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Viral respiratory diseases complex: Investigation on the relevance of Newcastle disease virus in vaccinated poultry flocks in Egypt and characterization of further detected viruses.

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aa	amino acid
AI	Avian influenza
AIV	Avian influenza virus
Bp	base pair
CAM	Chorioallantoic membrane
Con-A	Concanavalin A
CPE	Cytopathic effect
Ct	Cycle threshold
ELISA	Enzyme-linked Immunosorbent Assay
F	Fusion
FAO	Food and agriculture organization of the United Nations
HA	Haemagglutination
HI	Haemagglutination inhibition
HN	Haemagglutinin-neuraminidase
HPAIV	Highly pathogenic avian influenza virus
HR	Heptad repeat
ICPI	Intracerebral pathogenicity index
IF	Immunofluorescence
IFN	Interferon
IVPI	Intravenous pathogenicity index
L	large polymerase
LPAI	Low pathogenic avian influenza
M	Matrix
MAb	Monoclonal antibodz
MDT	Mean death time
NP	Nucleoprotein
NCBI	National center for biotechnology information
ND	Newcastle disease
NDV	Newcastle disease virus
OIE	World Organisation for Animal Health
P	Phosphoprotein
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SPF	Specific pathogen free
ARV	Avian REO virus
IBV	Infectious bronchitis virus
CAstV	Chicken astro virus
vvNDV	viscerotropic velogenic Newcastle disease virus
nvNDV	neurotropic velogenic Newcastle disease virus

Dedication

TO THE SOLE OF MY FATHER

TO MY MOTHER, LILA

TO MY LOVELY WIFE AND DAUGHTER

GEHAD & SARA

TO MY BROTHERS

MOHAMED & ABDEL RAHMAN

TO MY SISTER

RASHA

1 Chapter 1:

1.1 Introduction

Newcastle disease (ND) is a highly contagious viral infection of birds that is endemic in many regions of the world, caused by virulent strains of Newcastle disease virus (NDV) or Avian Paramyxovirus-1, a member of the genus Orthoavulavirus within the family of Paramyxoviridea [1, 2].

For Egypt, ND was ranked as one of the most important poultry diseases in the last decade especially in broiler flocks [3-5]. The predominant clinical finding observed in affected farms are respiratory distress and nervous disorders. This includes wet eyes, sneezing, rales and dyspnea. Nervous disorders like tilting of the head and torticollis are often observed [6, 7]. Mortality is variable and mostly around 15–20%[8], but can reach up to 80% or more in some reports even in vaccinated flocks [6, 9].

Because of the endemic ND situation in Egypt with expected high economic losses, intensive vaccination programs are in widely applied. For broilers, vaccination programs during the 35-40 days of the fattening period includes at least two applications of live vaccines, often with one an inactivated vaccine in between. For layers and breeders, an intensive vaccination scheme used during rearing the chicks with repeated application of live and inactivated vaccines that may be followed by another vaccinations during the production period according to the need [10, 11]. Nevertheless, continuous ND outbreaks are reported and linked to high losses especially in broiler farms. Detection of NDV in these farms are considered as indication for failure of vaccination and hygienic measures [6, 12]. This discussion is inspired by an observed phylogenetic distance between of vaccine type virus strains, that were established in the late 1940ies and current viruses associated to ongoing outbreaks. Even though, recent studies show to a great extent clinical protection from disease by established vaccines, regardless of the genotype.

In this context, it is important to note, that diagnosis of ND relies mostly on the clinical signs in conjunction with post mortem findings. However, virus detection is restricted to generic detection of NDV without differentiating the pathotype. In consequence, clinical suspicion for ND outbreaks in vaccinated farms might be superimposed or caused by other pathogens and detected NDV is the vaccine type virus.

The current study attempted to investigate the prevalence / relevance of virulent NDV in flocks that suffer from respiratory distress accompanied with elevated mortality in Egypt. Beside detection of NDV, relevant viral infections, i.e. IBV and Avian Influenza virus (AIV) should be included in the deferential diagnostic approach. The detected viruses should be used to determine the circulating virus strains and to investigate potential antigenic mismatch of obtained NDV field isolates. Appropriate monoclonal mouse antibodies should be established to further depict possible genotype specific antigenic sites of NDV. These results are mandatory to study potential antigenic drift of prevalent NDV in Egypt and should help to translate sequence information into information of the antigenic properties of NDV.

1.2 Review of literature

Definition of Newcastle disease

Newcastle disease is a highly contagious viral infection of birds that is endemic in many regions of the world. Clinical course may vary from per-acute to subacute-chronic and is characterized by manifestation in the respiratory- and digestive tract with involvement of the nervous system later during disease. Mortality can reach up to 100 %. It is recognized as notifiable disease by the world health organization of animal disease [13] and control measures include obligatory vaccination combined with stamping out policy.

1.2.1 Virus structure

1.2.1.1 Virion and genome organization

Newcastle disease virus (NDV) formally known as avian paramyxovirus one (APMV-1). Based on phylogenetic analysis of the L-protein, NDV has recently re-classified as avian orthoavulavirus-1 [1]. In the new taxonomy system, NDV is belonging to the family Paramyxoviridae, genera Orthoavulavirus, in the sub-family Avulavirinae, together with two other genera Metaavulavirus and Paraavulavirus (Figure.1).

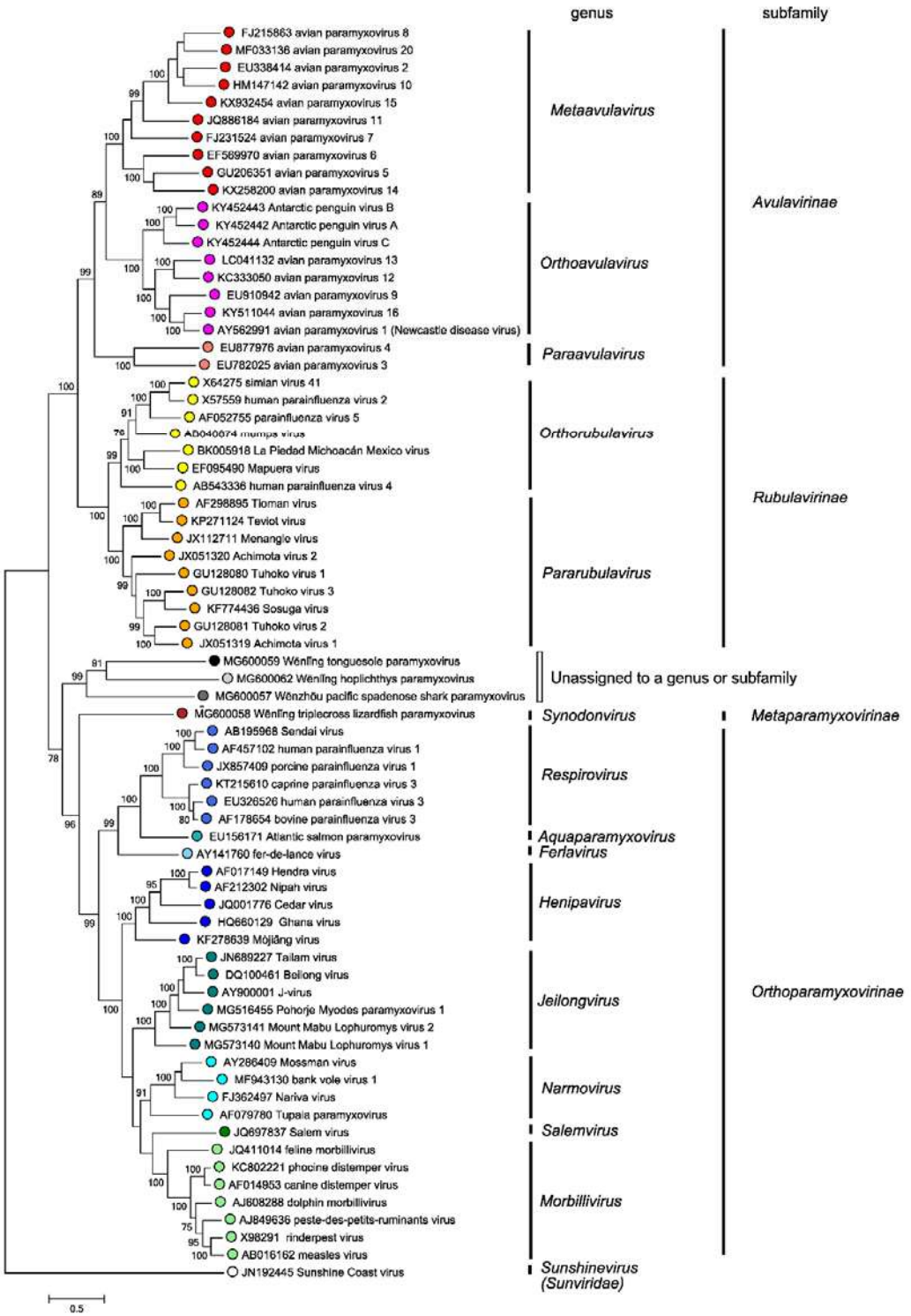

Figure 1: Classification of family Paramyxoviridae.

The family of paramyxoviridae is sub divided into the four subfamilies Avulavirinae, Rebulavirinae, Metaparamyxovirinae and orthoparamyxovirinae. Viruses from birds are within the subfamily avulavirinae, that is further subdivided in three genera. Newcastle disease viurs is belonging to the genus orthoavulaviruses. The classification is described according to [1].

NDV has a RNA genome of negative polarity, encoding six structural proteins [14, 15]. From 5´to 3´ end these include the genes for the nucleocapsid-protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and large RNA-dependent polymerase (L) [16, 17].

The F-protein is a type I transmembrane glycoprotein integrated in the viral envelope and mediates the fusion of the viral and host cell-membrane to facilitate the release of the RNA-NP-P-L complex into the cell cytoplasm. The F-protein is synthesized as an inactive precursor (F0) and has to be activated by proteolytic cleavage, transforming the protein into its mature fusion-competent form, with the two subunits (F1 and F2) that are linked by disulfide-bridge bindings. [18-21]. It consists of three monomers forming a homo-trimeric glycoprotein. Within each oligomer a head-, neck- and stalk-domain can be distinguished. The head- and neck-domains are composed of both the F2 and F1 polypeptides. The stalk region is a long coiled-coil trimer and includes the carboxyl terminal region of heptad repeat domains 1 (HR1) that play important role in fusion activity of the fusion protein [22]. In addition, a transmembrane domains (TM) and a cytoplasmic tail (CT) were identified in paramyxoviruses fusion proteins. While the TM is clearly necessary to anchor the protein in the membrane, the CT is thought to play a role in fusion activity. [23]. The main functional site is located in the amino terminus of the F1 polypeptide, composed of hydrophobic stretch of amino acids (aa, 117- 142) termed fusion peptide [22, 24]. Besides, a second fusion peptide with an internal location in F1 polypeptide was proposed in paramyxoviruses based on comparison to Sendai virus [25, 26]. The NDV F-protein contains further four heptad repeat (HR) domains that play a role in the folding of F-protein and the fusion activity as well [22].

The HN-protein is the second spike-protein of NDV, embedded in the virus membrane and exposed on the surface of the NDV virion. It has multifunctional properties: it is responsible for receptor binding, neuraminidase activity and promotes the fusion activity of the F-protein [27, 28]. The HN is a type II transmembrane glycoprotein. Two homo-dimers are forming a tetramer that consists of an ecto-, and transmembrane-domain as well as a cytoplasmic tail. The ecto-domain consists of a globular head, perched on top of a membrane-anchored stalk domain [29, 30]. The globular head is responsible for viral attachment to sialic acid-containing receptor(s). The function as neuraminidase (NA) is considered necessary to remove sialic acid from new progeny virions in order to prevent self-aggregation [31, 32]. The stalk interacts with the F-protein to promote membrane fusion [33-36]. The cytoplasmic tail of the HN-protein are

found to be critical for HN-protein specific insertion into the virus particle and modulates the fusion activity of NDV as well [37].

The **matrix protein (M)** forms a grid-like array on the inner surface of the viral membrane [38]. It is believed to play an important role in virus assembly as it interacts with both the cytoplasmic tails of HN and F-glycoproteins as well as NP-protein initiating virus assembly and budding [38-41].

The **nucleocapsid-protein (NP)** forms the nucleocapsid (NC) that encapsulates the genomic viral RNA, thus rendering the viral genomic RNA resistant to ribonucleases. Moreover, the viral polymerase complex, consisting of the phosphoprotein (P) and the L-protein (P-L) is associating to the nucleocapsid. Together they are facilitating viral RNA transcription and replication. In addition, NC interacts with the M-protein and both glycoproteins (F and HN) during the assembly of paramyxovirus virions [42, 43].

The **phosphoprotein (P)** is vital for viral RNA synthesis. It is part of the viral polymerase complex and enhances the interaction between L-protein and NDV RNA templates. Besides, The P-protein interacts with unassembled NP-protein to prevent uncontrolled encapsidation of non-viral RNA [44]. Besides the phosphoprotein (P), two another proteins (V and W) are encoded by the P-gene through a frame shift during P-gene transcription. The V protein plays a role in blocking the activation of the interferon pathway in the host cells and thereby enhances NDV replication [45, 46]. Moreover, the V-protein induces the host cell extracellular signal-regulated kinase (ERK), which subsequently enhance NDV replication [47]. On the other hand, the function of the W-protein is still unknown. The mRNA of a potential W-protein of NDV was first described in 1993 [48], but the expression and the nuclear localization of the W-protein was finally identified in 2018 [49]. W-protein was found to be expressed in both lentogenic as well as velogenic viruses. Moreover, it was demonstrated that there was non-essential functions of W-protein expression or localization in virus replication *in Vitro*. Compared to the function of W-protein in other paramyxoviruses as Nipah virus, it was suggested that the W-protein may play a role in the virus virulence [49].

The **L-protein (L)** is the largest viral protein. In conjunction with P-protein, the L-protein is mediating viral RNA synthesis and contains catalytic activities of a viral polymerase [50, 51].

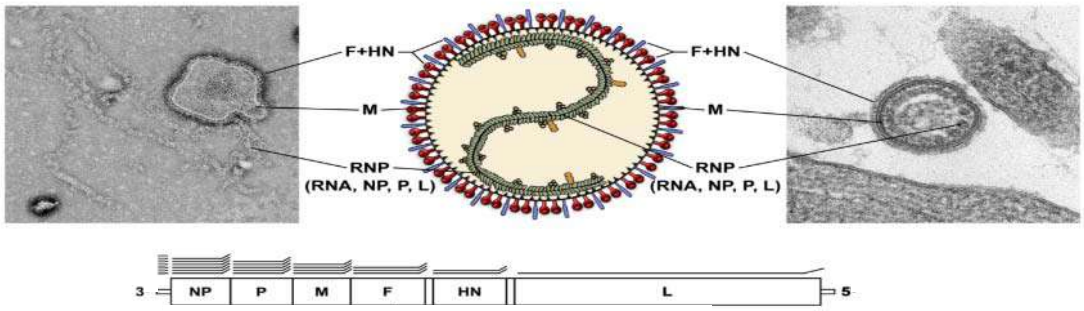

Figure 2. Newcastle Disease Virus and genome arrangement.

(FLI, 2006, electron microscopy according to Granzow, schematic representation after M. Jörn).

Figure 2 shows the localization of the structural viral proteins in a graphical model of the NDV particle and within electron micrographs. The two spike proteins, F- and HN- are embedded in the viral membrane and exposed to the outer surface of the viral particle. The M-protein is localized directly underneath the viral membrane and can be seen as electron dense layer by transmission electron microscopy (right panel). The nucleocapsid, enclosing the viral RNA, is visible close to the raptured particle as fish bone like string by negative staining electron microscopy (left panel) and is considered to fill the viral particle. Schematic organization of the genome is depicted underneath the model.

1.2.1.2 NDV antigenic sites of HN – and F-proteins

Monoclonal antibodies (MAbs) were used in identification and characterization of a panel of antigenic sites in NDV HN-and F-proteins. Besides, it was used in mapping of different NDV isolates and giving an idea about the variation in antigenicity between different NDV strains.

1.2.1.2.1 HN epitopes

Using neutralizing MAbs, seven antigenic sites were identified within the HN protein of NDV strain - Australia-Victoria (AV). The those antigenic sites were functionally sub-divided into three groups a)-MAbs that can inhibit both HA and NA (MAbs to sites 12, 2 and 23), b)-MAbs that that can inhibit only HA (MAbs to sites 14 and 1) and c)- MAbs that can inhibit neither function (MAbs to sites 3 and 4). Although all the MAbs recognized conformational sites, only site14 MAbs recognized a linear epitope. MAbs to sites 1, 4, and 14 were reactive with broad

panel of NDV strains, while antibodies to the other sites exhibit various degrees of strain specificity [52-57].

Moreover, four antigenic sites (I, II, III and IV) were identified by means of neutralizing antibodies against the a pathogenic strain of NDV (D26). The antibodies to sites I and III inhibited neuraminidase activity. Site II antibodies inhibited only HA activity, while site IV antibodies inhibited neither HA nor neuraminidase but inhibited fusion activity. Antibodies against site II have very week neutralizing ability compared to the other three sites [58]. The amino acids (aa) reactivity panel for site (I, III and IV) was further characterized by Gotoh and colleagues [59]. Furthermore, one MAb (AVS-I), raised against the avirulent LaSota strain and was considered specific for avirulent NDV viruses with neutralization and haemaglutination properties [60]. But it was identified to react with at least to one mesogenic and one virulent viruses [57].

The amino acids residues and biological activities corresponding to each antigenic site were summarized in (Table 1).

Table 1: **HN- protein antigenic sites and corresponding amino acid(s) and activity**.

Site[52, 56]	**23**	**23**	**23**	**23**		**3**	**3**	**3**	**4**	**4**	**1**	**14**	**14**	**14**	**4**		**14**	**12**		**2**	**2**	**2**	**23**	**2**
Site[58, 59]					**I**							**I***				**IV**			**II**					
Amino Acid residue	**193**	**194**	**201**	**203**	**225**	**263**	**287**	**321**	**332**	**333**	**345**	**347**	**350**	**353**	**356**	**481**	**493**	**494**	**495**	**513**	**514**	**521**	**547**	**569**
Activity	**N NA HA**	**N NA HA**	**N NA HA**	**N NA HA**	**N NA HA**	**N**	**N**	**N**	**N**	**N**	**N HA**	**N HA**	**N HA**	**N HA**	**N NA HA**	**F**	**N NA HA**	**N NA HA**	**N NA HA**	**N NA HA**	**N NA HA**	**N NA HA**	**N NA HA**	**N NA HA**

N= Neutralizing activity NA= Neuraminidase activity
HA= Hamagglutination activity F= Fusion activity
* MAb had with neuraminidase activity by [58] but without by [52]

1.2.1.2.2 Fusion protein epitopes

Using neutralizing anti-F- protein MAbs, at least eight epitopes and three antigenic sites were identified for NDV fusion protein. Through induction of resistant escape mutants for Beaudette C strain. Yusoff *et al.* [61] were able to characterize a panel of eight neutralizing anti-F MAbs that were produced by [62] against La Sota and NDV Italian strain . Similar results against Ulster strain were obtained by (Alexander & Collins, unpublished data) [61]. The MAbs were found to represent five epitopes (A1-A5). Furthermore, in the study conducted by Neyt *et al.*

[63] using the MAbs from [62] and through induction of escape mutants against NDV Italian strain, two antigenic sites (1 and 2) were identified. In another investigation, four antigenic sites (I to IV) were identified using a panel of 12 anti F-protein MAbs against NDV Sato strain [64]. These antigenic sites were further characterized to three specific F-protien and one specific to HN-protein [65]. Another three epitopes (E1-E3) were mentioned by Collins and colleagues [66], but with little information about their binding sites. The amino acids residues and biological activities corresponding to each antigenic site were summarized in (Table 2).

Table 2: **F- proteins antigenic sites and epitopes and corresponding amino acid(s) and activity.**

Site [64, 65]	**II**									**III**											**I**
Site [62, 63]	**1**	**1**	**1**																		**2**
Epitope[61, 62]	**A1**			**A2**	**A3**								**A4**								**A5**
Amino Acid residue	**72**	**74**	**75**	**78**	**79**	**157**	**158**	**159**	**160**	**161**	**162**	**163**	**164**	**165**	**166**	**167**	**168**	**169**	**170**	**171**	**343**
Activity	**N F**	**N F**	**N F**	**N F**	**N F**	**N F**	**N F**	**N F**	**N F**	**N F**	**N F**	**N F**	**N F**	**N F**	**N F**	**N F**	**N F**	**N F**	**N F**	**N F**	**N F**

N= Neutralizing activity F= Fusion activity

1.2.2 Virus classification

1.2.2.1 Serotyping

Originally avian paramyxoviruses (APMV-1) or now avian orthoavulavirus-1 were differentiated by haemagglutination inhibition test (HI) using reference sera [67, 68]. These subtype specific differences can be confirmed by neuraminidase inhibition- (NI) and serum neutralization test (SNT). According to this classification NDV was assigned as serotype 1, which can be confirmed by phylogenetic analysis (see above). Despite the growing evidence of genetic divergence with new evolving genotypes (see below). NDV is considered one serotype, with reactivity between all recognized strains. The majority of data are related to the titer differences in HI of below 2 (log_2), a finding that related to relationships value (R value) as defined by Archetti and Horsfall [69], within the range of $0.67 \leq R \leq 1.5$, that would indicates no significant antigenic differences between the two viruses.as those between genotype VII viruses [70]. However, other reported R-values less than 0.5 indicates major differences as reported for the newly identified avian paramyxovirus [71, 72]. Even though R-value less than 0.5 was reported even with APMV-1 field viruses and vaccines strains [70, 73]. The correlations between the R-value and HI are showing in (Table 3).

