – 07/2010	Master	LanZhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China
	Project	Evaluation of the adjuvant effect of CpG ODN and cytokine on the Foot-and-Mouth Disease Virus vaccine
09/2002 – 07/2006	Bachelor	College of Life Sciences, Northeast Agricultural University, China
	Project	Canine IL-2 gene expression in bacterial system.

List of publications

Publications during the phd study:

1. **Zhao N,** Grund C, Beer M, Wang G, Harder TC: Tetraplex Fluorescent Microbead-Based Immunoassay for the Serodiagnosis of Newcastle Disease Virus and Avian Influenza Viruses in Poultry Sera. *Pathogens* 2022, 11(9):1059.

2. **Zhao N**, Grund C, Beer M, Harder TC: Engineered recombinant protein products of the avian paramyxovirus type-1 nucleocapsid and phosphoprotein genes for serological diagnosis. *Virology journal* 2018, 15(1):8.

3. Henritzi D, **Zhao N**, Starick E, Simon G, Krog JS, Larsen LE, Reid SM, Brown IH, Chiapponi C, Foni E et al: Rapid detection and subtyping of European swine influenza viruses in porcine clinical samples by haemagglutinin- and neuraminidase-specific tetra- and triplex real-time RT-PCRs. *Influenza and other respiratory viruses* 2016, 10(6):504-517.

4. Steglich C, Grund C, Röder A, **Zhao N**, Mettenleiter T.C: Römer-Oberdörfer, A. Chimeric avian paramyxovirus-based vector immunization against highly pathogenic avian influenza followed by conventional Newcastle disease vaccination eliminates lack of protection from virulent ND virus. *Trials in Vaccinology* 2014, 3, 65-72

5. **Zhao N**, Lange E, Kubald S, Grund C, Beer M, Harder TC: Distinction of subtype-specific antibodies against European porcine influenza viruses by indirect ELISA based on recombinant hemagglutinin protein fragment-1. *Virology journal* 2013, 10:246.

Publications during the other scientific research:

6. Wang X, He S, **Zhao N**, Liu X, Cao Y, Zhang G, Wang G, Guo C: Development and clinical application of a novel CRISPR-Cas12a based assay for the detection of African swine fever virus. *BMC Microbio*l 2020, 20(1):282.

7. **Zhao N**, Wang G, Das AT, Berkhout B: Combinatorial CRISPR-Cas9 and RNA Interference Attack on HIV-1 DNA and RNA Can Lead to Cross-Resistance. *Antimicrobial agents and chemotherapy* 2017, 61(12).

8. Wang G, **Zhao N**, Berkhout B, Das AT: CRISPR-Cas9 Can Inhibit HIV-1 Replication but NHEJ Repair Facilitates Virus Escape. *Molecular therapy*: the journal of the American Society of Gene Therapy 2016, 24(3):522-526.

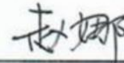
9. Wang G, **Zhao N**, Berkhout B, Das AT: A Combinatorial CRISPR-Cas9 Attack on HIV-1 DNA Extinguishes All Infectious Provirus in Infected T Cell Cultures. *Cell reports* 2016, 17(11):2819-2826.

10. **Zhao N**, Chen G, Fang Y, Li W, Jia H, Jing Z: Enhanced Immunity to Killed FMDV Antigen by Dual Factors Recombinant Plasmid of Porcine Interferon-gamma and CpG Motifs. *Acta Veterinaria et Zootechnica Sinica* 2010, 41(10):1281-1289.

Eidesstattliche Erklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Hilfsmittel benutzt habe. Die Stellen der Arbeit, die dem Wortlaut oder dem Sinn nach anderen Werken entnommen sind, wurden unter Angabe der Quelle kennt-lich gemacht.

GuangZhou, China 2022, 08, 23



Ort, Datum

Unterschrift der Doktorandin/des Doktoranden