Table 3: Relationship between NDV viruses [69]

	R-Value[69]	HI
No significant difference	$0.67 \leq - \leq 1.5$	
Minor difference	$0.67 \leq - \leq 1.5$	
Major differences	< 0.5	>2 wells (4-fold difference)[74]

1.2.2.2 Pathogenicity

Although the NDV viruses are one serotype, they differ in virulence and can be classified into into five different pathotypes (Table 4) [13, 75]. NDV pathotypes could be differentiated from others by their ability for rapid killing of the chick embryos and the chickens [76, 77]. NDV virus pathogenicity and virulence are very fundamental factors that determine not only the

economic losses from NDV infection but how to deal with the infection as well. As it is common to isolate lentogenic viruses as well as velogenic viruses especially in endemic countries that use intensive vaccination programs for protection from losses caused by NDV infection. Pathogenicity determination is very important as only NDV strains with ICPI of 0.7 or more beside the presence of the polybasic cleavage site are defined to be notifiable [13].

Table 4: Pathotypes and virulence of Newcastle disease virus strains [13, 75]

Pathotype	**Virulence**
Asymptomatic	low-virulent (loNDV)
Low pathogenic/ lentogenic	
Mesogenic	virulent (vNDV)
Velogenic viscerotropic	
Velogenic neurotropic	

Multicycle replication of low virulent NDV (loNDV) like vaccine strains, is restricted to the intestinal and respiratory epithelium, hence facilitating localized infections in the upper respiratory and intestinal tracts. In contrast, virulent viruses are fostering a systemic NDV infection: spreading after primary replication in the respiratory tract to other organs. The velogenic virus could be detected caecal tonsil, liver, bursa of Fabricius, intestine, proventriculus, lung, spleen, thymus, kidney, heart, and brain [78].

The different pathotypes can be differentiated by testing pathogenicity for embryonated chicken eggs (mean death times; MDT), or for chicken after intracerebral inoculation (intracerebral pathogenicity index; ICPI) or intravenous inoculation (intravenous pathogenicity index; IVPI). [79]. Relevant test according to international standards (OIE) is the ICPI as it is used as one determinant of NDV that requires reporting. Applying standardized scoring systems NDV strains are pathotyped into velogenic, mesogenic and lentogenic (Table 5).

Table 5: NDV pathogenicity indices

	Non/Low-virulent	**Mesogenic**	**Velogenic**
MDT	> 90 h	60- 90 h	< 60 h
ICPI	<0,7	0.7-1.5	>1,5
IVPI	0	0.5-2	2-3

1.2.2.2.1 Molecular basis of pathogenicity

The F- protein is considered as a major determinant for virulence of Avulaviruses, as it is responsible for differences in tissue tropism of a particular strain. Differences in a stretch of amino acids at the C-terminus of the F2 protein and the first amino acid motif at the N-terminus of the F1 protein (aa 112-117) determine the recognition pattern of cellular proteases and subsequent cleavage in two biological active fragments of the F-protein. NDV strains that are low-virulent have only two basic amino acids within the recognition motif and a leucine at the N-terminus of the F1 protein (^{112}G-R/K-Q-G-R↓L^{117}). This motive within the F0 of loNDV are cleaved extracellularly by trypsin-like proteases that are present only in the respiratory and intestinal tract. On the other side, virulent NDV strains have multi-basic amino acids at the C-terminus of the F2 protein and a phenylalanine at the N-terminus of the F1 protein (^{112}R/G/KR-Q/K-K/R-R↓F^{117}). F0 of vNDV are cleaved intracellularly by ubiquitous furin-like proteases, found in most host tissues. This multibasic recognition pattern can be found in mesogenic as well as velogenic NDV strains. Accordingly, determination of the cleavage site can`t distinguish between these mesogenic and velogenic. For pigeon type paramyxovirus (PPMV-1) it has even been described that some PPMV-1 strains with a proteolytic cleavage site resembling a virulent pathotype are low pathogenic when tested by ICPI [80]. This highlights, that other parameters could determine the pathogenicity in addition to F-protein cleavage site. As shown for PPMV-1, these include the replication complex NP-P_L [81]. In addition, a strain specific balanced interaction of F-and HN influences pathogenicity of recombinant NDV [82]. Besides, noncoding regions of different genes (M-, NP-, F- and HN-protein) [83], the length of the HN-protein [21] and the abundance of the V-protein [45] influence pathogenicity of NDV.

1.2.2.3 Phylogenetic classification by genotype

Genetic classification of NDV was initially established based on partial sequences of F2–gene segment, including the proteolytic cleavage site and the fusion peptide [84-86]. Recently, an international working group established a new system for NDV genotyping based on the full F-gene sequences [87]. Different genotypes are defined to have on average a nucleotide distance of above 10%, when at least four independent viruses from distinct outbreaks are used for the analysis. A distinction between the sub-genotypes was considered, when nucleotide-distance

was above 5%. Each node had to be supported by bootstrap values of 70% or higher, when analyzing at least 1000 bootstrap replicates [87].

The new nomenclature system verifies two major classes of NDV / Avian orthoavulavirus-1, termed class 1and class 2 (Figure. 3) [87].

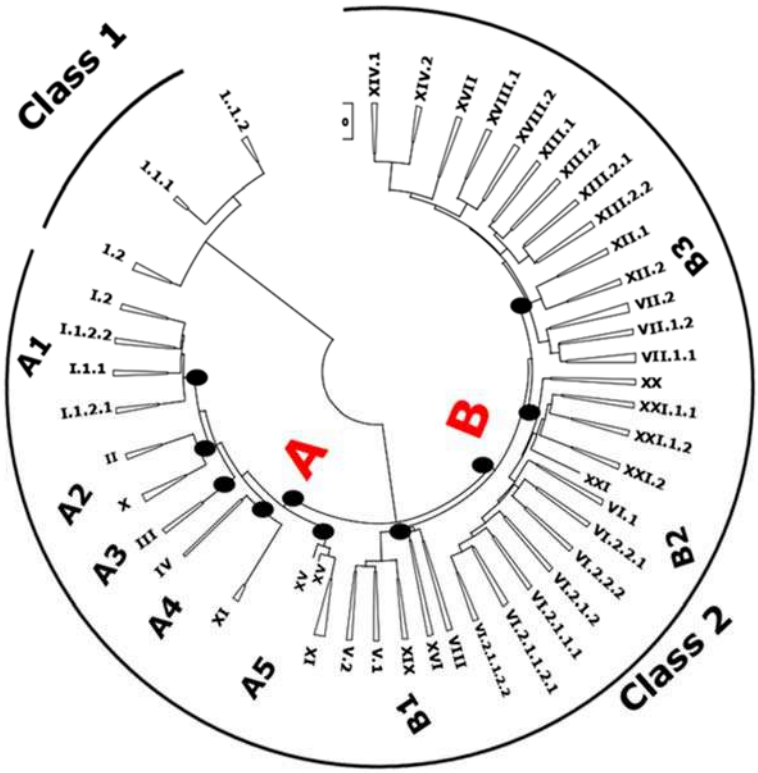

Figure 3: Phylogenetic tree of Newcastle disease virus.

NDV is phylogenetically subdivided into classes 1 and 2. Class 2 represents historical and recent viruses from poultry and can be separated to two major branches. ***Branch A*** holds five sub-branches (A1-A5) and include with 2.II within A2 the commonly used vaccine viruses. ***Branch B*** is separated into three sub-branches with branch B2 representing the so called pigeon type (PPMV-1) as genotype 2.VI and branch B3 the currently circulating genotype in Asia and Middle East (2.VII) and Africa. The figure was designed using sequences data collected by [87].

Class 1 are generally endemic in wild water birds or shore birds with described spill over to domestic poultry. These viruses are lentogenic and have been reported to be isolated from live bird markets worldwide [88, 89]. This class comprise one genotype that is subdivided into three sub-genotypes **1.1.1, 1.1.2** and **1.2**.

Class 2 viruses are subdivided in twenty-one genotypes (2.I to2.XXI), encompassing strains relevant for poultry and include strains causing the epidemics of the last century as well as vaccinal strains. Two major ***branches (A, B***) can be distinguished within class 2 viruses. ***Branch A*** holding 8 genotypes, separated into five branches (***A1-A5***).

Branch A1 is formed by genotype **2.I** and this genotype is further subdivided into four sub-genotypes, **I.1.1, I.1.2.1, I.1.2.2** and **I. 2**. Australian vaccine strain QV-4 is belonging to sub-genotype **I.1.1.** ***Branch A2*** includes two genotypes: Genotype **2.II** include historical NDV strains that are associated to ND outbreaks in the USA in 1940s. During that time, lentogenic genotype **2.II** viruses were discovered, that subsequently were the basis for LaSota and B1 vaccine strains. Within the same ***branch (A2),*** genotype **2.X** is arranged. Genotype **2.III** is forming ***branch A3*** while ***Branch A4*** includes two genotypes **2.IX** and **2.VX**. The last ***branch, A5*** includes two genotypes as well (**2.IV** and **2.XI).**

Branch B of class 2 viruses is sub divided into three branches ***B1-B3*** and includes 14 genotypes. ***Branch B1*** is encompasses four genotypes that are mainly first detected in America, with genotype 2**.V, 2.VIII, 2.XIX** and **2.XVI.** Genotype **2.V** is further subdivided into three sub-genotypes **V, V.1** and **V.2**. Viruses of genotype **2.XIX** are, despite their velogenic pathotype, circulating in apparently healthy double-crested cormorants in the USA and Canada.

Branch B2 of class 2 NDV is representing the former genotype **2.VI** that is now separated into 3 different genotypes. Viruses of genotype **2**.**XX** are representatives for NDV of chicken origin that emerged in epizootics in the Middle East and Asia in the 1960s [90, 91]. Genetic evidence support the hypothesis that chicken-to-pigeon transmission of NDV strains resulted in a new variant, termed pigeon type paramyxovirus (PPMV-1) that presumably is of African origin [84, 85]. This group of viruses is today enclosed in genotype **2**.**VI** that is highly divergent and includes viruses circulating nearly all over the world. Genotype **2**.**VI is** further sub divided in altogether eight sub-genotypes, **1, 2.1, 2.1.1.1 , 2.1.1.2.1, 2.1.1.2.2, 2.1.2, 2.2.1** and **2.2.2.** Genotype **2**.**XXI** is sub divided in three sub-genotypes with one unassigned the branch of viruses that include viruses isolated mainly from chicken from Ethiopia that might resemble an introduction of ancestral class II genotype **VI** viruses from Columbidae birds into poultry [92].

The last ***branch B3*** is separated in six genotypes and includes the currently most prevailing genotype **2.VII** associated with the majority of the recent ND outbreaks worldwide. It has been

recognized to be involved in fatal infections of different poultry species since the early 1990s in the Far East. Since 2000s, genotype **2.VII** viruses have gradually become the predominant strains in poultry flocks in Asia, Europe, Middle East, South Africa and South America. **2.VII** viruses can be further subdivided in three sub-genotypes Sub-genotype **2.VII.1.1**, **2.VII.1.2** and **2.VII.2**.

Genotype **2.XII** was further sub divided into two sub-genotypes **2.XII.1 and 2.XII.2** with third an unassigned branch comprising viruses recovered from chickens in Vietnam starting in 2008. Genotype **2.XIII** was further sub divided into five sub-genotypes **2.XIII.1.1, 2.XIII.1.2, 2.XIII.2, 2.XIII.2.1** and **2.XIII.2.2**.

The last three genotype (**2.XIV, 2.XVII, 2.XVIII)** are restricted to some African countries. Genotype **2.XIV** is further divided into two sub-genotype, **2.XIV.1** and **2.XIV.2**. Genotype **2.XVIII** as well is subdivided in two sub-genotypes with sub-genotype **2.XVIII.1** and **2.XVIII.2**.

Using the sequences data collected by [87], detailed information about NDV different genotypes and sub-genotypes, pathogenicity, types of birds detected in and countries recovered from were summarized in (Table 6).

Table 6: Genotypic classification of NDV viruses

Genotype				Year	Affected birds	Geographical distribution	Pathotype		
C	B	G	S				A	B	C
Classs 1		I	1.1	2005	chickens, ducks and geese	China	×		
			1.2	2009	chickens, ducks, black swan, peafowl, egret, heron	China	×		
			2	1997	chickens, ducks,teals, swans, geese,shore birds, wild waterbirds including ducks	USA, Europe, Asia	×		
Class 2	A1	I	1.1	1966	chicken, wild birds	Australia,vietnam			×
			1.2.1	1994	wild birds	Sweeden, Japan, Russia, USA	×		
			1.2.2	1962	chickens , wild birds	Japan, Russia, USA	×		
			2	2001	cickens, ducks and wild birds	USA, Africa, Asia, Europe			
	A2	II		1944	chickens	USA, Mexcico, Palastine	×	×	×
		X		1986	ducks, turkeys, wild birds	USA, Argentina			×
	A3	III		1932	chickens, pigeons	Australia,Asia, Bulgaria		×	
	A4	IX		1948	chickens, wild birds	China	×		
		XV		1997	chickens, geese	China			×
	A5	IV		1933	chickens, turkeys, ducks, pigeons	Europe, Africa, Russia, Asia			×
		XI		2008	chickens and wild birds	Madagascar			×
	B1	V	V	1971	chickens, pigeons, pistacine	Europe, North - and South America, Bulgaria, India, Kenya, Uganda		×	×
			V.1	1981	chicken, turkey, parrots	central and North America			×
			V.2	1973	chickens,caged-birds, tree-ducks, quails, doves	Mexico and Central America			×
		XIX	XIX.1	1992	double-crested cormorants, turkeys, wild birds	USA and Canada			×
		XVI		1947	chickens	Mexico, Dominican Republic			×
		VIII		1960s	chickens	Asia, Argentina, south Africa			×
	B2	VI	1	1982	pigeons	Japan, Argantiena, USA, Europe		×	
			2.1	2011	pigeons and doves	Israel		×	
			2.1.1.1	1996	pigeons(a), doves(b), chicken (c,), turkey (d), pheasant-(e)	USA(a-e) and Europe(a-b)		×	
			2.1.1.2.1	2000	pigeon (a), chickens (b), ducks (c) doves (d)	China (a-c), Namibia (d), USA, Europe (a, d)		×	
			2.1.1.2.2	1998	pigeons(a), doves(b), (chicken, ducks Grey heron) (c), rosella (d)	Europe(a,d), China(a,-c), India (a), Egypt(a)		×	
			2.1.2	1997	pigeons (a), doves(b) and quails (c)	Brazil (a), Argentina(a), Kenya(b), Nigeria (a,c)		×	
			2.2.1	1991	pigeons, chickens, duck	USA		×	
			2.2.2	1996	pigeons	mainly China, one sequence india		×	
		XXI	not	2011	chickens	Ethiopia			×
			1.1	2007	pigeons (a), doves (b), ducks (c)	Russia (a, c), Kazakhstan(a), Ukraine (a), Pakistan (a), Egypt (a), Nigeria (b)		×	
			1.2	2014	pigeons	Pakistan		×	
			2	2010	doves (a), Pigeons (b)	Italy (a), Israel (a), Nigeria (b), Iran (b)		×	
		XX		1985	chickens	Asia and Europe			×
	B3	VII	1.1	1997	chickens,geese, ducks, quail and wild birds	Venezuela Asia, Europe, middle east, Africa			×
			1.2	1996	chickens, pigeon and geese	China			×
			2	1993	chickens, ducks, turkeys, geese	Netherland, Asia, middle east, Africa			×
		XII	not	2008	chickens	Vietnam	×		
			1	2004	chickens, peafowl	Colombia , Peru	×		
			2	2010	geese and ducks	China	×		
		XIII	1.1	1995	chickens, Ostrich, wild birds	Sweden, India, Africa			×
			1.2	2008	chickens	Iran			×
			2	2013	chickens	India			×
			2.1	2007	chickens	Pakistan			×
			2.2	2003	chickens, quails, Redlori	india			×
		XIV	not	2007	chickens	Mali			×
			1	2006	chickens, turkeys	Nigeria, Niger			×
			2	2007	chickens, turkeys, ducks, Guina fowls, Pigeons, wild birds	Nigeria, Benin			×
		XVII		2002	chickens, pigeons, quails	West and central Africa(Benin, Burkina Faso, Cameroon, Ivory Coast, Mali, Niger, Nigeria			×
		XVIII	1	2006	chickens, guinea fowls, green wood hoope	Ivory Coast, Mali, Mauritania			×
			2	2006	chickens, ducks, and wild birds	Ivory Coast, Mali, Nigeria, Togo			×

A: Lentogenic **B**: Mesogenic **C**: Velogenic

1.2.3 NDV disease and clinical signs

1.2.3.1 Natural and Experimental Hosts

Infection with NDV has been reported in at least 241 species of the class *Aves* [93] and it is assumed that species of the entire class are susceptible to NDV infection. However, clinical signs may vary widely depending on several factors. Beside differences between different species [94, 95] In addition, the immune status of the bird can play a very important role In infected SPF turkeys showed earlier onset clinical signs compared to commercial turkeys [96]. Also breed of the birds could be a limiting factor such as infected Mandarah breed chickens found to be resistant to VNDV infection compared to Sina, Dandrawi and Gimizah chickens breeds [97], Fayoumi breed considered resistant to NDV infection compared to Leghorn [98, 99]. In ducks Pekin was more resistant to VNDV infection while, Mallard was the most susceptible [100]. Moreover, virulence of specific NDV isolates can affect the clinical outcome for example NDV strain ZA/04 was found to be the more virulent compared to other velogenic viruses as Te/48, Ca/71 and NL/93 [101].

In general, chickens and turkeys are considered most susceptible poultry species. However, for turkey it has been reported that infection with high dose (10^6 EID_{50}) of NDV strain APMV-1/chicken/Great Britain/1453/96 (lineage 5b/ genotype 2.XVIII) did not induce mortalities, even though animals shed virus for several days [95]. In contrast, the authors in the same study demonstrated that chickens were extremely sensitive to the same virus with a lethal dose 50 LD_{50} of $10^{1.9}$ and death of infected animals within 7 days. On the other hand, water birds are considered to be resistant in general to clinical sings [95, 100].

1.2.3.2 Incubation period

The incubation period for NDV infection varies depending on the host species, age, immune and health status of the infected birds. In addition, virulence, dose of virus received, the route of exposure as well as secondary infections may influence the onset of clinical signs and course of the disease. Experimentally infection of chicken, induce clinical signs within 1-4 days post infection (dpi) [102]. This is in agreement with experimental infection in SPF turkeys, where

clinical disease started 2 dpi [103]. However, in the same study infection of commercial turkeys the incubation period was 6 dpi. In an early experimental study, onset of mortality was one to two days earlier in chickens than turkeys [94]. After natural exposures to NDV, incubation period is considered to be between 2 and 15 days with average 5–6 days in most of gallinaceous birds [2, 104, 105]. In experimentally infected pigeons clinical signs start from 4 to 7 dpi [106, 107].

1.2.3.3 Geographic distribution of NDV.

NDV is widely distributed all over the world, since the most of countries are using 2.II vaccines for poultry vaccination even in non-endemic countries. Similarly, Mesogenic NDV viruses (PPMV) have a wide distribution among pigeon population with sporadic cases in poultry even in those countries considered NDV free as in Europe. In the other hand, vNDV viruses is endemic in many countries all over the world especially in Asia, Africa, Central and South America as well as in the US and Canada [104]. Even though outbreaks occur in in poultry in NDV free areas from time to time as in Belgium in 2018 and USA in 2018 to 2019. Figure 4.

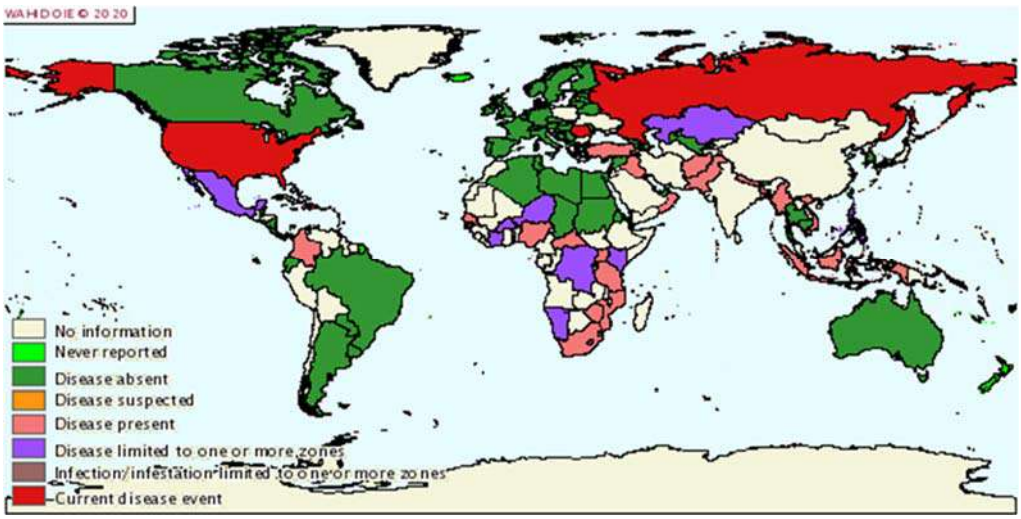

Figure 4: Geographic distribution of the NDV (OIE map-2019).

Showing NDV reports in different countries with current out breaks (red colour) in Asia, Europe and North America. While the virus is considered endemic (pink and violet colour) in many other countries in Asia, Africa and Central America. Even though some areas known to

be Endemic like Egypt appears to be free from the disease (green colour) in the map that could be regarded to the absence of the sufficient reports.

1.2.3.4 Clinical signs in chickens

Neither clinical signs nor gross lesions are pathognomonic for ND infection. Several factors can influence the, course of the disease as well as the clinical signs. Infection with velogenic ND viruses is lethal with high mortality rates of up to 100%. Depending on the tissue tropism of the particular NDV strain, two different clinical entities can be distinguished: **Velogenic-viscerotropic** ND is characterized as acute severe systemic disease. The chicks become listless and depressed within 2 dpi, with 100% mortality after 3-5 days [108, 109]. After oculo-nasal inoculation, birds may show respiratory signs including bilateral conjunctivitis with some facial swelling, nasal discharge and gasping. In case they are dropping their head, clear mucus may pour from the beak of affected chickens. In some cases, watery green feces may be observed. The comb may become cyanotic, but hemorrhages are only seen after infection with some isolates [102]. On the other side, **velogenic-neurotropic** ND is characterized by nervous disorders leading to paresis and paralysis of the limbs. Birds usually may become unusual alert as early as 2 dpi, then become excitable and hypermetric 3 to 4 dpi. Approximately 5 dpi, birds show head or muscular tremors and/or torticollis. Paralysis of one wing or one leg may occur as late as 10 dpi. These birds are alert and bright and usually die from the inability to reach food and water after 9 or more dpi. Mortality is usually up to 50% in adult birds, with higher mortality in younger birds [2, 108].

In contrast, infection of adult chicken with **mesogenic strains**, like the pigeon type (PPMV-1) may not result in clinical signs in adult chicken [108, 110], but can lead to a drop of egg production as well as nervous manifestation such as head tremors, torticollis, and paralysis.[109]. Five weeks old SPF chicks experimentally infected with mesogenic NDV virus showed only conjunctivitis [111]. Natural infection of 14-day old broilers with mesogenic NDV (ICPI 1.18) was associated to 70 to 80% morbidity and 10% mortality. The birds showed respiratory signs as difficult breathing and gasping. Some birds had nervous signs as torticollis and ataxia, some others had unilateral or bilateral paralysis of wings or legs [112].

In layers infected with NDV showed drop of egg production and drop internal and external quality. Eggs might display abnormal shells or are shell less. Internal abnormalities of the egg include poor albumen quality, visible as decreased albumen height and is an indication that both magnum and uterus are affected. Egg production returns to normal within 4 to 5 weeks, but the total recovery may take up to three months [113, 114]. In vaccinated layers, clinical sings may be confined to decrease in egg production one week after NDV infection, reaching its peak of egg reduction after two to three weeks. After that, the number of eggs produced will start to increase [102].

1.2.3.5 Clinical sings in other species

Generally clinical signs of ND in turkey are similar to those in chickens. Turkeys infected with vvNDV show different degrees of depression, nasal discharge and conjunctivitis. Nervous manifestations include incoordination, tremors, head shaking and paralysis of legs and wings [94, 96, 103]. However, unlike chickens, infected turkeys can show oral exudate mixed with blood [94] or bloody droppings [103]. The clinical signs in commercial turkeys were less severe and of later onset than those observed in experimentally infected SPF turkeys [96].

For a long time ago ducks were considered to be resistant to disease and to be act as a natural reservoir or carriers of NDV[75]. For example, Aldous *et al.,* [95] reported on complete resistance of the Pekin ducks (Anas platyrhynchos) to infection with velogenic NDV strain APMV-1/chicken/Great Britain/1453/96 (Lineage 5b/2.XIII) even at a dose of 10^6 EID50 [95]. Further study reporting on the resistance of Pekin ducks to disease after i.m. infection with velogenic NDV strain JSD0812 (genotype VII). In contrast, comparing six different duck breeds, the authors found Mallard ducks seem to be the most susceptible with 100 % mortality [100]. Differences in clinical manifestation in relation to the infected strains is reported for waterfowl as well [115]. Dai and colleagues stated that such waterfowl pathogenic strains emerged in China, can induce an economical important disease for ducks and geese [100].

1.2.3.6 Clinical signs of PPMV-1 in pigeon

Clinical signs in pigeons are generally similar to the ones observed for neurotrophic-velogenic ND in chicken. However, some viscerotropic strains exhibit specific affinity for the kidneys, so that the first sign is polyuria, followed by nervous signs that appear only in some individual birds. The incidence of the infection varies from 30 to 70%, with mortality not exceeding 10%. In case of co-infections with bacteria or parasites, mortality may reach to be more than 30%. The most common nervous manifestations are head and neck 180º twists (torticollis), imbalance, circling movement, paralysis of wings and legs, which is accompanied with difficulties in food intake. Infected birds sometimes have polyuria or bloody diarrhea, however naturally infected pigeon also exhibit respiratory symptoms such as sneezing, coughing and nasal discharge [107, 116, 117].

1.2.3.7 Pathology

1.2.3.7.1 Gross pathology

Similar to the clinical signs, gross lesions are highly variable. Virulent NDV causes haemorrhagic lesions in the respiratory and the intestinal tract. Lesions in the respiratory tract are dominated by haemorrhagic lesions / congestion of the lung and airsacculitis. Most prominent are petechia in the coronary fat of the heart and in proventriculus. In addition, haemorrhagic lesions in the caecal tonsils and Peyer's patches are prominent, often associated with an enlarged spleen [103, 109]. Gross lesions in the central nervous system (CNS) are seldom even in birds showing neurological signs before death. Laying hens infected with virulent NDV often show egg peritonitis [102, 118]. Kidneys might be pale with multifocal small whitish areas [119]. Naturally PPMV-1 infected pigeon showed hyperemia in the proventriculus and small intestine serosa. Besides, markedly enlarged and hyperemic spleen and liver, with light discoloration of the liver with small areas of necrosis were observed. The pancreas was a mildly increased with multifocal white[120].

1.2.3.7.2 **Histopathology**

The most prominent histopathologic changes associated with vvNDV infection are severe necrosis of the lymphoid tissues especially spleen and gut-associated lymphoid tissue, caecal tonsil, thymus, and bursa. In the beginning lesions are dominated by lymphoid depletion and hyperplasia of macrophages that have large vacuolated cytoplasm. Later lymphoid cells are replaced by fibrin and filled with karyorrhectic debris. The later stage might be associated with extensive ulceration of intestinal epithelium and rapture of myocardial fibers. Lesions in the central nervous system (CNS) consists of neuronal degeneration and gliosis in the brain and perivascular cuffing, most prominent in infection with velogenic-neurotropic strains. Infection and or vaccination with mesogenic NDV is associated with hyperplasia and lesions in lymphoid tissues and inflammation of the myocardia with myofiber degeneration and infiltration of macrophages. Also, Lentogenic stains can cause splenic lymphoid hyperplasia [102, 108, 121]. In addition, Lymphocytic or lymphoplasmacytic airsacculitis of different degrees was reported in ND infection of different pathotypes in turkeys [96].

El Bahrawy and colleagues [119] reported that chickens infected with vvNDV had congestion, haemorrhages, and marked mononuclear cell infiltrations, followed by mild multifocal lymphohistiocytic tubulointerstitial nephritis that gradually increased to moderate multifocal lesions with cortical tubulointerstitial nephritis. Later marked degeneration, necrosis, and apoptosis of the renal tubular epithelium with severe cortical tubulointerstitial nephritis was observed.

Laying hens show histopathological lesion after vvNDV infection stating with scattered necrotic lesions and lymphocyte infiltration in the mucosal epithelia of the infundibulum, magnum, isthmus, and uterus. Followed by sever oedema in the uterus with severe necrosis and lymphocytic infiltration in the vagina. Further on, the lesions become more sever in isthmus and vagina represented in necrosis and massive lymphocytic infiltration [122].

1.2.3.8 NDV clinical problem in Egypt

Despite intensive vaccination with different types of vaccines, NDV infection is associated high mortalities especially in broiler farms in Egypt [3, 4]. The predominant clinical finding

observed in affected farms are respiratory distress and nervous disorders. [6, 7]. In laying hens drop of egg production is the predominant clinical finding [123]. Pathological findings may include Catarrhal tracheitis with haemorrhagic mucosa and hemorrhages in the proventriculus and caecal tonsils [124, 125]. Mortality is variable and mostly around 15–20% [8], but can reach up to 80% or more in some reports [6, 126]. Clinical signs of NDV could be modified by mixed infections with other pathogens especially other avian respiratory viruses and bacteria. In practice, contribution of different pathogens to disease is difficult to assign [12, 127-130].

1.2.3.9 Respiratory virus mixed infection

As many pathogens can infect the respiratory tract of the poultry (Table 7) concurrent infection with one or more pathogen could be non-avoidable. Many studies were concerned with respiratory virus mixed infection and its contribution in complicating the situation in Egypt. For example, out of 150 swabs and organ samples from broilers and layers flocks suffering from respiratory manifestation, 70 samples (46 %) were positive for both IBV and NDV. However, NDV pathotype was not determined [131]. Similarly nine out of 20 IBV positive samples (45%) were also positive for NDV [129]. In the study done by [132], NDV/H9N2 was the most prevalent mixed infection in broilers (7.7%) while H5N1/ND, H5N1/H9N2/ND and H5N1/H9N2/ND/IB mixed infection revealed (2.6%). As well, in layer NDV/H9N2 mixed infection was the most prevalent (27.3%). and H9N2/ND/IB was (9.1%). Furthermore, in the study done by Setta and colleagues, out of 1722 investigated organ samples collected from commercial chicken flocks suffering from respiratory manifestations. The incidence of mixed IBV/vNDV was 27.9%. IBV, vNDV and H9N2 mixed infection incidence was 12.2%. IBV, vNDV and H5N1 mixed infection incidence was 10.4%. VNDV, H5N1 and H9N2 mixed infection incidence was 2.3% [133]. In further study, it was reported that out of 36 broiler flocks positive o for H9N2, eight flocks (11.1%) were co-infected with NDV[134]. Finally, In recently published study by Hassan and colleagues [135] , nine out 39 test flocks (23%) were positive for vNDV. Four flocks were positive for vNDV, H5N1, H5N8 and H9N2, two flocks were positive for vNDV, H5N1, H5N8, H9N2 and IBV. While, one flock was positive for vNDV, H5N1, H9N2 and IBV, one flock was positive for vNDV, H5N8, H9N2 and IBV and one flock was positive for vNDV, H5N8 and H9N2.

1.2.3.10 History of Newcastle in Egypt

Presence of ND in Egypt is established at least since 1948 [136], and it is considered to have been prevalent in this region since that time [4, 137, 138]. Beside domestic poultry, NDV infection have been reported from pigeons (pigeon) and different wild bird species in Egypt like quail (Coturnix coturnix), coot (Fulica atra), and in song birds like Flycatcher (Ficedula), western yellow wagtail (Motacilla flava), Willow warbler (Phylloscopus trochilis) and Common whitethroat (Sylvia communis) [139, 140].

Currently there are at least three NDV genotypes circulating in Egypt: beside vaccine type genotype 2.II virus, virulent genotype 2.VIb, 2.VIg and 2.VIIb were identified. Genotype 2.VIb and 2.VIg were isolated mainly from infected pigeons and regarded as pigeon paramyxovirus (PPMV) [141, 142]. Genotype 2.VIIb, in contrast, is relevant for poultry, with numerous outbreaks of ND in all chicken production sectors. Despite intensive vaccination attempts with genotype 2.II vaccines, high mortality is observed especially in broiler farms [3-5].

1.2.4 NDV diagnosis

As the clinical signs and lesions related to vNDV infections are not pathognomonic, accurate virological diagnosis is essential for accurate diagnosis to be able to differentiate between virulent and non-virulent strains or class I virus that circulate in wild birds. This discrimination between pathogenic and non-pathogenic NDV is not only relevant for specific flock diagnosis, but is relevant for restriction that might apply on the national and international level [2, 13]. The techniques used for NDV diagnosis could be direct detection of virus antigen and or molecular techniques in aim to detect viral RNA or indirect techniques to detect antibodies produced as a result for virus infection or vaccination in the blood serum.

1.2.4.1 Substrate for direct virus detection

Virus detection can be achieved from oropharyngeal and/or cloacal swabs from suspected live birds and should be combined if available with other diagnostic approaches to detect other respiratory agents, tracheal swabs are also suitable. From necropsy, organ samples should include the trachea, brain, lung and intestine. kidney liver and other lymphoid organs such as spleen, caeacal tonsils and bursa of fabricius. Swabs should be fully submerged in media and both type of samples should be kept at 4°C for short time storage or frozen until processing and further using [2, 118, 143].

1.2.4.2 ND Virus isolation and Identification

The most sensitive isolation system for NDV are embryonated chicken eggs, where the virus is inoculated into the allantoic cavity of 9 to 11 day old SPF chicken eggs. Harvesting the amnio-allantoic fluid will harvest a heamagglutinating agent that has to be further characterized and distinguished from possible bacterial contaminants. NDV also replicates in a variety of avian and non-avian cell cultures, such as e.g. primary chicken embryo liver cells (CEL), chicken kidney cells (CEK), chicken embryo fibroblasts (CEF) and African green monkey kidney cells (Vero), QM5 and CER cells [144]. After infection of the cell cultures, viral growth could be recognized from a visible cytopathogenic effects (cpe) as syncytia or plaque formation.

Effective virus replication in chicken embryonic cells depends on the $Mg2^{+}$-Ions content and diethylaminoethylene dextran or trypsin in the cell culture medium [121]. Newcastle disease viruses with low virulence require the addition of exogenous trypsin to spread from cell to cell and form syncytia in cell cultures, In contrast virulent strains do not need this addition [76, 145]. Like in the egg culture, supernatant from inoculated eggs have to be further characterized.

1.2.4.3 Molecular identification

Application of molecular techniques allow timely detection and further characterization of NDV.[2]. Diagnostic protocols use reverse transcription (RT) to convert viral RNA into DNA that is subsequently amplified by polymerase chain reaction (PCR) Conventional RT-PCR assays that depend on agar gel electrophoresis for detection of virus specific products, have mostly been replaced for primary diagnostic by real time RT-PCR protocols. Most established methods use fluorogenic taqman probes to detect NDV specific amplificants. Protocols that target conserved sites are designed to detect all NDV strains, independent of the pathotype and are described for the M-gene [146], the NP-gene[147] or L-gene [148]. With respect to improve detection of class I viruses, primers and probes were targeted at the L gene such that primer/probe sequences and assay conditions were compatible with the M gene RT-PCR, allowing for a multiplex RRT-PCR [149]. RRT-PCR that targets the fusion gene at the cleavage site could be used to detect and pathotype the NDV isolate in a single assay. Furthermore, the investigators designed different probes to detect loNDV or vNDV [150, 151]. Moreover, genotype specific could be beneficial in suspected cases as in case of PPMV in pigeon isolates [142]. Unfortunately, there still some problems related to sensitivity as the detection limits of all tests varied between 10^1 and 10^3 EID_{50}. Problems related to specificity can play role that the detection limits may vary with viruses belonging to other genotypes depending on how probe and primer sequences match the target sequences. In addition, the probe specific for loNDV used in this assay does not detect all class I viruses [102].

1.2.4.4 F-gene sequencing

Although RRT-PCR can distinguish between velogenic and lentogenic viruses. Complete or partial F-gene is essential for strain determination and epidemiological studies. Initially partial F-gene sequencing including the cleavage site was done for such purposes [152-155]. Meanwhile the partial F-gene sequencing is still sufficient for diagnostic purposes, recently complete F-gene sequencing becomes more efficient in epidemiological studies and strains classification [87-89, 142].

1.2.4.5 Serology

The serological procedures are usually used for indirect detection of the antibodies, which could be related to viral infection and/or vaccination in blood serum [13]. Yolk samples can be also used [156]. The Haemagglutination Inhibition Test (HI), the enzyme-Linked Immunosorbent Assay (ELSIA) and the Virus Neutralization Test (VNT) have largely been used for diagnostics, monitoring and vaccination control. A discrimination of the pathotypes that induced antibodies in not possible.

1.2.4.6 Differential diagnosis of NDV

The clinical signs of ND is mainly in the respiratory, digestive, CNS, and urinary system. In terms of differential diagnosis, there are many more diseases in question. Basically, NDV should be differentiated from other causes that can result in respiratory symptoms (Table 7). However, high loss rates in stocks can also be technical and structural defects (failure of air, water or feed supply as well as various Intoxication [157]. VNDV Salmonellosis, yersiniosis, Borrelia infection, intestinal parasitosis could induce digestive manifestation that should be excluded during NDV diagnosis. Finally, NDV should be differentiated from several poisonings that can cause sudden death [104, 157, 158].

Table 7: **possible cause of respiratory disease in poultry [159]**

Infectious		**Non infectious**	
Viral agents		***Management***	
	ND, PMV3, IB, aMPV,		Litter quality
	Influenza A, Pox		Stocking density
Bacterial agents			Ventilation rate
	ORT, P. multocida, Mycoplasma,		Temperature
	C. psittaci, E. coli,		High ammonia level
	Bordetella avium		High dust concentration
Mycotic agents		***Feed***	
Aspergillus fumigatus			High dust content
Parasites			Vitamin A deficiency
Syngamus, Cryptosporidium			

1.2.5 Protection against NDV

1.2.5.1 Vaccines against NDV

Vaccination together with good biosecurity measures could provide an effective protection against clinical signs and mortalities resulting from vNDV infection. Different kinds of vaccines (live, inactivated and vector vaccines) are available and used commercially for protection against ND [2]. The aim of immunizing of the herd is to get the antibody titer that is homogeneous as possible in the entire flock [182]. Good flock immunity can only be achieved when high homogenous antibody titer in the majority of birds within the flock s that mean more than 85% have a high antibody titer (HI titer higher than 3 $\log_2$) after vaccination [183]. From the early 1950's until the late 1990's live and inactivated ND vaccines were the only vaccines available that were used to decrease economic losses resulting from morbidity and mortality [184, 185]. More recently genetic engineered vaccines became available for commercial use [186].

For assessing a live vaccine efficiency, at least ten SPF chickens or other susceptible birds should be vaccinated at the minimum recommended age with the minimal recommended dose. After 14-28 day the birds are challenged with 10^4 EID_{50} or 10^5 LD_{50} from the challenge virus by intra muscular route. As controls, ten non vaccinated birds should be challenged as well. The birds are observed for 14 days. The vaccine is considered to be efficient, if 90-95% of the birds remain survive and free from clinical signs while all the control birds died in 6 days.

For assessing an inactivated vaccine, in Europe 21 to 28-day-old SPF chickens should be vaccinated with 1/25, 1/50 and 1/100 of a vaccine dose. 17–21 days later the birds are challenged with 10^6 LD_{50} from the challenge virus by intra muscular route. Ten non vaccinated birds should be challenged as controls. The birds are observed for 21 days The PD_{50} (50% protective dose) is calculated by standard statistical methods. The test is considered to be valid only if all the control birds died in 6 days. The vaccine is valid, if the PD_{50} is not less than 50 per dose and if the lower confidence limit is not less than 35 PD_{50} per dose. In USA it much similar for the live vaccine. The challenge virus strains used in USA and Europe are GB Texas and Herts 33, respectively. [13].

1.2.5.1.1 **Live vaccines**

Live NDV vaccines are originated from lentogenic strains isolated starting from 1940's. These viruses that were circulating in poultry farms were the source of the LaSota, B1, and VG/GA vaccines. Most of those vaccine viruses belong to genotype 2.II and are genetically and antigenically highly related (>98% nucleotide identity) [160]. Some lentogenic vaccines have been cloned for selection of viruses which produces less post vaccination reactions than the original virus [161, 162]. Those cloned viruses vaccines as (clone 30), are widely used nowadays for commercial poultry production [163]. For the European Union (EU) only vaccines containing lentogenic strains are licensed that fulfill the criteria of an ICPI of < 0.4 when tested with not less than 10^7 EID_{50} /bird or < 0.5 when tested with not less than 10^8 EID_{50} /per bird [164]. Live NDV vaccines have the advantage of mass application by drinking water, sprays. This is less labor intensive and inexpensive compared to administration of inactivated vaccines that have to be injected to individual birds [102]. More recently live vaccines of other genotypes mainly genotype 2.VII were generated using reverse genetics, some of them are used at a commercial basis nowadays [123, 165, 166]

1.2.5.1.2 **In-activated vaccines**

The same NDV strains used as live vaccine are also used in inactivated vaccines. The viruses are propagated in chicken embryos and harvested allantoic fluid is inactivated by formalin, beta-Propiolactone (BPL) or binary ethylenimine (BEI) retaining the antigenic properties of the vaccine virus. Modern vaccine preparations are oil emulsions, that proved to be more effective[102]. The in-activated vaccines are applied intramuscular or subcutaneously mainly in laying hens and in farms in order to achieve long-lasting high antibody titers, which can be passed also for offspring [167]. Also according to the directive 93/152/EEC of the Commission of the European Council, inactivated vaccines are produced according to the guideline from ND virus strains, whose original seed virus at testing has an ICPI of < 0.7 when administered no less than 10^8 EID_{50} to each bird in the ICPI test [164].

1.2.5.1.3 Recombinant vaccines

Commercially available recombinant ND vaccines are based mainly on fowl pox (rFPV) or herpes virus of turkeys (rHVT), as live-vector. They express the F-glycoprotein and have to be delivered also by injection in one day old chicks (commonly in hatcheries) or even in ovo. Both types of recombinant vaccines have no side effects and can be successfully administered to chicks with maternal antibody. A disadvantage for rFPV vaccine is interference of MDA [168]. While this is not affecting rHVT vaccines, the vector virus is cell associated and requires to be kept in liquid nitrogen, and to be administered within an hour of being thawed. But, only one rHVT vaccine can be applied otherwise there would be interference between the different HVT vaccines [169, 170].

1.2.5.2 Vaccination against NDV in Egypt

A series of different types of ND vaccines are licensed in Egypt. That includes classical ND vaccines containing genotype 2.II strains for live (B1, LaSota, Clone 30, and V4) and inactivated (LaSota, Clone 30) ND-vaccines. In addition, recombinant HVT-NDV vaccine expressing F gene from genotype 2.I (D26) strain are commercially available [7, 125, 171]. Recently, live and inactivated genotype 2.VII vaccines have been licensed as well [123, 172]. Because the ND endemicity in Egypt and Middle East, virus burden is expected to be high and therefor vaccination programs are intense. Vaccination programs in broilers based on at least two live vaccines or one recombinant and one live vaccine and in the majority of farms additional one inactivated vaccines, while in layer and breeders Multiple vaccination live and in activated vaccines are used [10, 11]

1.2.5.3 Vaccination failure

Although live and inactivated vaccines protect against clinical disease in SPF chickens, there are continuous reports of vaccinal breaks under field conditions [12, 173]. These vaccinal breaks could be regarded to either insufficient vaccination or vaccination failure. Insufficient vaccination should to be clearly differentiated from vaccination failures and could be defined

as the result of mistakes during the vaccination procedure. There are many causes for insufficient vaccination that include inactivation of live vaccine during transport, handling or application. For example, residues of disinfectant in the water supply systems or the water quality could inactivate the vaccine. Technical problems related to the intake / application of the vaccine have to be considered. Vaccine failure on the other hand would summarize inadequate protection of a proper applied vaccine. This might be caused by the immune status of the birds. Birds might be immune compromised due to previous infection with immunosuppressive pathogens like infectious bursal disease virus (IBDV), chicken infectious anemia virus (CIAV), Marek's disease virus (MDV), (MDV-induced lymphomas), Reovirus, reticuloendotheliosis virus (REV), and the avian leukosis virus (ALV) complex. Some other infections as mycoplasma spp. or presence of mycotoxins were associated with immune suppression as well [170, 174] or by subclinical intoxications by Mycotoxins [174, 175]. In addition chicks, might be to young i.e. the immune system is premature and not able to respond adequately to vaccination [176] or vaccination is hampered by maternal derived antibodies [176] that was shown for IBDV and Influenza as well [177, 178]. A final explanation for vaccine failure might be antigenic mismatch. This has been shown for AIV [179].

In recent years, phylogenetic mismatch of vaccine virus strain and field strain have been considered as cause for ongoing outbreaks despite vaccination. This discussion was sparked by an early study [180] describing that protection rate of SPF-chicken was only 40% after vaccination with inactivated LaSota vaccine and subsequent challenge infection with genotype 2.VII NDV, isolated in 1996 in China. Subsequent studies however, proved that poor flock immunity due to inadequate vaccination rather than antigenic variation account for field outbreaks [101, 181]. In this respect type of bird, i.e. broiler or layer chicken and/or pre-existing immunity like maternal derived antibodies might contribute to vaccine potency. Very impressive was study by Sediek and colleagues that demonstrated that only 37.5-46.7 % of commercial broiler, vaccinated on day 7, were protection against challenge infection 3 weeks after the vaccination [172]. In addition, new evidence indicates that clinical protection does not include the oviduct: In layers a drop of egg production down to 76 % from 88% was observed even though animals did not suffer other apparent clinical signs and no mortality was evident [123]. However, beside clinical protection, viral shedding might contribute to dissemination of certain genotypes.

Majority of the studies were conducted to test/compare efficacy of genotype 2.II vaccines against different genotype 2.VII challenge models, the predominant genotype in poultry in Asia

and Middle East. In this respect, conflicting evidence is provided. A number of groups report significant reduction of virus shedding by homolougous vaccines, both by number of shedders or by amount of virus shed [165, 166, 182, 183]. This is contradicted by other groups that do not observe significant difference between homolouges or heterolougoud vaccines [101, 181, 184]. Even though, this might be partly been due to limited numbers of birds within tested groups.

1.3 Outline of the publications in the current dissertation

1.3.1 Publication 1

Ibrahim Moharam, Alaa Abd el Razik, Hesham Sultan, Mohammed Ghezlan, Clement Meseko, Kati Franzke, Timm Harder, Martin Beer & Christian Grund. **Investigation of suspected Newcastle disease (ND) outbreaks in Egypt uncovers a high virus velogenic ND virus burden in small-scale holdings and the presence of multiple pathogens.** Avian pathology 2019. 48(5), 406–415. https://doi.org/10.1080/03079457.2019.1612852

In this publication, we were interested to verify clinical diagnosis by investigating 26 flocks with clinical manifestation suggestive for ND by specialized laboratory diagnostics. In addition, epidemiological links between ND-positive farms were further analysed phylogenetically. The samples were divided into two groups: 1) sector 3, i.e. commercial small scale poultry farms with low to minimal biosecurity (n=16), 2), sector 2 comprises commercial broiler farms, with moderate to high biosecurity (n=10) . In 23 of a total 26 investigated flocks with suspected ND. Virulent NDV was detected by species specific RT-qPCR´s in farms of sector 3 only. From sector 2 only three broiler flocks were NDV negative. In addition, in both small and large scale farms IBV (n=10), Avian influenza virus (AIV), subtype H9 (n=3) and Avian Reovirus (ARV, n=5) were identified. From one large broiler farm, chicken Astrovirus **(CAstV)** was isolated.

Phylogenetic analysis of 11 sequenced ND virus from sector 3 revealed that all the viruses belonged to sub-genotype 2.VII.1.1 (formerly 2.VII-b), the dominant NDV genotype in the Middle East and northern Africa. However, analysis of the fusion (F) protein gene, revealed that virus present in these farms clustered in four different branches, although they are located in close proximity to each other. This finding, pointed that four independent introductions of viruses from other close governorates, with subsequent transmissions between farms, regardless of the poultry type.

1.3.2 Publication 2

Ibrahim Moharam, Hesham Sultan, Kareem Hassan, Mahmoud Ibrahim, Salama Shany, Awad A. Shehata, Mohammed Abo-ElKhair, Florian Pfaff, Dirk Höper, Magdy EL Kady, Martin Beer, Timm Harder, Hafez M. Hafez, Christian Grund. **Emerging infectious bronchitis virus (IBV) in Egypt: Evidence for an evolutionary advantage of a new S1 variant with a unique gene 3ab constellation**. Infection, Genetics and Evolution 85 (2020) 1044332. https://doi.org/10.1016/j.meegid.2020.104433

In the second publication were interested in characterizing IBV strains circulating in small-scale farms in Egypt, identified in the first publication study in which, IBV was detected in 3 out of 16 farms from sector 3, i.e. commercial poultry production systems with low to minimal biosecurity farms. From the same study, five IBV positive samples were derived from sector 2 farms, i.e. commercial poultry production system with moderate to high biosecurity. Poultry in all farms suffered from respiratory distress with mortality rates varied from 10 to 70%. Characterization of detected IBV revealed that one of the three viruses from small scale holdings represents a new subclade of genotype GI (GI-23.3), whereas the other two viruses match with GI-23.2, formerly designated as GI-23 subgroup EGY-var2. Full genome analysis for the 23.3 isolate from a small scale flock revealed intra-genotypic gene exchange for partial fragments of the S1 gene and complete switches for gene 3ab and E and inter-genotypic recombination for gene 6b. This particular gene 3a/b was present also in subsequently investigated historical samples from 2013 derived from large broiler farms, and the new S1:3a/b constellation dominated in samples taken three years later, i.e. 2019. In silico analysis of the S protein demonstrated an accumulation of point mutations over time within the hypervariable regions (HVR) of the S1 protein. Compared to vaccine type virus striking mutations in at least four of seven defined epitopes within the HVR3 region were present in the new GI-23.3 strain from small scale farms of the year 2016 and all subsequent related viruses, indicating a possible antigenic drift of these new variants.

1.3.3 Publication 3

Ibrahim Moharam, Olayinka Asala, Sven Reiche, Timm Harder, Hafez M. Hafez, Martin Beer, Christian Grund. **Monoclonal antibodies specific for the hemagglutinin-neuraminidase protein define neutralizing epitopes specific for Newcastle disease virus genotype 2.VII from Egypt**. Submitted Manuscript to virology journal

In order to characterize antigenic sites of circulating NDV genotype 2.VII, we generated genotype-specific **Monoclonal antibodies** (MAbs), which are able to block biologically active sites of the HN-protein, i.e. antibodies that, are able to neutralize infectivity and/or block HA activity. For this approach biophysically enriched HN-protein fraction of purified virus proved to be an efficient antigen source and the applied Concavalin A (ConA)-ELISA technique, that binds antigen not directly on the plate, but coupled by the lectin, provided a high throughput system suitable to detect antibodies to conformation sensitive sites. The resulting MAbs were used to recognize unique neutralizing epitopes of NDV genotype 2.VII. Out of six MAbs that were able to neutralize and / or block HA activity, three MAbs were reacting specifically with genotype 2.VII strains and one MAb differentiated even between two distinct genotype 2.VII viruses. On the other hand, one MAb had residual cross reactivity to three other genotypes (2.0, 2.VI and 2.XIV) and one MAb reacted with all eight genotypes tested (class 1, 2.0, 2.I, 2.II, 2.III, 2.VI, 2.VII and 2.XIV). These results pointed to the concurrent presence of flexible and conserved epitopes within the HN-spike protein.

2 Chapter 2: Results

2.1 **Publication1:** Investigation of suspected Newcastle disease (ND) outbreaks in Egypt uncovers a high virus velogenic ND virus burden in small-scale holdings and the presence of multiple pathogens.

Ibrahim Moharam, Alaa Abd el Razik, Hesham Sultan, Mohammed Ghezlan, Clement Meseko, Kati Franzke, Timm Harder, Martin Beer & Christian Grund

Avian pathology 2019. 48(5), 406–415

https://doi.org/10.1080/03079457.2019.1612852

2.2 **Publication 2:** Emerging infectious bronchitis virus (IBV) in Egypt: Evidence for an evolutionary advantage of a new S1 variant with a unique gene 3ab constellation.

Ibrahim Moharam, Hesham Sultan, Kareem Hassan, Mahmoud Ibrahim, Salama Shany, Awad A. Shehata, Mohammed Abo-ElKhair, Florian Pfaff, Dirk Höper, Magdy EL Kady, Martin Beer, Timm Harder, Hafez M. Hafez, Christian Grund.

Infection, Genetics and Evolution 85 (2020) 1044332.

https://doi.org/10.1016/j.meegid.2020.104433

2.3 **Publication 3:** Monoclonal antibodies specific for the hemagglutinin-neuraminidase protein define neutralizing epitopes specific for Newcastle disease virus genotype 2.VII from Egypt

Ibrahim Moharam, Olayinka Asala, Sven Reiche, Timm Harder, Hafez M. Hafez, Martin Beer, Christian Grund.

Submitted Manuscript to Virology Journal

Virology Journal

Monoclonal antibodies specific for the hemagglutinin-neuraminidase protein define neutralizing epitopes specific for Newcastle disease virus genotype 2.VII from Egypt.

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Abstract:	Newcastle disease is a devastating disease in poultry caused by Newcastle disease virus (NDV), a paramyxovirus endemic in many regions of the world despite intensive vaccination. Phylogenetic analysis revealed ongoing evolution in the predominant circulating genotype 2.VII, and the relevance of potential antigenic drift is under discussion. To define neutralization-sensitive epitopes within the major immunogenic Heamagglutinin-Neuraminidase (HN) spike protein and subsequently investigate variation within these sites, we established genotype-specific monoclonal antibodies (MAbs). Immunizing mice with an HN-enriched fraction of a genotype 2.VII gradient-purified virus preparation, successfully induced antibodies to conformationally intact sites reactive by haemagglutination inhibition (HI). The screening ELISA system used to select mouse hybridoma cultures employed Concavalin A (ConA-ELISA) coupled antigen and proved to present conformation-dependent epitopes. Six out of nine selected MAbs showed HI-activity, with four of them reacting specifically with the genotype 2.VII virus NR730 but not with the vaccine type virus of genotype 2.II nor six additional tested viruses representing genotypes 1.I, 2, 2.I, 2.III, 2.VI and 2.XIV. While three of these MAb reacted with an additional genotype 2.VII strain, one MAb recognized an epitope only present in the homologue virus. On the other hand the cross-reacting MAb reacted with all genotypes tested and resembled the reactivity profile of genotype specific polyclonal antibody preparations. These results point to the concurrent presence of variable and conserved epitopes within the HN-molecule. The described protocol should help to generate MAbs to a variety of NDV strains and enable in depth analysis of the antigenic profiles of different genotypes.
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Question	**Response**

Monoclonal antibodies specific for the hemagglutinin-neuraminidase protein define neutralizing epitopes specific for Newcastle disease virus genotype 2.VII from Egypt.

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Keywords:

monoclonal antibody, Newcastle disease virus, genotype 2.VII, antigenicity, Haemagglutinin-Neuraminidase protein, conformational epitopes

Highlights:

- First description of specific MAbs to NDV genotype 2.VII from Egypt
- The combination of HN-enriched virus antigen and ConA-ELISA represents a fast and convenient approach to generate MAbs to biological relevant antigenic sites of NDV
- HN-specific MAbs recognize strain-specific and cross reacting neutralizing epitopes.
- NDV genotype 2.VII from Egypt expresses unique epitopes interfering with receptor binding

Abstract:

Newcastle disease is a devastating disease in poultry caused by Newcastle disease virus (NDV), a paramyxovirus endemic in many regions of the world despite intensive vaccination. Phylogenetic analysis revealed ongoing evolution in the predominant circulating genotype 2.VII, and the relevance of potential antigenic drift is under discussion. To define neutralization-sensitive epitopes within the major immunogenic Heamagglutinin-Neuraminidase (HN) spike protein and subsequently investigate variation within these sites, we established genotype-specific monoclonal antibodies (MAbs). Immunizing mice with an HN-enriched fraction of a genotype 2.VII gradient-purified virus preparation, successfully induced antibodies to conformationally intact sites reactive by haemagglutination inhibition (HI). The screening ELISA system used to select mouse hybridoma cultures employed Concavalin A (ConA-ELISA) coupled antigen and proved to present conformation-dependent epitopes. Six out of nine selected MAbs showed HI-activity, with four of them reacting specifically with the genotype 2.VII virus NR730 but not with the vaccine type virus of genotype 2.II nor six additional tested viruses representing genotypes 1.I, 2, 2.I, 2.III, 2.VI and 2.XIV. While three of these MAb reacted with an additional genotype 2.VII strain, one MAb recognized an epitope only present in the homologue virus. On the other hand the cross-reacting MAb reacted with all genotypes tested and resembled the reactivity profile of genotype specific polyclonal antibody preparations. These results point to the concurrent presence of variable and conserved epitopes within the HN-molecule. The described protocol should help to generate MAbs to a variety of NDV strains and enable in depth analysis of the antigenic profiles of different genotypes.

Introduction

Newcastle disease virus (syn. avian ortho Avulavirus-1; APMV-1, NDV) is a member of the family of Paramyxoviridae within the genus Orthoavulavirus which together with the genera Meta- and Paraavulavirus form the subfamily Avulavirinae [1]. Like other members of the order of Mononegavirales, NDV is an enveloped virus with an RNA genome of negative polarity. The genome size of either 15,186, 15,192 or 15,198 nucleotides [2] encodes for six structural proteins [3, 4](Cambers et al., 1986; Wilde et al., 1986) with two outer spike glycoproteins, the hemagglutinin-neuraminidase- (HN) and fusion protein (F) that facilitate attachment on and subsequent entry into the host cell. The latter protein is translated as a precursor molecule (F0) and cleaved by cellular proteases into disulfide bond-linked subunits F1 and F2 [5, 6]. Both spike proteins are immunogenic and induce protective immunity in the host [7-9] . Dominating structural protein of the virion however, is the nucleoprotein (NP) which enwraps the viral RNA into a helical capsid and together with the phospho (P)-protein and the large (L)-protein forms the viral polymerase complex. The matrix (M-) protein is positioned underneath the viral membrane and is considered to stabilize the virion architecture. In addition two regulatory proteins, V and W, are integrated into the virion [10, 11].

In poultry virulent NDV strains induce Newcastle disease, a systemic infection with mortality of up to 100% in chickens. Introducing avirulent APMV-1 strains as ND-vaccines in the late 1940ies was a hallmark for protecting chickens and turkeys from disease [12], and today ND-vaccination is fundamental for protecting poultry worldwide. Nevertheless, the disease is endemic in many regions of the world. Lately inadequate protection by established but old vaccine strains against current circulating NDV strains has been proposed [13-15]. This hypothesis is supported by genetic analysis: Circulating NDV strains reveal a tremendous heterogeneity and strains can be divided in two genetic classes (1-2) with respectively three (1.I.1.1, 1.I.1.2 and 1.I.2) and 21 (2.I – 2.XXI) recognized genotypes [16]. Whereas the established vaccine strains belong to genotype 2.II, genotype 2.VII is dominating the current panzootic in Asia and the Middle East. Homology between the two genotypes for F- and HN-spike proteins, responsible to elicit protective immune response in the host [17], is low: 75-79% and 71-75% on the nucleotide level and 85-89% and 84-88% on the level of amino acids. Despite the genetic variation, antigenically NDV still forms a single homogenous serotype [18, 19]. Based on the haemagglutination inhibition test (HI) that detects HN-specific antibodies that block virus binding to sialic acid receptors on erythrocytes or serum neutralization tests, polyclonal sera can differentiate between serotypes of the subfamily Avulavirinae [20, 21] but

cannot distinguish between NDV gentoypes [19]. However, by monoclonal antibodies (MAbs) differences in specific epitopes have been recognized [22-24]. Profiling of a battery of MAbs raised against NDV-Ulster 2 C (genotype 2.I.2) and pigeon type paramyxovirus (genotype 2.VI) to a total of 1526 NDV-isolates, allowed distinction of different groups but also revealed considerable heterogeneity within groups [25]. Further studies with neutralizing MAbs to NDV strain Australia-Victoria (AV) (genotype 2.I.1.1) recognized seven different conformation dependent antigenic sites within the HN-protein. Two sites conveyed virus neutralization only (sites 3 and 4), while MAbs binding to other sites inhibited HA-activity only (sites 1 and 14) or both HA- and NA-activity (sites 2, 12 and 23) [26-31]. Studies with MAbs established with apathogenic strain NDV-D26 (genotype 2.I.1.1) established three different epitopes sensitive for both HI and NI activity of MAbs and mapped these sites to different amino acids when escape variants were analyzed [32]. Likewise HI-positive MAb AVS-I, raised against the avirulent LaSota vaccine strain (genotype 2.II) [33] mapped to aa residue 570, that was close to but not part of the other described epitopes [34]. This indicates, that NDV strains might express at least slightly different neutralizing epitope patterns. Information for such epitopes of NDV genotype 2.VII are not available.

In order to characterize antigenic sites of circulating NDV genotype 2.VII, we generated genotype-specific MAbs that are able to block biologically active sites of the HN-protein, i.e. antibodies that are able to neutralize infectivity and/or block HA activity. For this approach biophysically enriched HN-protein fraction of purified virus proved to be an efficient antigen and the applied Concavalin A (ConA)-ELISA technique, that binds antigen not directly on the plate but coupled by the lectin, provided a high throughput system suitable to detect antibodies to conformation sensitive sites. The resulting MAbs were used to recognize unique neutralizing epitopes of NDV genotype 2.VII.

Material and Methods

Viruses and sera:

NDV strain chicken/EGY/NR730/2016 (NR730; GenBank Acc. no. MH899939; genotype VII.1.1) was isolated from an ND vaccinated layer flock in Egypt suffering respiratory distress. The virus was characterized as velogenic having an intracerebral pathogenicity index (ICPI) of 1.8 and belonged to genotype 2.VIIb [35]. Pigeon type paramyxovirus-1 (pigeon/Germany/R75/1998), genotype 2.VI, was derived from the repository of the ND reference laboratory (Acc. No. KJ736742) and NDV/clone 30, genotype 2.II, was derived from a commercial vaccine (MSD, New Jersey, USA). As a source of polyclonal reference antibodies, the watery egg yolk preparation from eggs was used of specific pathogen free (SPF) chickens, immunized repeatedly with a commercial inactivated ND-vaccine containing NDV clone 30. A monospecific rabbit α-NDV-HN serum and α-NDV-F serum [36] was used for specific detection of NDV-HN protein by western blot (WB) analysis. Immunizations were carried out in accordance with the legally approved protocol (MV-LALLF- 7221.3-2.5-010/10).

Virus propagation and purification.

Virus was propagated in embryonated SPF chicken eggs (ECE) as described [37] (VALO BioMedia GmbH, Osterholz-Scharmbeck , Lower Saxony, Germany). Amino-allantioc fluid (AAF) was harvested on day 3 post infection (dpi) and purified by sucrose gradient ultra-centrifugation. Briefly, debris was cleared from AAF by low speed centrifugation (30 min at 10,976 x g; 10,000 rpm Rotor JA-10; Beckman Coulter, Brea, California, USA). Then virus was spun down by ultra-centrifugation (1.5 hour at 96,281 x g; 28,000 rpm, 32Ti Rotor, Beckman Coulter). Virus pellets from 6 tubes were re-suspended in a total of 45 mL of phosphate buffered saline (PBS, pH 7.2) containing 1 M KCl (KCl-PBS) before adding 15 mL of the virus suspension on top of a discontinuous sucrose gradient (30-60%, in KCl-PBS). Visible bands forming after ultra-centrifugation overnight (96,281 x g; 28,000 rpm, 32Ti Rotor; Beckman Coulter) were collected and diluted 1:5 in KCl-PBS before pelleting the virus by ultra-centrifugation for 1.5 h (96,281 x g; 28,000 rpm, 32Ti Rotor; Beckman Coulter). The final virus pellets, representing a total of 228 mL AAF were collected and resuspended in 1.5 ml KCl-PBS. The protein concentration of the obtained virus suspension was determined according to Bradford using the Roti®-Quant protein quantitation assay (Carl

Roth GmbH, Karlsruhe, Baden-Württemberg, Germany) following the producer's instructions. Haemagglutination activity was determined using the HA test according to standard procedures [37]. Purification for pigeon type paramyxovirus (PPMV-1) R75/98 and vaccine type APMV-1 clone 30 was done accordingly, but virus was resuspended in PBS.

Enrichment of HN-protein

Separation of NDV spike proteins was done as described by [38]. Briefly, protein concentration of purified virus was adjusted to 1.5 mg/mL in KCl-PBS before 0.1 mL Triton X-100 in PBS (20% (v/v)) was added, gently mixed and kept at room temperature (RT) for 20 min. The suspension was centrifuged (20 min at 10,000 x g, 9,703 rpm, A-4-81-11 Rotor, (Eppendorf, Hamburg, Germany)) and the obtained pellet (p1) was resuspended in 1 mL PBS (0.01 M, ph 7.2) and kept for analysis whereas the supernatant was further cleared by ultra-centrifugation at high speed (1 h at 200,000 x g, 55,000 rpm Rotor TSL 55 (Beckman Coulter, Brea, California, USA)) for 1 h. Again the pellet (p2) was kept for analysis after resuspension in 0.2 mL PBS. The supernatant was collected and dialyzed against 0.01 M phosphate buffer in order to remove the potassium chloride from the buffer used during purification. Any precipitate that formed during dialysis was sedimented by centrifugation (20 min at 10,000 x g, 9,703 rpm, A-4-81-11 Rotor (Eppendorf, Hamburg, Germany)) and the pellet was resuspended in 0.1 mL PBS (p3). The supernatant of the dialyzed material (s3) was the fraction that was subsequently used as antigen for immunization.

SDS-PAGE and Western blot

Proteins were separated under denaturing conditions in 10% sodium dodecyl sulphate (SDS) polyacrylamide gels using a minigel system (Biorad, Hercules, California, USA) according to standard guides (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6040.pdf). Shortly, samples were diluted in sample buffer (Roti load® (Carl Roth GmbH, Karlsruhe, Baden-Württemberg, Germany)) heated at 100°C for five minutes before adding 16 µL per lane into the gel pocket. Protein separation was conducted applying constant voltage setting (200 V) and the gel was either stained by Coomassie blue (Biorad, Hercules, California, USA) or proteins were blotted on a nitrocellulose membrane (Amersham™ Protran, Cytiva, Marlborough, MA, USA) applying constant voltage setting (15 V) for 1.5 hours. For western

blot analysis the membrane was blocked for one hour with 1% skimmed milk powder in 0.025% Tween 20 in PBS (PBS-Tween) and subsequently incubated with target specific antibodies over night at 4°C. After washing three times with PBS-Tween, blots were incubated with peroxidase (POD) labeled species-specific anti-immune globulin G (IgG) or immune globulin Y (IgY) conjugates (Sigma-Aldrich, St. Louis, Missouri, USA) for 1 h at RT. After washing three times, peroxidase activity was visualized by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific™. Waltham, Massachusetts, USA) and the Chemi Doc XRS+ imaging system (Bio-Rad, Hercules, California, USA).

Mouse inoculation and monoclonal production

Two female BALB/c mice were immunized five times intraperitoneally with 20 µg of purified protein fraction (S3) mixed with an equal amount of GERBU Adjuvant MM (GERBU Biotechnik, Heidelberg, Germany) over a period of 26, at weeks 4, 7, 11 after the first immunization, and the last boost was administrated four days before extraction of the spleen. Blood samples were taken on days 35, 49, and 84 after first immunization (dpi) from the submandibular vein and at 183 dpi, at the end of the experiment. Four days after the final boost mice were euthanized, their spleen removed under aseptic conditions and splenocytes harvested into serum-free RPMI-1640 medium (Invitrogen, Carlsbad, California, USA/Thermo Scientific™. Waltham, Massachusetts, USA) by using a cell strainer (BD Biosciences, Franklin Lakes, New Jersey, USA). In presence of polyethylene glycol 1500 (Roche Applied Science, Penzberg, Germany) the isolated splenocytes were fused with murine myeloma SP2/0 cells following a slightly modified standard protocol [39] by using a cell-to-cell ratio of 1:4. Fused spleen cells were seeded in three different cell densities (30,000, 15,000, and 7,500 spleen cells per well, two plates per density) in 96well flat-bottomed plates (Greiner bio-one, Kremsmünster, Austria) and incubated for 10 days (37 °C, 90% RH, and 5% CO2) by using complete RPMI-1640 culture medium 10% FCS (Fischer Scientific, Hampton, New Hampshire, USA), 1x MEM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate (Invitrogen, Carlsbad, California, USA/Thermo Scientific™. Waltham, Massachusetts, USA) supplemented with 1x BM Condimed H1 (Hybridoma Cloning Supplement, Sigma-Aldrich). For selection of growing hybridoma clones the complete medium was additionally supplemented with 1x HAT Media Supplement (50×) Hybri-Max™ (Sigma-Aldrich). Growing cultures were screened for specific antibodies by Con-A ELISA, IF and HI. The haemaglutination inhibition test (HI) was used to determine

the haemagglutination inhibition activity according to standard procedures [37]. For generating MAb producing cell clones, cells from positive cultures were cloned at least twice by limiting dilution (0.1 cells per well) in complete RPMI-1640 medium supplemented with 1x HT Media Supplement (50×) Hybri-Max™ (Sigma-Aldrich). Final clones were adapted to complete RPMI-1640 medium without any further supplements.

Con-A ELISA

The ELISA procedure was done according to a previously published protocol [40, 41]. Briefly, ELISA plates (Immunolon II, Thermo Scientific™. Waltham, Massachusetts, USA) were pretreated with Concavalin A (ConA) (Carl Roth GmbH) by adding 50 µl of Con-A (50 µg/mL in PBS) to each well. After incubation for 1 h at RT plates were washed three times using PBS-Tween and coated with 50 µL (20 µg/mL) pre-treated antigen. For preparation of the antigen, gradient purified virus stock at a concentration of 200 µg/mL was incubated with TritonX100 (1% v/v) for 45 min at RT and subsequently diluted 1:10 with PBS for coating the plates for 1 h. Thereafter, plates were washed three times with PBS-Tween and blocked with 1% FCS in PBS for 30 min at RT. Test sera (50 µL/well) at indicated dilutions were incubated for 30 min at RT and after three washings with PBS-Tween, plates were incubated with POD labeled species specific anti-IgG or IgY conjugates (50 µL/well) for 30 min at RT. After washing the plates three times with PBS-Tween, bound antibodies were visualized by incubation with o-Phenylenediamine dihydrochloride (Sigma-Aldrich). (50 µL/(100 µL/well, 1 mg/mL in 0.05 M phosphate-citrate buffer) for 20 min. Colour reaction was stopped with 2.5 M H_2SO_4 well density was measured at 492 nm using Sunrise™, Tecan`s microplate readers (Männedor) and the optical f, Zürich, Switzerland).

Indirect immunofluorescence (IF)

Immunofluorescence test was done with NDV/NR730/16 infected LMH cells (ATCC CRL-2117™) cultivated in 96 well plates (Corning, Corning, New York, USA) grown to 70-80% confluency before infection. Infected plates were fixed with 3.7% formaldehyde after incubation for 24 hours and stored at 4 °C for up to four weeks. For use, plates were emptied and treated with TritonX-100 (1% v/v) in PBS (100 µL/well) for 30 minutes at RT and after flicking off the supernatant, blocked with 1% fetal calf serum in PBS for 30 minutes at RT.

Subsequently, cells were incubated with MAb or indicated sera (50 µL/well) for 1 h at RT. After washing the plates three times with PBS-Tween, wells were incubated with FITC-conjugated anti-species specific IgG or IgY conjugate (Sigma-Aldrich, country) for 1 h at RT. After final washing for three times with PBS-Tween, wells were mounted with glycerol in water 1:5 and inspected under the microscope (Eclipse TS100, Nikon, Minato, Tokyo, Japan) with the appropriate filter (excitation 495 nm, emission 525 nm).

Serum neutralization test (SNT)

Serial dilutions (log_2) of MAb´s, starting with concentrated supernatants, were mixed with equal volume of NDV/NR730/16 (100 µL) containing 400 KID_{50}. For each dilution row, the last well was kept without serum to serve as virus control. For each test a heat inactivated (56 °C, 30 min) NDV reference serum was included as positive neutralization control. After incubation for 30 min at 37 °C, 50 µL of antibody / virus mixture was transferred in triplicate to 100 µL LMH cells that were cultivated without fetal calf serum but with TPCK treated Trypsin (2 µg/mL) (Sigma-Aldrich) in 96well plates (Corning) at a density 10^6 cells/cm^2. The plates were incubated at 37 °C with 5% CO_2 atmosphere for four days and SNT-titer was determined by determining the last dilution without presence of a cytopathic effect (cpe) and calculated according to Reed and Muench [42].

Haemagglutination inhibition test (HI)

Haemagglutination inhibition test was done according standard procedures, applying four haemagglutinating units and HI-titer are given as last serum dilution (log2) that inhibit agglutination [37]. For analyzing antigenic differences by polyclonal sera, the test were done in triplicate and repeated in three independent experiments. Results were analyzed by Sigma Plot 11 (Systat Software, INC) applying Kruskal-Wallis One Way Analysis of Variance on Ranks.

Results

HN-antigen preparation.

For the antigen preparation, gradient purified virus, harvested form AAF (228 ml) was used, that yielded 1.5 mL with an HA activity of 15 (log_2) and a total protein content of 1.9 mg/mL. Purified virus preparations were dominated in SDS-PAGE and subsequent Coomassie staining by a band at 55 kD, likely representing co-migrating proteins of NP (53 kD), P (53-56 kD) and F1 (55 kD) (Fig. 1A). This protein fraction is recognized as a major immunogenic fraction in the virion also by antibodies of NDV vaccinated chicken (Fig. 1B). In addition, the HN-protein is apparent as a ~70 kD band by Coomassie staining (Fig 1A) and in western blotting (Fig. 1B) as indicated by * in the figures. The identity of the HN-protein with the observed ~70 kD band could be confirmed using a HN-monospecific hyper-immune serum in WB analysis (Fig. 1C). Likewise a minor band at ~40 kD representing the M-protein, was detected by Coomassie staining (Fig 1A) and polyclonal chicken antibodies in western blot analysis (Fig 1B). After disruption of the virus with Triton and subsequent centrifugation (p1), proteins of the 55 kD bands were the dominant fraction of the pellet. Embedded in this band is the F1-protein that was specifically detected by an F-specific hyperimmune serum (Fig 1D). Subsequent pellets (p2 and p3) and also the final supernatant (S3) still contained proteins of the 55 kD band, detected by Coomassie staining (Fig 1A) and western blot analysis (Fig 1B). However, compared to the other proteins in these fractions, the 55 kD band was less prominent. During all centrifugation steps also HN-protein was pulled down, but gradually became the dominant fraction in the final supernatant that was used for the immunization (S3) and retained an HA activity of 13 (log_2). On the other hand, the western blot analysis with the F-specific serum (Fig 1D) indicated that the F1-protein was no longer present in these later fractions including the final supernatant.

Establishing the ConA ELISA test system

Already after the first immunization of two BALB/c mice with the S3 fraction (20 µg/mouse), NDV specific antibodies were detected in blood samples (Fig 2A). Reactivity by HI (Fig 2A, filled symbols) indicated that induced antibodies were able to block receptor mediated binding of the virus to erythrocytes, demonstrating that the antigen preparation represents conformationally intact epitopes of the HN-protein. Antibody reactivity was also detected by ConA ELISA (Fig 2A, transparent symbols). However, to verify that the ConA ELISA is

detecting also antibodies to conformation dependent epitopes, the test system was checked with MAb 617/161 (Collins et al., 1989). This MAb has HI activity against genotype 2.VI but not genotypes 2.II or 2.VII, and does not react with denatured viral protein in western blot analyses. Hence, it is directed against a conformation-dependent epitope. In ConA-ELISA of plates coated with NDV genotype 2.II (clone 30), genotype 2.VI (pigeon/ DEU/R75/98) or genotype 2.VII chicken/EGY/ NR730/2016), the polyclonal reference antibody preparation, was reactive with all three antigens (Figure 3A). In contrast, but in agreement with the HI data, MAb 617/161 reacted only with genotype 2.VI antigen (Figure 3B). Having established that the ConA-ELISA is suitable to detect antibodies to conformational epitopes within the HN-protein of NDV, this assay was chosen for screening of supernatants from hybridoma cultures.

Preparation of monoclonal antibodies

For the preparation of hybridoma cells, splenocytes of both mice were harvested four days after the 4th booster-immunization, pooled and half of the cells were used for the fusion. From a total of ten seeded 96well plates, 30 wells yielded supernatants reactive in the Con A-ELISA with an OD reading above 0.2 (0.2.1-0.7), and virus-specific reactivity could be confirmed by IFT. Supernatants of 6 of the 30 wells also showed reactivity by HI to the homologue NDV NR730 (genotype 2.VII) (HI-titer (log2) between 1-5 and were chosen for subcloning and further characterization. In addition, three HI-negative cultures where chosen. Final supernatants from hybridoma cultures obtained after two rounds of cloning by limited dilution were subsequently used for the characterization. The 6 HI-positive hybrdidoma colonies showed reactivity by ConA-ELISA (Figure 2B) and retained HI reactivity after single cell cloning (Table 1).

Analysis of cross reactivity reveals unique epitope patterns in the HN of genotype 2.VII

Initial analysis of the MAbs addressed the reactivity in different test systems and considered cross reactivity to NDV vaccine strain clone 30, genotype 2.II. The majority of MAbs reacted specifically with the homologues genotype 2.VII virus (NR730) (Table 1). In addition one of the HI-positive MAb (4E11) was able to block haemagglutination of clone 30 as well, although at two log_2 titer steps lower, compared to the homologous NR730 antigen. Reactivity was confirmed by IFT with specific reactivity with NR730 infected cells (Figure 4B) and cross

reactivity to clone 30 for MAb 4E11, respectively (Table 1). By ConA-ELISA, the HI-positive MAbs showed a strong reactivity to the homologous NR730 genotype VII virus (Figure 2B) and again only MAb 4E11 cross reacted with heterologous vaccine strain clone 30 (Table 1). In contrast, ConA-ELISA reactivity of the three HI-negative MAbs was low (Figure 2C). Interestingly, two MAbs (5B4, 6F2) were reacting only with the homologous genotype 2.VII virus (NR730/16) while the third MAb (1B6) was also reacting with a genotype 2.II vaccine strain (clone30). This reactivity profile could be observed by ELISA (Table 1) and confirmed by western blot analysis (Figure 4A): All three HI-negative MAbs reacted with virus proteins migrating at ~55 kD of NR730 and, analogous to the ELISA, only MAb 1B6 showed cross reactivity to clone 30. However, as NP-, P- and F1 are co-migrating on SDS-PAGE gels, it is not possible to conclude from our western blot results on the specificity of the MAbs. In contrast, none of the HI-positive MAbs were reactive by western blot analysis (Figure 4A). The final test explored whether the MAbs were able to block infection of NR730 in LMH cells. By SNT four out of the six HI-positive MAbs neutralized viral infectivity at titers comparable to the HI-test. In contrast none of the three MAbs reactive with the 55 kD proteins were able to neutralize homologous NDV NR730.

For the six HI-positive MAbs we extended our analysis of cross reactivity with six additional strains, representing class 1 and 2 viruses (Table 2). It became clear, that the epitope recognized by MAb 4E11 was present in all genotypes tested and represents a well conserved site. For the other five HI-positive MAbs, reactivity profiles were highly specific for genotype 2.VII, even though one MAb (5C2) had residual reactivity to viruses from other genotypes. This includes Herts 33/56, a very early NDV strain not assigned to a specific genotype within class 2 viruses and hence termed genotype 2.0. In addition low level reactivity was observed with the PPMV-1 virus (genotype 2.VI) and a recent virus from Nigeria of genotype 2.XIV. Apparently also within genotype 2.VII there are antigenic differences: While three MAb (5C2, 5D1 and 7C12) were reactive with a second genotype 2.VII virus tested, reactivity of MAb 5C9 was four fold lower with the heterologous virus. For MAb 7C12 reactivity to heterologous dropped to 1 log2. In contrast, polyclonal antibody preparations raised against genotypes 2.I, 2.II, 2.VI, 2.VII and 2.XIV were reactive with all antigens tested (sFig1). For the majority of antigens, titer differences of sera were in the range of 2 $\log_2$ steps. However, sera, raised against genotype 2.I, 2.VI and 2.XIV, respectively, had HI-titer differences (dHI) of >2 ($\log_2$) to individual antigens, a discrimination not present with other sera. This is reflected when analyzing the antigenic relation of the five antigen / serum pairs (genotype 2.I, 2.II, 2.VI, 2.VII and 2.XIV) by calculating the R values according to Archetti and Horsfall [43]. The majority of R-values were

between 0.9 (genotype 2.I /2.II) and 0.25 (genotype 2.VI / 2.XIV) (supplemental Table 1). The highest R-value was 0.15 (genotype 2.I /2.VI), resembling dHI of almost 3.

Discussion

HN-enriched virus antigen induced antibodies to conformational epitopes

We report on the generation of MAbs to NDV that recognize genotype 2.VII specific epitopes on the HN-protein. By enrichment of the HN-protein fraction in the antigen preparation used for immunization of mice, we were able to obtain six hybridoma cultures that are reactive by HI, i.e. are able to block the receptor binding site of the HN-protein on erythrocytes, and four of them neutralized viral infectivity in LMH cell cultures. In addition, three further selected hybridoma cultures neither showed HI nor neutralizing activity. By western blot analyses, these latter MAbs, revealed reactivity against viral proteins that migrate at 55 kD and, hence, do not target HN. In the past antibodies to NDV have been prepared by using whole, mostly gradient-purified virus preparations [22, 24, 44]. With this approach Lana and colleagues reported that five hybridoma fusion experiments yielded 20 NDV specific MAbs, of which only three were reactive by HI. Earlier reports [33] obtained a single MAb from a total of 184 maintained hybridoma cultures that was specifically reactive by HI with the homologous virus. The described problems to obtain sufficient numbers of MAbs reactive with ND spike proteins might in part be explained by the composition of the viral particle, which is dominated by the highly immunogenic NP-protein that fills the entire inner virion [45]. In addition the M-protein, forming an array underneath the viral membrane is abundant in the virion [46]. Both proteins account for ~30% of the virus-specific molecular masses in gradient-purified viral preparations when analyzed by nano-LC MALDI-TOF/TOF mass spectrometry and only about 10% of the virion are HN molecules [11; Karger personal communication]. By disrupting gradient-purified virus by Triton X-100 (2%) and 1 M KCl, Nishikawa and colleagues could increase the fraction of HI reactive HN-specific MAbs, retrieving 9 out of a total of 21 [47]. Our method also used Triton X-100 and KCl disruption, but we subsequently applied ultracentrifugation to enrich HN and deplete M and NP proteins. This procedure was able to pull down a high proportion of the proteins that are migrating as a 55 kD band including the NP. The subsequent steps reduced the proportion of the 55 kD proteins further, but did not achieve to remove all of the proteins: A slight band was still visible by Coomassie-staining (Fig 1A) and was reacting with the polyclonal antibody preparation form a ND vaccinated chicken (Fig 1B). This might be a result of incomplete

separation of the interaction of the HN-protein with the NP-protein via the matrix protein [48]. Accordingly, three of the selected MAbs were reactive with the 55 kD band by western blot (Fig 3). However, the western blot with an F-specific serum (Figure 1D) identified that a large proportion of the F1-protein was in the very first pellet of the antigen preparation, indicating that F-protein was not part of the antigen. This would be in line with results of co-immunoprecipitation experiments, showing a direct interaction of F and NP and HN and M [48]. Thus, it is likely that the three HI-negative MAbs are directed against NP- or P-protein. Further studies on protein specificity have to address this question and evaluate potential potency in diagnostic approaches. Harsher conditions for particle disruption might have yielded an improved depletion of nucleocapsid proteins but, at the same time, may have denatured HN and destroyed HI epitope conformation.

Overall our aim to produce MAbs capable to block biologically functional sites of the HN-protein of genotype 2.VII NDV by using an HN-enriched fraction of purified virus proved to be successful. We obtained a sufficient number of HI-positive MAbs already with a single fusion experiment, and two third of the selected hybridoma cultures yielded HN-specific MAbs that were able to block receptor binding.

ConA-ELISA presents biologically intact and relevant antigenic sites of NDV

Due to the complex structure of biologically relevant epitopes of the homo-tetrameric HN-protein [49-51], the approach to generate biological functional MAbs depends on native HN-protein with the correct conformation, ideally from virus produced in eukaryotic cells ensuring bona fide glycosylation and folding. Reassuring evidence was obtained through the HI-positive antibody response of two mice immunized with enriched HN preparations. However, in order to select the appropriate antibodies, the screening system has to present the test-antigen in its native conformation. When using the indirect ELISA techniques immobilizing antigen directly to the plastic surfaces of the plates likely interferes with the conformational integrity of the antigen, and important epitopes may be unpredictably masked or exposed [52]. To avoid such structural alterations caused by the coating, Russel and colleagues used NDV infected and formalin fixed cells with indirect immunoperoxidase test to visualize specific reactivity [23]. Having the advantage that already staining pattern in this test may give an indication whether MAbs are directed to outer glycoproteins, the test depends on manual inspection of the plates and is more difficult to standardize; in addition

use of formalin may denature sensitive epitopes. To be able to use ELISA technique for high throughput screening, investigators have used antibody- or poly L-lysine-coated plates to capture and thereby preserve the conformation of a viral protein [53, 54]. Our test system relied on the lectin properties of ConA as an anchor for the antigen, a system previously shown to preserves conformational epitopes of viral glycoproteins [40, 41] By using a genotype 2.VI specific monoclonal antibody that is highly reactive by HI [55], we could demonstrate, that the ConA-ELISA preserves NDV epitopes sensitive to receptor blocking. Applying the Con-A-ELISA for screening of the initial hybridoma supernatants and subsequent cloning, we obtained six MAb that were reactive by ELISA and HI, and all six clones could be confirmed by IFT. However none of the six HI-positive MAb were reactive by WB, which is in line with previous studies that denaturing WB conditions destroyed the conformation of HI sensitive epitopes of the HN-molecule. Overall, comparing ELISA-reactivity to HI-titers, it became apparent that ConA-ELISA was far more sensitive producing titer of well above 1:3200 whereas the highest HI-titer was 1:256 (8 $\log_2$) but without a direct correlation between the two values. It is striking, that the MAb with the lowest HI titer (5C2) had comparable or even higher ELISA-reactivity than the other MAbs. The ConA-ELISA, in addition, was able to present strain specific epitopes, as five out of six HI-positive MAbs reacted only with NDV genotype 2.VII strain NR730 but not with the vaccine strain clone 30, genotype 2.II, by HI. Thus, using HN-enriched virus preparation in conjunction with ConA-ELISA system that preserves conformational epitopes proved to be efficient to produce specific MAbs that are suitable to identify biological relevant targets in specific NDV genotypes.

HN-specific MAbs distinguish strain-specific and broadly cross reacting epitopes

In order to investigate the putative conservation of HI-sensitive epitopes of HN proteins of ND viruses of different genotypes we extended the analysis on cross reactivity using NDV strains representing distantly related class 1 NDV as well as seven additional class 2 genotype viruses. Only MAb 4E11 was reactive with all 8 different NDV genotypes tested, including the class 1 strain. On the other hand, the majority of HN specific MAbs predominantly reacted with the homologous NDV strain NR730 although within this group of MAbs three distinct reactivity profiles were discernable: a) MAb 5C2 showed residual cross reactivity to three other genotypes, b) three MAbs (5C9, 5D1, 7C12) reacted specifically with two different genotype 2.VII strains, and c) MAb 7A8 differentiated between the two genotype 2.VII

viruses. This points, in summary, to a high degree of flexibility of some HI epitopes, whereas other epitopes seem to be very well preserved. This is corroborated by observations derived from structural analysis of the HN molecule: Crenell and colleagues found that MAbs could interfere with receptor binding even though the target was not the receptor binding pocket itself [49]. In consequence such sites might not be crucial for the function of the molecule and are allowed to have more structural flexibility. Intrinsic properties of antibodies targeting these sites might then influence the specificity of the reaction, i.e. antibodies with high affinity might be able to bind a broader spectrum of virus strains. On the other side, epitopes adjacent or directly at the receptor binding site are part of functionally pivotal structures and have to stay conserved. These epitopes would represent conserved antigenic sites across the genotypes as recognized by the MAb 4E11 which showed HI and neutralization activity. In the viral HN-antigen used for immunizing the mice, both types of sites were apparently immunogenic. Considering the number of obtained MAb, i.e. five strain specific versus one MAb specific for a conserved site, the variable sites might have been more immunogenic for mice. In contrast, polyclonal antibody preparations did reveal only minor antigenic differences, indicating a balanced presentation of conserved and strain-/genotype-specific antigenic sites for chickens. In consequence polyclonal sera are of limited value when addressing variability of antigenic sites. On the other hand, polyclonal sera raised after immunization of chickens are capable to react to (and neutralize) a broad range of different NDV genotypes; this in turn may also relate to cross protection against current circulating field strains after vaccination with genotypically distant strains.

Further studies on epitope mapping of MAbs will help to elucidate these specific epitopes. It will be interesting to learn whether genotype specific antibodies recognize known binding sites but have a different amino acid configuration or whether the genotype 2.VII specific epitopes are formed by different sites. Overall our studies highlight the value of MAb to dissect antigenic sites. The established MAb are the first step to define genotype 2.VII specific neutralizing sites of NDV based on sequence information and should enable phylogenic analysis of antigenic sites in the future, a prerequisite to elucidate whether antigenic drift was the driving factor for evolution of NDV and define critical sites.

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Declarations

Ethics approval and consent to participate

Immunizations were carried out in accordance with the legally approved protocol (MV-LALLF- 7221.3-2.5-010/10).

Consent for publication

Not applicable

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

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Olayinka Asala	Investigation
Sven Reiche	Investigation; Writing - review
Hafez Hafez	review & editing
Martin Beer	Funding acquisition; Writing - review
Timm Harder	Conceptualization; review & editing
Christain Grund	Conceptualization; Supervision; Validation; Visualization; Writing

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Figure 1: Preparation of HN-enriched fraction from purified NDV/NR730/2016.

Purified virus and fractions of the preparation were subjected to SDS-PAGE and subsequent Coomassie staining (A) and Western blot analysis using egg yolk preparation of a vaccinated chicken (B) HN specific rabbit hyperimmune serum (C) or F-specific rabbit hyperimmune serum (D). Beside the original gradient purified virus from AAF (V), the discarded pellets after Triton-100 treatment and two successive centrifugation steps (p1 and p2) are shown, together with the insoluble fraction after dialysis (p3) and the final supernatant used for immunisation (s3).

Figure 2: Antibody response of immunized mice and reactivity of obtained MAb

Antibody response of two mice immunized with the S3 antigen preparation were tested at indicated times after the first immunisation by HI (filled symbols) and by ConA ELISA (white symbols) (A). Time of booster immunizations are given as triangles (▲). In addition, ConA ELISA reactivity of HI positive MAbs (B) or HI-negative cloned MAbs (C) is given.

Figure 3: ConA-ELISA presents conformational dependent HN-epitope.

Reactivity of MAb 617/161 recognizing specifically genotype 2.VI (PPMV-1) by HI, was tested against different antigens (PPMV-1: R75/98, 2.VII: NR730/16; 2.II: clone 30) by ConA- ELISA (B). Coating of plates was verified by homologue polyclonal chicken antibodies (A).

Figure 4: Analysis of reactivity profile of MAbs

All MAbs were tested by WB-analysis (A). Boxes are giving results of the HI-reactive MAbs (left) and HI-negative MAbs (right). Beside testing with homologous virus antigen (NR750/16 genotype 2.VII), cross reactivty of MAb (1B6) to genotype 2.II NDV was confirmed with heterologous clone 30 antigen. Besides reactivity was tested by IFT with the homologous virus NR750/16 (B).

sFigure 1: Antigenic profiling of different NDV genotypes

Sera against five different genotypes were tested by HI against nine different antigens representing eight different NDV genotpyes.. Boxplots represent results of three independent tests with three replicates each. Significant differences ($p<0.05$) to homologues serum, marked by red boxes, are indicated (*).

Table 1: Reactivity of obtained monoclonal antibodies (MAbs)

Mab	HI (log2)		IF		ConA ELISA [OD 492]		WB		SNT (log2)	Specificity
	NR730	clone 30	NR730	clone 30	NR730	clone 30	NR730	clone 30	NR730	
4E11	6	5	pos	pos	1.79	1.19	neg	n.d.	8	HN
5C2	4	neg	pos	neg	1.69	0.17	neg	n.d.	neg	HN
5C9	4	neg	pos	neg	1.86	0.08	neg	n.d.	7	HN
5D1	3	neg	pos	neg	1.74	0.09	neg	n.d.	8	HN
7A8	5	neg	pos	neg	1.55	0.07	neg	n.d.	neg	HN
7C12	5	neg	pos	neg	2.04	0.08	neg	n.d.	8	HN
1B6	neg	neg	pos	neg	0.18	0.18#	55 kD	55 kD	neg	NP/P
5B4	neg	neg	pos	neg	0.16	0.06#	55 kD	neg	neg	NP/P
6F2	neg	neg	pos	neg	0.16	0.05#	55 kD	neg	neg	NP/P

NR730: virulent Egyptian NDV isolalte of genotype 2.VII, directly; clone 30: avirulent NDV vaccine strain of genotype 2.II

(#): directly coated ELISA plates; pos: postive reaction; neg: negative reaction

Table 2: Cross reactivity by HI assay of HN-specific MAbs compared to polyclonal NDV reference sera

			HI (log2)								
			Antigen								
		Genotype[c]	1.I[1]	2.0[2*]	2.I[3]	2.II[4]	2.III[5]	2.VI[6]	2.VII[7]	2.VII[8]	2.XIV[9]
Mab	4E11	2.VII	5	6	4	5	4	5	6	7	6
	5C2	2.VII	neg	3	neg	neg	neg	2	4	7	3
	5C9	2.VII	neg	neg	neg	neg	neg	neg	4	2	neg
	5D1	2.VII	neg	neg	neg	neg	neg	neg	3	4	neg
	7A8	2.VII	neg	neg	neg	neg	neg	neg	5	**1**	neg
	7C12	2.VII	neg	neg	neg	neg	neg	neg	5	4	neg
	617/161	2.VI	2	10	<1	<1	<1	13	1	1	<1
Sera[a]	a-Ulster	2.I	8.2	9.2	10.1	10.3	9.5	**6.3**	7.4	**6.1**	9.5
	a-clone 30[b]	2.II	7.0	8.8	8.3	8.8	8.3	7.0	7.6	7.7	8.2
	a-R151/94	2.VI	6.2	8.5	6.3	5.9	6.2	8.1	7.8	7.7	7.0
	a-NR 730/16	2.VII	10.9	10.0	10.2	10.2	10.2	9.9	11.2	11.0	10.4
	a-NR 81/18	2.XIV	5.9	7.8	6.3	6.6	6.1	7.4	6.0	8.1	8.5

a: Sera were raised in SPF-chicken by immunizing with ß-Propiolacton inactivated virus preparations from SPF-eggs

b: yolk preparations form imunized SPF-chicken; c: genotype nomenclature according to Dimitrov et al. 2019

1: mallard/GER/R2919/2006; 2: Herts33/56 (PEI: PEI-AY170140)* 3: chicken/IRL/Ulster/1967; 4: vaccine/clone 30; 5: JPN/Miyadera/1951;6: pigeon/GER/R75/1998; 7: chicken/EGY/NR730/2016; 8: chiccken/IRN/NR1468/2012; 9:chicken/NGA/NR81/2018; (country code alpha-3 according to ISO 3166)

bold boxes indicate homologues serum / antigen pairs

* unassigned class 2 virus (Czeglédi et al. 2003)

differences between HI-titer to homologous antigen (dHI)

≤1	1- ≤2:	>2- ≤3	**<3**

Supplemental Table 1: Antigenic relation of NDV genotypes

		Antibody titer Ratio (R) [a]				
Sera[b]	genotype[d]	Antigen				
		2.I[1]	2.II[2]	2.VI[3]	2.VII[4]	2.XIV[5]
a-Ulster	2.I	X	0.90	0.15	0.28	0.38
a-clone 30[c]	2.II		X	0.25	0.47	0.41
a-R151/94	2.VI			X	0.58	0.46
a-NR 730/16	2.VII				X	0.31
a-NR 81/18	2.XIV					X

a: the antobody titer ration (R) was calculated according to Archetti and Horsefall, 1950; b :Sera were raised in SPF-chicken by immunizing with ß-Propiolacton inactivated virus preparations from SPF-eggs; b: yolk preparations form imunized SPF-chicken;
c: genotype nomenclature according to Dimitrov et al. 2019

1: chicken/IRL/Ulster/1967; 2: vaccine/clone 30; 3: pigeon/GER/R75/1998;
4: chicken/EGY/NR730/2016; 5:chicken/NGA/NR81/2018;
(country code alpha-3 according to ISO 3166)

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Figure 1

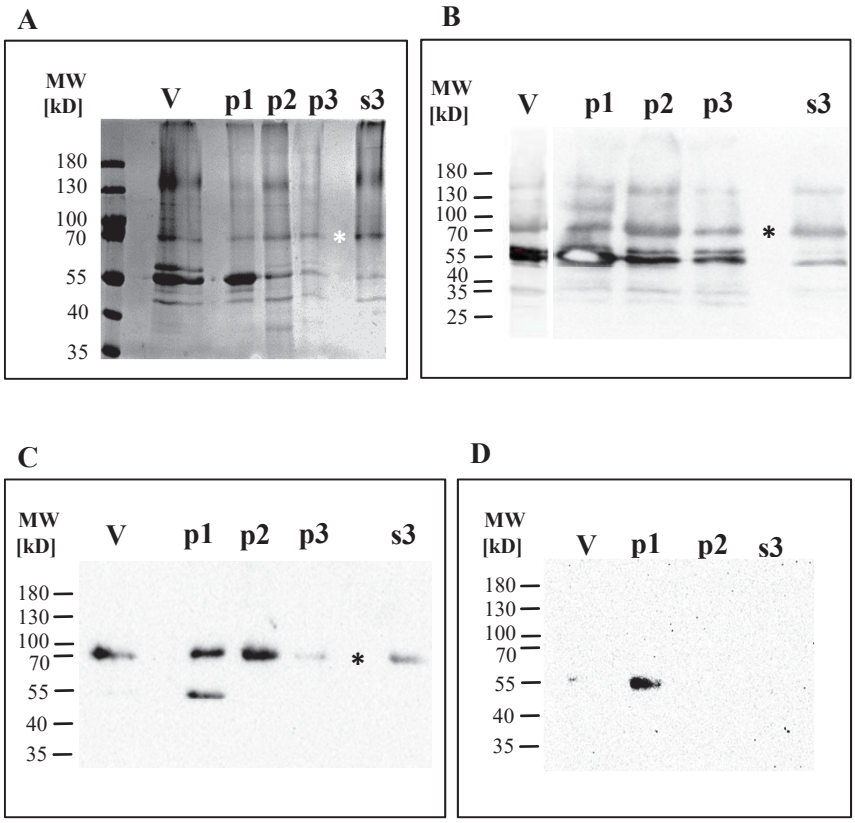
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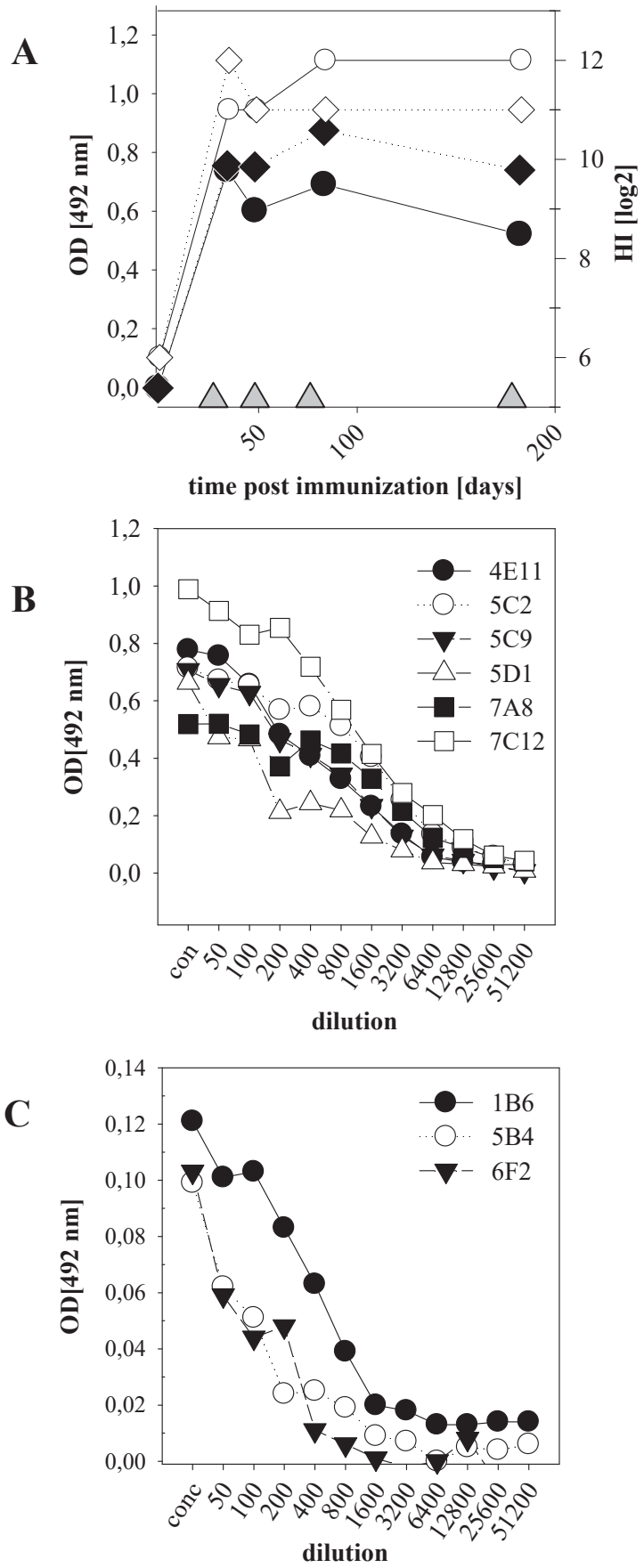
Figure 2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6F2
dilution

Figure 3

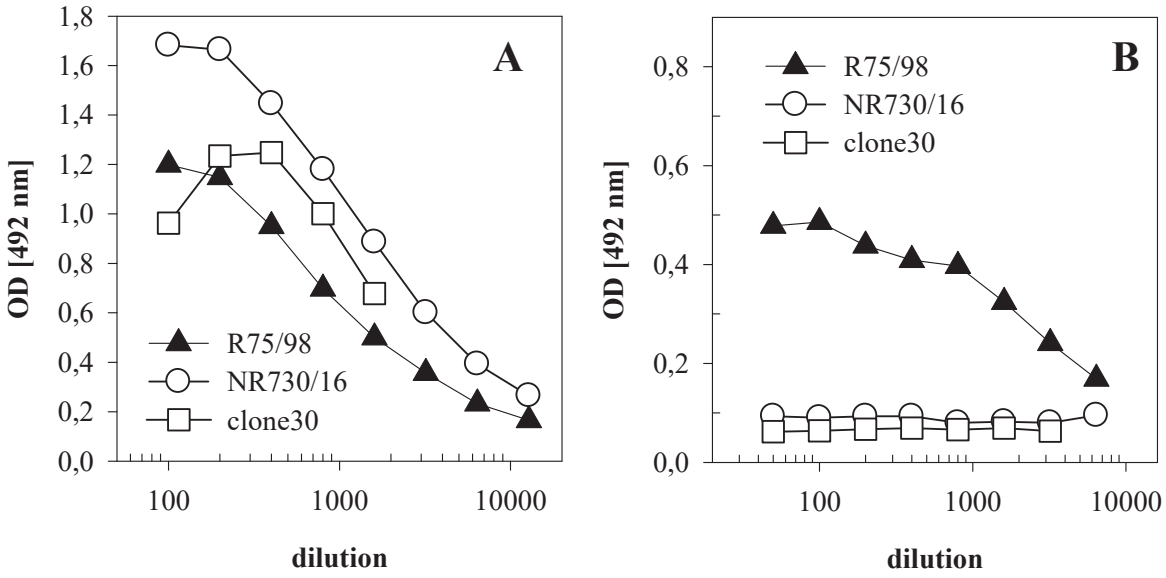
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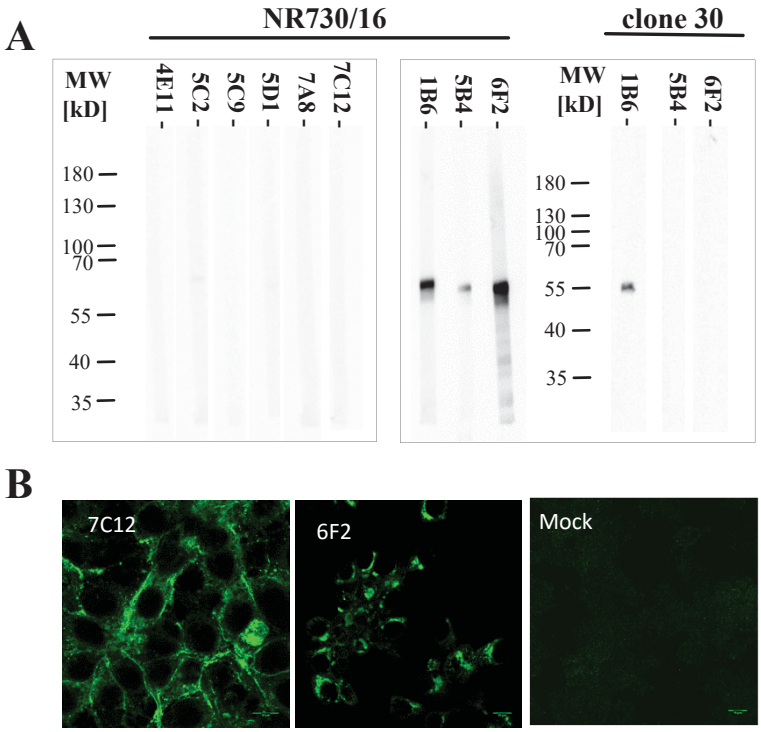
Figure 4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7C12 -
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1B6 -
5B4 -
6F2 -
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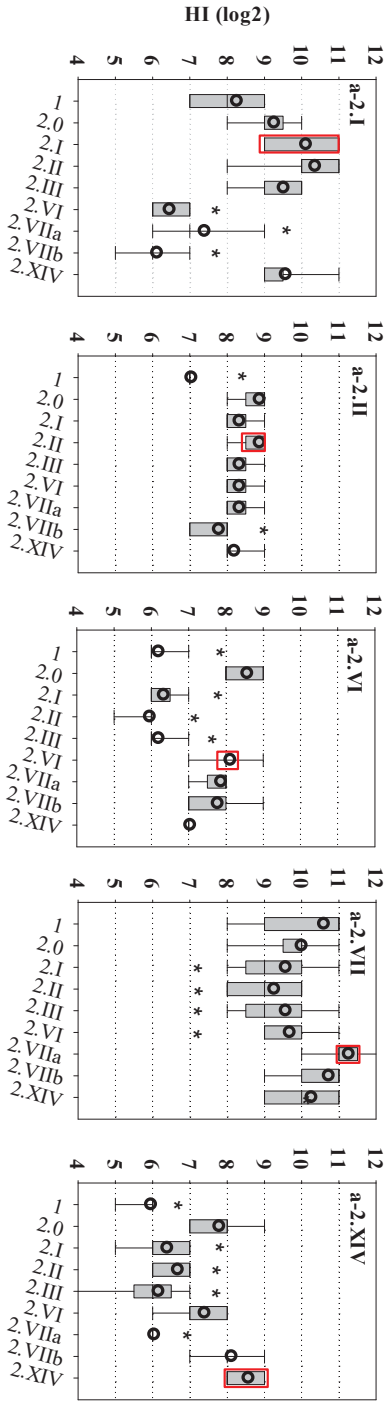
Mock

Supplemental Figure 1



3 Chapter 3: General discussion

The current work aimed to investigate antigenic mismatch of Newcastle disease virus vaccine -strains and field strains circulating in Egypt. The initial survey examined the prevalence of NDV in vaccinated poultry flocks in Egypt that suffered from clinical sings indicative for ND and generated NDV isolates that were the basis to explore potential antigenic drift. Infectious bronchitis virus as important differential diagnosis for respiratory disease was included in the diagnostic approach of the study and an obtained IBV isolate served as second approach to investigate virus evolution in a vaccinated poultry population.

The result of the initial study supported the notion that vNDV is regular associated with respiratory distress in conjunction with high mortality in Egyptian poultry holdings. However, from 26 farms examined, only 16 farms from small scale producers with low to minimal biosecurity were positive for vNDV despite the reported ND vaccination history. This could indicate, that ND is putatively relevant in particular, for small scale producers with insufficient biosecurity would play the major role for spread of NDV infection. However, the notion of lateral spread in between farms of close proximity was only partially emphasized by phylogenetic analysis. Even though all eleven viruses obtained from farms located within radius of approximately 15 km in one governorate (El Beihera) belong to the same sub-genotype 2.VII.1.1 (formerly VII-b), viruses clustered in four different branches, indicating four independent transmission chains. Remarkably, one branch enclosed viruses from different types of poultry holdings i.e. layers, Baladi and broiler farms. These results certainly demonstrate, that biosecurity in place was not able to prevent entry of the pathogen and further spread of infection from farm to farm, regardless of the type poultry. Moreover, in two other branches, viruses were related to viruses identified in another Egyptian governorate (Ismailia). In addition, in the last branch, viruses were not only related to viruses from other Egyptian governorates as Dakahlia and Qalyubia, but were also related to viruses detected in Israel in the same year (2016). These data mean that transmission over long distance is a regular event and point altogether to the suspected high virus burden in poultry holdings in Egypt. In contrast, all ten large broiler farms were negative for vNDV. It is tempting to speculate, that the more sophisticated biosecurity level in these farms could be one of the major determinants for preventing entry and further spread of NDV among the farms. On the other hand, further viral

respiratory pathogens like IBV and AIV H9 have been detected in this group and clearly demonstrate that biosecurity was indeed incomplete. Considering endemicity of ND, it would be likely that also these farms were exposed to vNDV.

In this context, the absence of vNDV in large farms could be regarded as the result of good vaccination program that reduce the shedding of NDV. This conclusion would be in line with experimental data, demonstrating clinical protection and restriction of virus shedding in vaccinated chicken, regardless of the genotype [181, 184]. On the other hand, the high burden of the vNDV in small scale farms would than point to insufficient vaccination or vaccination failure. Insufficient vaccination should to be clearly differentiated from vaccination failures and could be defined as the result of mistakes during the vaccination procedure. There are many causes for insufficient vaccination that include inactivation of live vaccine during transport, handling or application. For example, residues of disinfectant in the water supply systems or the water quality could inactivate the vaccine. Technical problems related to the intake / application of the vaccine have to be considered. Vaccine failure on the other hand would summarize inadequate protection of a proper applied vaccine. This might be caused by the immune status of the birds. Birds might be immune compromised due to previous infection with immunosuppressive pathogens like infectious bursal disease virus (IBDV), chicken infectious anemia virus (CIAV), Marek's disease virus (MDV), (MDV-induced lymphomas), Reovirus, reticuloendotheliosis virus (REV), and the avian leukosis virus (ALV) complex. Some other infections as mycoplasma spp. or presence of mycotoxins were associated with immune suppression as well [170, 174] or by subclinical intoxications by Mycotoxins [174, 175]. In addition chicks, might be to young i.e. the immune system is premature and not able to respond adequately to vaccination [176] or vaccination is hampered by maternal derived antibodies [176] that was shown for IBDV and Influenza as well [177, 178]. A final explanation for vaccine failure might be antigenic mismatch. This has been shown for AIV [179]. To verify vaccine failure due to antigenic mismatch in the field this would need the proof of adequate vaccine application and proper immune response to the vaccine. On the other hand, such study would also need to correlate observed pathogenicity to NDV infection. This became apparent in particular by the finding that mortality levels were up to 60% irrespective of the detection of vNDV. These observations clearly highlight the well-known notion that clinical signs are not sufficient to diagnose ND. Infection with other pathogens inducing respiratory distress and/or viruses induce immunosuppressive have to be considered. In this context it is interesting to note, that in seven out of the ten large scale broiler farms vaccine type NDV was detected. It is

well known, that certain vaccine strains can induce inflammations of the air sacs and induce respiratory distress. For example, aerosol application of the lentogenic vaccine strain B1 to 3-week- old chicks induced respiratory distress in 60% of the birds. Histological alterations included lymphoid infiltrates in air sacs and parabronchi as well as tracheitis and has been observed as long as 16 days after the application [185] with detection of viral RNA by in situ hybridization associated to the inflammation [108]. Pathogenesis by lentogenic NDV will be even amplified by co-infections with Mycoplasma and/or E. coli [186, 187] or other viral pathogens like IBV, Adeno- and Reovirus [188] and Avian meta pneumovirus [189].

Concerning mixed infections with NDV, IBV and AIV H9 have been detected in eight and three farms respectively, with one farm experiencing mixed infections with all three viruses. The observation of mixed infections has been associated with increased loses [12, 128, 129]. However, for IBV several live vaccines are widely used, In consequence, appropriate diagnostic needs typing of the detected strain. This has been investigated in detail and is described in the second publication of the dissertation (see next paragraph).

Concerning the relevance of the detected AIV H9, only inactivated vaccines are applied, routinely on day 7-10 of age. In consequence, the detected virus or virus RNA has to be field virus. AIV H9N2 of the G1-like clade was detected in Egyptian poultry farms in late 2010 [190]. Since this time, it became endemic and cause high economic losses to the poultry industry. The infection of broilers with AIV H9 in the field is associated with respiratory signs and depression accompanied with mortality rate ranging between 7-12% in the flocks. The postmortem examination revealed tracheitis, aerosacculitis, and pneumonia.[190]. We could induce unilateral or bilateral sinusitis and chronic aerosacculitis in female young and adult turkeys after aerosols infection using AIV H9N2 (A/chicken/Saudi-Arabia/R61/02[191]. Applying this infection route as challenge model it was illustrated that inactivated virus preparations of the homologous AIV H9N2 strain induced only partial protection and did not prevent virus replication. Even though not proven as primary pathogen for broilers the detection of AIV-H9 has to be of concern as co-factor to exacerbate clinical disease. The situation of two other viruses, Avian Reovirus and (ARV) and chicken Astroviurs (CAstV) is a further indication for the complex situation in the field. To study their pathogenic effect of the pathogenesis in the filed situation and to verify “vaccine failure” for ND, histological investigations in conjunction with immune-histochemical detection of the specific pathogen would be necessary.

Altogether, the study could generate defined NDV isolates feasible for in depth analysis. In addition, the data illustrate the multifactorial problems of preventive medicine for the Egyptian poultry sector that cannot be solved by conventional vaccination strategies alone. Improved management of hygienic measures for the specific farm as well as for the community are mandatory and have to include small scale production units and back yard holdings. Ongoing review on the efficacy of vaccines is an integral part of such prevention programs.

Detection of a new IBV variant.

In the initial study, IBV was frequently detected in large poultry farms (5/10) and in three cases in small-scale farms. IBV, an avian coronavirus, replicates in several organs and like NDV in the upper respiratory tract, and induce respiratory disorders. Depending on the synergistic co-infections clinical signs may vary from mild to severe respiratory infection with mortality varied between 0% and 82%, depending on the age and the immune status of the birds, the virus strain, and secondary bacterial or viral infections [192]. This would be in agreement with observed high variability in mortality ranged between 7 to 60 %. IBV strains differ in pathogenicity and low virulent strains are used as live vaccines. In order to elucidate the currently circulated type of IBV in the different farms, the second publication focused on the characterization of the eight detected IBV viruses. In particular, we were interested whether the IBV isolates differ between the two types of poultry holdings, i.e. small scale and large scale production units. Initial comparison between the IBV isolate from a small-scale broiler farm (EGY/NR 725/2016) with four samples from large scale broiler farms indicated that all five viruses are belong to genotype G1-23, which is clearly distinct from classical vaccine strains Mass H120, Ma5 (GI-1), 4/91 GI-13 and D274 (GI-12). However, isolate EGY/NR725/16 from the small-scale farm, did not group with the four other viruses or previously described EGY-var1 nor EGY-var2 subgroups [193-195]. Based on standardized criteria as established for NDV[87] a new nomenclature was proposed, where the virus from small scale flock formed a separate branch in genotype S1:GI-23 and was considered as a prototype for newly emerged subclade designated as S1:GI-23.3. Comparison of the complete genome of EGY/NR725/16 revealed intra-genotypic gene exchange partially for the S1 gene and complete switches for gene 3ab and E. In addition, gene 6b was raised from inter-genotypic recombination. Further analysis of the gene 3a/b constellation could elucidate, that the 3ab: genotype found in the small

scale farm was present already in samples from large scale farms in 2013 and dominated in samples taken three years later i.e. 2019 in conjunction with the new genotype S1:GI-23.3.

These data indicate that newly emerged subclade S1:GI23.3, first recognized in during our initial study emerged by successive recombination events at least since 2013 and included viruses that were not confined to a specific sector. This would lead to the conclusion, that there has to be a wide exchange between the different sectors with pathogens transmitted from small farms to large scale production units and vice versa. Although the large farms in Egypt have a more sophisticated biosecurity system in place this system is not sufficient to prevent entry of pathogens into the farm nor transmission of pathogens from the farms.

Another striking observation was, that the viruses with the new S1:3ab constellation were apparently successful to spread in vaccinated poultry population. In silico analysis of the S protein demonstrated an accumulation of point mutations within the Hypervariable regions (HVR) of the S1 protein. The S protein facilitates the attachment and invasion of IBV into the host cell and is of uppermost importance for the protective immune response [196-198]. For IBV specific neutralization sensitive epitopes have been defined by monoclonal antibodies (MAb) [199] and were subject for our in analysis. Compared to vaccine strains, the original strain, obtained isolate AGY/ NR 725/16 as well as all subsequent viruses of the year 2018 had mutations in at least four defined epitopes, within the HVR3 region, indicating a possible antigenic drift of the new S1:GI-23.3 variants. For IBV, the importance of antigenic mismatch has been described in detail and lead to the introduction of a variety of several IBV vaccines (H120, Ma5, 4/91 and D274). The ongoing circulation of NDV in Egypt and in other regions of the world in the recent years, raised the concern whether antigenic variations as observed for IBV, are also accountable for vaccination failure in ND outbreaks.

The third part of the thesis was dedicated to establish respective tools for defining genotype 2.VII specific epitopes and enable analysis on possible antigenic drift of NDV.

Antigenic characterization of genotype 2.VII virus from Egypt

In order to characterize antigenic sites of circulating NDV genotype 2.VII, we were interested to generate genotype specific monoclonal antibodies (MAbs) that are able to block biological active sites of the HN-protein, i.e. antibodies that are able to neutralize and / or block HA

activity. A representative isolate (EGY/NR730/16) was chosen from the closely related viruses obtained during the initial study and used for the subsequent analysis. Applying an HN-enriched virus preparation as antigen and a con-A ELISA test system that preserves and presents conformational intact sites, six HN-specific MAb were established. One MAb was reactive with tested eight different NDV genotypes, including the class 1 strain. On the other hand, the majority of HN specific MAbs were reactive predominantly with the homologous NDV strain EGY/NR730/16. Within this group of MAb, we could observe three distinct binding patterns: a) one MAb (5C2) having residual cross reactivity to three other genotypes, b) three MAbs reacting specifically with two genotype 2.VII strains (5C9, 5D1, 7C12) and c) one MAb (7A8) differentiating between the two genotype 2.VII viruses. These results clearly point to high flexibility of some epitopes, whereas other epitopes seem to be very well preserved. These results coincide with results obtained with polyclonal antibodies raised against different genotypes. All sera strongly reacted with all NDV strains tested, but results of cross-HI revealed antigenic differences. In consequence the presence of these broadly cross -reacting epitopes are in line with the protection studies demonstrating cross protection against current circulating field strains after vaccination with genotypically distant strains [101, 184]. On the other hand, vaccines that present matching genotype might provide a benefit when clearing an infection. In vivo protection studies with different MAb, i.e. type specific and broadly cross -reacting MAb might help to elucidate that question. In addition, it remains unclear whether observed minor antigenic differences provide a selection advantage in a vaccinted population or whether other/additional factors contribute to the dominance of genotype 2.VII viruses. In this context, observations from infection studies, with juvenile and adult turkeys might point to a different explanation. In this study the genotype 2.VII strain (chicken/Iran/R1468/12) was less pathogenic compared to a classical NDV strain Herts 33/56. It was striking that in adult turkeys the genotype 2.VII strain did not induce clinical signs even though the shedding was comparable to the historical NDV strain. [200]. From the epidemiological point of view, a lower virulence of NDV genotype 2.VII might be an advantage to become endemic with more efficient unnoticed spread within and in between farms. Facing a high virus burden like NDV in Egypt, it likely that other factors than vaccine failure by antigenic mismatch contribute to the problems. In particular for Broilers it should be considered that the rearing period with the physiological gap between protection by maternal derived antibodies and active immunity provide a gate for entry of pathogens into poultry flocks and that this gap can´t be closed only by active vaccination. To prevent ND outbreaks and manifestation of other infectious disease, flanking measures beside vaccination seem indispensable.

4 Chapter 4: Summary

The dissertation focused on Newcastle disease virus (NDV) in vaccinated poultry flocks in Egypt and explored potential antigenic drift of circulating NDV strains. The investigations, were mirrored by analysis on the evolution of Infectious bronchitis virus (IBV), another important pathogen inducing respiratory distress in poultry and known to be antigenically variable

The initial field study detected virulent NDV (vNDV), in all of 16 examined small-scale poultry holdings suffering from respiratory distress and high mortality. Genetic characterization confirmed NDV genotype 2.VII.1.1 (formerly 2.VII-b), the dominant NDV genotype in the Middle East and northern Africa. However, analysis of the fusion (F) protein gene revealed that virus present in these farms which are located in close proximity to each other clustered in four different branches. That means four independent introductions of viruses with relatives in other governorates, with subsequent transmissions between farms, regardless of the poultry type. In the second group enclosing large broiler farms (n=10) only vaccine type NDV was detected (n=6).

In addition, in both small and large scale farms IBV (n=8), Avian influenza virus (AIV), subtype H9 (n=3) and Avian Reovirus (ARV, n=5) was present in those holdings. From one large broiler farm, chicken Astrovirus (CAstV) was isolated.

Characterization of detected IBV revealed that one of the three viruses from small scale holdings represents a new subclade of genotype GI (GI-23.3) whereas the other viruses match with GI-23.2, formerly designated as GI-23 subgroup EGY-var2. Full genome analysis for the 23.3 isolate from a small scale flock found intra-genotypic gene exchange for partial fragments of the S1 gene and complete switches for gene 3ab and E and inter-genotypic recombination for gene 6b. This particular gene 3a/b was present also in subsequently investigated historical samples from 2013 derived from large broiler farms in Egypt, and the new S1:3a/b constellation dominated in samples taken three years later, i.e. 2019. In silico analysis of the S protein demonstrated an accumulation of point mutations over time within the hypervariable regions (HVR) of the S1 protein. Compared to vaccine type virus striking mutations in at least four of seven defined epitopes within the HVR3 region were present in the new GI-23.3 strain from

small scale farms of the year 2016 and all subsequent related viruses, indicating a possible antigenic drift of these new variants.

Antigenic differences were also evident for NDV genotype 2.VII using Hemagglutin-neuraminidase (HN) spike protein specific monoclonal antibodies (MAbs) that were raised in this study against a representative Egyptian NDV isolate. Out of six MAbs that were able to neutralize and / or block HA activity, two MAbs were reacting specifically with genotype 2.VII strains and one MAb differentiated even between two distinct genotype 2.VII viruses. On the other hand, one MAb had residual cross reactivity to three other genotypes (2.0, 2.VI and 2.XIV) and one MAb reacted with all eight genotypes tested (class 1, 2.0, 2.I, 2.II, 2.III, 2.VI, 2.VII and 2.XIV). These results pointed to the concurrent presence of flexible and conserved epitopes within the HN-spike protein. This notion was corroborated by results obtained with polyclonal antibodies that indicated different degrees of antigenic differences between different genotypes but clearly demonstrated strong cross reactivity between all eight genotypes tested. The importance of these broadly cross-reacting epitopes was in line with reports on vaccine protection studies, demonstrating clinical protection against genotypically distant strains when using genotype 2.II vaccine strains.

Overall, the work shown here provided evidence for a high burden in Egyptian poultry holdings of viruses, in particular NDV and IBV, associated with respiratory disease. The phylogenetic analysis of IBV pointed to a flourishing viral exchange between different sectors of poultry production and ongoing antigenic drift. For NDV the data confirmed prevalence of vNDV, genotype 2.VII in broiler flocks with respiratory distress and elevated mortality rates. However, the minor antigenic differences found for NDV using newly established MAbs did not support the notion of vaccine failure. Irrespective of possible technically insufficient vaccination in small scale flocks, the physiological gap between protection of the offspring by maternal derived antibodies and active immunity has to be considered as an intrinsic gate for entry of pathogens into poultry flocks during the rearing period. To prevent ND outbreaks and manifestation of other infectious disease, flanking biosecurity and management measures beside vaccination seem indispensable.

5 Chapter 5: Zusammenfassung

Virologisch bedingte Resprirations-Erkrankungen: Untersuchungen zur Relevanz des Newcastle Disease Virus in geimpften Geflügelbeständen in Ägypten und Charakterisierung zusätzlich nachgewiesener Viren.

Die Dissertation untersuchte einen potentiellen „Antigendrift“ des Virus der Newcastle Krankheit (NDV) in geimpften Geflügelbeständen in Ägypten. Parallel wurde die Evolution des infektiösen Bronchitis-Virus (IBV) analysiert, ein weiterer wichtiger Erregers, der bei Geflügel Atemstörung hervorruft und von dem bekannt ist, dass er variabel ist.

In einer ersten Feldstudie wurden Geflügelbestände in Ägypten untersucht, die unter Atemstörungen litten und eine hohe Mortalitätsrate aufwiesen. In allen 16 untersuchten Kleinbeständen wurde virulentes NDV nachgewiesen und als Genotyp 2.VII.1.1 (früher 2.VII-b) identifiziert, dem dominierenden NDV-Genotyp im Nahen Osten und in Nordafrika. Bemerkenswerter Weise wies die Analyse des Fusionsprotein-Gens (F), obwohl die Farmen sich in enger Nachbarschaft befunden haben, vier verschiedene Cluster nach. Dieser Befund deutet auf vier unabhängige Eintragungen von verwandten Viren aus anderen Ägyptischen Distrikten, mit anschließenden Übertragungen zwischen Betrieben, unabhängig vom Geflügeltyp. In der zweiten Gruppe der untersuchten Farmen, die große Broilerfarmen umfasste (n = 10), wurde nur NDV vom Impfstofftyp (n = 6) nachgewiesen. Sowohl in kleinen als auch in großen Betrieben war allerdings IBV, Aviäres Influenzavirus (AIV) des Subtyps H9 (n = 3) und das Aviäre Reovirus (ARV, n = 5) nachweisbar. Zusätzlich wurde von einer großen Broilerfarm Hühner Astrovirus isoliert.

Die Charakterisierung des nachgewiesenen IBV ergab, dass eines der drei Viren aus kleinen Beständen einer neuen Unterklasse des Genotyps GI (GI-23.3) angehört. Die anderen IBV Stämme sind dem Genotyp GI-23.2 zu zuordnen, der früheren GI-23-Untergruppe EGY-var2. Eine vollständige Genomanalyse für das GI-23.3 Isolat aus dem Kleinstbetrieb wies intra-genotypische Genaustausche für Teilfragmente des S1-Gens und vollständige Austausche für Gen 3ab und E nach, sowie eine inter-genotypische Rekombination für das Gen 6b. Dieser spezielle Gen 3a / b Genotyp war auch in nachfolgend untersuchten historischen Proben aus dem Jahr 2013 vorhanden, die aus großen Broilerfarmen stammten. In Proben aus dem Jahr

2019 von Broilerfarmen in Ägypten war die neue S1: GI-23.3 Variante in der spezifischen 3a / b-Gen-Konstellation schließlich dominant. Eine in-silico-Analyse des S-Proteins wies eine Akkumulation von Punktmutationen innerhalb der hypervariablen Regionen (HVR) des S1-Proteins nach. Im Vergleich zu Viren vom Impfstofftyp waren traten bei den neuen GI-23.3 Varianten in mindestens vier von sieben definierten Epitopen der HVR3-Region, Mutationen auf, was ein Hinweis auf einen möglichen Antigendrift darstellt.

Auch für den NDV-Genotyp 2.VII konnten mit Hilfe von in dieser Arbeit etablierten monoklonaler Antikörpern (MAbs) antigene Unterschiede aufgezeigt werden. Von sechs MAbs, die in der Lage waren, die Virus-Rezeptor vermittelte Agglutination Erythrozyten (HA) zu neutralisieren und / oder zu blockieren, reagierten zwei MAbs spezifisch mit Stämmen des Genotyps 2.VII und ein MAb unterschied sogar zwischen zwei verschiedenen Viren des Genotyps 2.VII. Andererseits wies ein MAb eine Restreaktivität mit drei anderen Genotypen (2.0, 2.VI und 2.XIV) auf und ein MAb reagierte mit allen acht getesteten Genotypen (Klasse 1, 2.0, 2.I, 2.II, 2.III, 2.VI, 2.VII und 2.XIV). Diese Ergebnisse weisen auf das gleichzeitige Vorhandensein flexibler und konservierter Epitope im Hämagglutinin Neuraminidase (HN)-Spike-Protein hin. Diese Annahme wurde durch Ergebnisse bestätigt, die mit polyklonalen Antikörpern erhalten wurden und nur geringfügige antigene Unterschiede zwischen verschiedenen Genotypen nachwiesen bei starker Reaktivität zwischen allen acht getesteten Genotypen. Dieser Nachweis breit reagierender Epitope stützt Untersuchungen über den klinischen Schutz gegen genotypisch entfernte Stämme bei Verwendung von Impfstämmen des Genotyps 2.II.

Insgesamt lieferten die Arbeiten Hinweise auf eine hohe Viruslast in ägyptischen Geflügelbeständen mit Atemwegserkrankungen, insbesondere von NDV und IBV. Die phylogenetische Analyse von IBV belegt einen regen Virus-Austausch zwischen den verschiedenen Sektoren der Geflügelproduktion mit Auftreten von neuen Antigen-Varianten. Für NDV bestätigten die Daten die Prävalenz von virulentem NDV des Genotyps 2.VII in Ägypten. Die geringfügigen antigenen Unterschiede, die unter Verwendung neu etablierter MAbs festgestellt wurden, stützten jedoch nicht die Hypothese von Versagen der ND-Impfung aufgrund eines „Antigendriftes“. Zusätzlich zu einer unzureichenden Impfung aufgrund technischer Impffehler in kleinen Herden, muss die physiologische Lücke zwischen passiver und aktiver Immunität berücksichtig werden: In der Zeit des Absinkens des maternalen Antikörperspiegels und der noch nicht ausgeprägten Impfantwort, sind Küken besonders

anfällig für Krankheitserreger. Daher sind flankierende Maßnahmen der Biosicherheit essentiell um ND-Ausbrüche und andere Infektionskrankheiten wirksam zu bekämpfen.

6 Chapter 6: References

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7 Curriculum Vitae

For reasons of data protection, the curriculum vitae is not published in the electronic version

8 Publication list

Moharam, I., M. Gebauer, S.-E. Behrens, H. Hennemann, C. Grund. Increased pathology of subtype H9 Avian Influenza virus after aerosol infection of turkeys. In: Proceedings of the 12th "Hafez" International Symposium on Turkey Diseases, (Ed. Hafez, H.M), Mensch & Buch Verlag. ISBN. 978-3-86387-958-7. Institute of Poultry Diseases, Free University Berlin. pp. 21 - 22. Berlin 31st May – 2nd June 2018.: oral. Pres. 31.05. -06.02.2018

Moharam, I, C. Grund. Longitudinal study on viral evolution demonstrates conservation of antigenic sites in Heamagglutinin Neuraminidase (HN) -attachment protein of pigeon Avulavirus-1, FLI Junior Scientist Symposium ; 7 (Greifswald - Insel Riems): poster pres.. 24-2609. 2018

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Moharam I., A. Abd el Razik, H. Sultan, M. Ghezlan, M. Meseko, K. Franzke, T. Harder, M. Beer, C. Grund (2019). Investigation of suspected Newcastle disease outbreaks in Egypt uncovers a high virus burden in small-scale holdings and the presence of multiple pathogens Avian Pathol. 48:406-415. 415 https://doi.org/10.1080/03079457.2019.1612852

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Moharam I, O. Asala, S. Reiche, T.Harder, H. Hafez, M. Beer, C. Grund. Monoclonal antibodies specific for the hemagglutinin-neuraminidase protein define neutralizing epitopes specific for Newcastle disease virus genotype 2.VII from Egypt. Submitted to Virology Journal

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Increased pathology of subtype H9 Avian Influenza virus after aerosol infection of turkeys.

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Avian Influenza viruses comprise important pathogens for poultry, that can be classified based on the heamagglutin (H) outer envelope protein, into 16 subtypes. Only for two AIV subtypes, H5 and H7, mutants within the proteolytic cleavage side arise, that are able to replicate efficiently in multiple organs including the brain and transforming the mutated virus from low to highly virulent pathotypes for gallinaceous birds. The other subtypes are considered low pathogenic, as virus replication is restricted to epithelial layers of the respiratory and digestive tract. Even though these viruses are not inducing systemic infections, they can be associated with dramatic clinical disease: Beside AIV-H6, subtype AIV-H9 has become an economical very important disease that is endemic in numerous regions of the world. Clinical signs in turkeys are dominated by respiratory signs and swelling of infra-orbital sinuses. Pathological alterations include exudative sinusitis and fibrinous aerosacculitis. Concomitant infections with other microorganism colonizing the respiratory tract, like Ornithobacterium rhinotracheale, Bordetella avium or Mycoplasma species are often observed and considered to deteriorate disease into chronic forms, with intermediate mortality rates.

Clinical protection against AIV H9N2 is attempted by vaccination with inactivated vaccines, preferentially with antigenically matched preparations. To verify efficacy of vaccination, adequate challenge models are needed. Beside the dose of a specific virus, route of infection is a critical parameter. To mimic a more natural route of infection, virus inoculation was done

by aerosolization of AIV H9N2. First titration experiments in turkeys established the minimal infectious dose and could characterize viral replication kinetics inducing mild aerosacculitis in infected animals. Applying this infection model to vaccinated adult turkeys, only partial protection could be achieved with inactivated virus preparations of the homologous AIV H9N2 strain. Based on the results, including data on viral shedding and serological responses, implication for AIV H9N2 prevention will be discussed.

FLI Junior Scientist Symposium; 7 (Greifswald - Insel Riems): Poster pres. 2018.09.24-26

Longitudinal study on viral evolution demonstrates conservation of antigenic sites in Heamagglutinin Neuraminidase (HN) -attachment protein of pigeon Avulavirus-1

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Abstract

Newcastle disease virus (NDV) or Avian Avulavirus -1 (AAvV-1) is inducing a fatal poultry disease. Despite vaccination, disease outbreaks are reported and prompted the question of antigenic drift in APMV-1. This question was addressed for PPMV-1, a specific AAvV-1 subtype endemic in pigeons (PPMV-1).

Genetic characterization of 223 PPMV-1 isolates obtained between 2008 and 2017 revealed, that one particular PPMV-1 subgroup was predominantly circulating in Germany (2.VI.7.2.2). Sporadic introduction of 2 other subgroups i.e. 2.VI.5 and 2.VI.8.2, were recognized. Comparing the virus attachment protein (HN) of 32 selected PPMV-1 isolates, genetic but not antigenetic-drift became evident: Early and late PPMV-1 isolates have an indistinguishable reactivity pattern with polyclonal sera and are reactive with a specific monoclonal antibody (mab) defining a conformational dependent, neutralization sensitive epitope. This mab binding site is conserved in predominant PPMV-1 viruses (2.VI.7.2.2), as well as in 2.VI.5 viruses. However, viruses of 2.VI.8.2 that emerged sporadically, have a point mutation and lost mab-reactivity but did not outcompete 2.VI.7.2.2 viruses.

These data indicate, that epitopes relevant for viral attachment of AAvV-1 are well conserved and circulation in a pigeon population that is partially immune, was not sufficient to promote emergence of antigenic drift variants.

Key words: Newcastle disease, PPMV-1, antigenicity, epitope mapping

10th Hafez" International Symposium on Turkey Production; 10 (Berlin): oral pres. 2019.06.06-08

Decreased virulence of Newcastle disease virus genotype VII in turkeys: a possible key to success.

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Highly contagious Newcastle disease virus (NDV) is causing devastating outbreaks with highly variable clinical signs among gallinaceous birds. Turkeys are susceptible to disease, but compared to chickens clinical outcome is considered milder (Beaudette, 1943). Studies with different NDV lineage 5b isolates pointed to strain specific differences (Alexander et al., 1999; Aldous et al., 2010. This prompted the question on pathogenicity of currently circulating NDV genotype 2.VII viruses for turkeys. To address this question, juvenile (28 days) and adult (22 weeks) turkeys were infected in parallel with a classical genotype 2.IV strain (Herts33/56; ICPI: 1.76) and a genotype 2.VII isolate from the middle east (R1468/12 (chicken/middle east/R1468/2012; ICPI: 1.85); both viruses considered as velogenic by intra cerebral pathogenicity index in chickens (ICPI).

In juvenile turkeys, onset of disease was between 3-7 days post infection (p.i.) and morbidity rate was 100 % for almost all groups, except turkeys receiving 10^6 EID_{50} intra muscularly (i.m.). Clinical signs included apathy, uncontrolled movements and subsequently tremor and paralysis of wings and/or legs. Mortality became evident between day 5 and day 8 p.i. and reached 100 % for both genotypes after i.m. infection. After oculo-nasal inoculation however, 100% mortality was observed only for the Herts 33/56 infected groups receiving 10^5 or 10^6 EID_{50}, but did not exceed 66% after R1468/12 infection, regardless of the virus dose (10^4-10^6 EID_{50}).

For adult turkeys, clincal signs were in general milder and mortality was observed only in the groups infected with Herts 33/56: With morbidity rates between 50% to 80%, depending on the virus dose (10^4-10^6 EID_{50} respectively), mortality never exceeded 30 %. After infection of adult turkeys with R1468/12 10^6 EID_{50}, 9 of 10 birds showed mild clinical signs for up to 3

days, but in contrast to Herts 33/56 infection, none of the animals died. However, regardless of the clinical outcome animals infected with both genotypes shed virus, detected in pharyngeal as well as cloacal swab samples.

The data confirms that young turkeys are more susceptible to Newcastle disease and clearly indicate strain specific differences. In both age groups, the genotype 2.VII strain R1468/12 was less virulent for turkeys. It was striking that in adult turkeys, classical signs of ND were absent, a point that impedes with clinical ND diagnosis under field conditions. As infected turkeys shed virus, turkeys should be considered as potential unrecognized virus carriers. In this respect lower virulence of NDV genotype 2.VII might be an advantage to become endemic.

World Veterinary Poultry Association Congress (WVPAC); 21 (Bangkok, Thailand): Poster pres. 2019.09.16-20

Prevalence of Newcastle disease and other respiratory pathogens in farms with respiratory distress in Egypt.

Ibrahim Moharam Mahmoud. Ibrahim, Alaa. Abd el Razik, Mohammed. Ghezlan, Dirk. Höper, Kati. Franzke, Timm. Harder, Martin. Beer, Christian. Grund.

Highly contagious Newcastle disease (ND) causes devastating outbreaks with highly variable clinical signs among gallinaceous birds. Considering endemicity of ND in Egypt, we aimed to verify clinical ND suspicions in two ND-vaccinated cohorts and analyzed the regional virus spread: Cohort A (n=16) combined small-scale holdings which were confirmed to suffer from virulent Newcastle disease virus (NDV). Phylogenetic analysis of the fusion protein gene of 11 NDV-positive samples obtained from this cohort assigned all viruses to genotype 2.VIIb, representing the currently dominating genotype in Egypt. Further genetic analysis revealed four distinct virus populations that were related to contemporary viruses in other governorates, pointing to both direct and indirect spread of NDV in these cases. Cohort B showing diffuse respiratory signs and elevated mortality rates comprised large commercial broiler farms (n=10). In these farms virulent NDV was not present, although, in 8 farms NDV vaccine type virus (genotype 2.II) was detected, and co-infections either by infectious bronchitis virus (IBV, n=5), avian influenza virus (AIV, n=2), avian reovirus (ARV)- (n=3) or avian astrovirus (n=1) could be identified, indicating multifactorial processes contributing to clinical disease in this cohort. IBV sequencing show emerging of a variant strain within the GI-23 which is circulating in the midel east with full S1 gene identity 94% with the nearest GI-23 sequence in the database (Gene bank). Similar ARV that indicate a totally newly emerged variant with σ c gene similarity of 86% with S1130 vaccine used in Egypt, while it is the first report for avian astrovirus virus isolation in Egypt

The results highlight that clinical ND suspicion has to be verified by pathotype-specific diagnostic tests. In addition, the results point to an insufficient ND vaccination practice in cohort A. To improve vaccination in this sector, verification of proper vaccination response is

indicated. Additionally, increased biosecurity measures are highly recommended, that aim not only to exclude introduction into but also hamper spread from infected farms, aiming to lower pathogen burden in general and NDV in particular

American Association of Avian Pathologists (AAAP) Virtual Annual Meeting. 2020. Poster pres. 07.31-08-8.

Characterization of variant infectious bronchitis virus from a small scale poultry holding in Egypt

Ibrahim Moharam, Hesham Sultan, Kareem Hassan, Mahmoud Ibrahim, Awad shehata, Salama Shany, Florian Pfaff, Dirk Höper, Magdy Ek Kady, Timm Harder, Hafez M. Hafez, Christian Grund

Avian infectious bronchitis (IB) is an economically important infectious disease caused by infectious bronchitis virus (IBV). In this study, we compared an IBV strain obtained from a small-scale poultry farm (NR 725/16, Moharam et al. 2019) to IBV-isolate from large-scale poultry farm (USC-5/2013, Sultan et al. 2015) in Egypt. Full genome sequencing of both viruses revealed close relationship to other Egyptian viruses, and phylogeny of the S1-gene confirmed genotype GI-23 for both. However, the IBV-strain NR 725/16 showed 6 % diversity compared to strain USC-5/2013, indicating a new variant. Analysis of possible recombination, revealed a switch of genes 3ab, E as well as 6b in NR 725/16 with genes present in viruses within subclade GI-23 and GI-19 respectively. Subsequent analysis of historical IBV-positives samples from large-scale poultry farms in Egypt revealed that NR 725/16 3ab gene was already present in strains from 2013, but at that time, IBV was carrying a distant S1, pointing to a recombination event where IBV from poultry farms was donating the 3ab gene cassette to an unknown ancestor. Analysis of current IBV-positive samples from 2018-19 demonstrate homology on the S1 and 3ab gene to the NR 725/16 recombinant, indicating that the strain first detected in a small scale holding became a representative for newly emerging IBV strains in Egypt. In this respect, the role of transmission in-between different poultry sectors, i.e. small scale- vs. industrialized poultry-holdings will be discussed.

Infectious bronchitis, Recombination, Variant

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10.2 Conflict of Interest

All authors and co-authors disclose any potential conflicts of interest when they submitted the different Publication. No potential conflict of interest was reported by the authors when they submits the thesis.

11 Declaration of authorship / Selbstständigkeitserklärung

Declaration of authorship

I hereby confirm that the present work was solely composed by my own. I certify that I have used only the specified sources and aids.

Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Ort, Datum	Unterschrift
Berlin, 08.01.2021	Ibrahim Moharam