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2. Abbreviations

Amp	Ampicillin
ATP	Adenosin triphosphat
BAC	Bacterial artificial chromosome
BHV	Bovine herpesvirus
bp	Base pairs
BSA	Bovine serum albumin
Cam	Chloramphenicol
CEC	Chicken embryo cells
CKC	Chicken kidney cells
CVI-988	Attenuated vaccine strain of MDV
coint.	Cointegrate
DdDp	DNA dependent DNA polymerases
ddH2O	Double distilled water
DEF	Duck embryo fibroblast
DEV	Duck enteritis virus
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dATP	Deoxyadenosine triphosphate
dTTP	Deoxythymidine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanine triphosphate
dpi	Days post infection
dpt	Days post transfection
dsDNA	Double strand deoxyribonucleic acid
E. coli	*Escherichia coli*
EBV	Epstein-Barr virus
EB	Elution buffer
EDTA	Ethylendiamine tetraacetic acid
EHV	Encephalomyocarditis virus
EMCV	Equine herpes virus
ER	Endoplasmic reticulum
ERAP	ER associated protease
Exo	Exonuclease
FBS	Fetal bovine serum
FFE	Feather follicle epithelium
For	Forward
GAG	Glycosaminoglycans
gB	Glycoprotein B
gC	Glycoprotein C
gD	Glycoprotein D
gH/gL	Glycoprotein H and L complex
GaHV-2	Gallid herpesvirus 2
GaHV-3	Gallid herpesvirus 3

GFP	Green fluorescent protein
GRE	Glucocorticoid responsive element
h	Hour
HCMV	Human cytomegalovirus
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HSV-1	Herpes simplex virus 1
hpt	Hours post transfection
hpi	Hours post infection
HVT	Herpesvirus of turkeys
HVEM	Herpes virus entry mediator
ICP0	Infect cell protein 0
ICP47	Infect cell protein 47
ICTV	International Committee on Taxonomy of Viruses
IE	Immediate early
IFN	Interferon
ILTV	Infectious laryngotracheitis virus
INM	Internal nuclear membrane
IR	Internal repeat
IRL	Internal repeat long
IRS	Internal repeat short
JM/102W	vMDV strain (Prototype)
Knm	Kanamycin
kb	Kilobases
kbp	Kilo base pairs
kDa	Kilo Dalton, non SI unit for molecular mass
LB	Luria-Bertrani medium (lysogeny broth)
LMB	Leptomycin B
LPP	Lambda protein phosphatase
LPS	Lipopolysaccharide
M	Marker
Mb	Mega bases
MD	Marek’s disease
Md5	vvMDV strain (Prototype)
MDV	Marek’s disease virus
MEM	Minimum essential medium (Eagle)
MHC	Major histocompatibility complex
min	Minutes
mut	Mutant
NBD	Nucleotide binding domain
NLS	Nuclear localization signal
NES	Nuclear export signal
NGS	Next generation sequencing
o/n	Overnight
OD600	Optical density, 600 nm wavelength
PAMPS	Pathogen associated molecular patterns
PBS	Phosphate saline buffer

PCR	Polymerase chain reaction
PFU	Plaque forming unit
p.i.	Post-infection
PLC	Peptide loading complex
Pol	Polymerase
PRV	Pseudorabies virus
P/S	Penicillin/streptomycin
RdRp	RNA dependent RNA polymerases
rev	Reverse
Rev	Revertant
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Rotations per minute
RT	Reverse transcriptase
SB-1	Strain of GaHV-3
SD	Standard deviation
SDS	Sodium dodecyl sulfate
s	Seconds
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SPF	Specific-pathogen-free
TAE	Tris-acetate-EDTA buffer
TAP	Transporter associated with antigen processing
Temp.	Temperature
TE	Tris-EDTA buffer
T'N'T	Transcription and translation
TRL	Terminal repeat long
TRS	Terminal repeat short
U_L30	Unique long 30, encoding alphaherpes DNA Pol
U_L	Unique long
U_S	Unique short
v	Reconstituted virus
vIL-8	Viral interleukin 8
vMDV	Virulent Marek's disease virus
vvMDV	Very virulent Marek's disease virus
vv+MDV	Very virulent plus Marek's disease virus
VSV	Vesicular stomatitis virus
VZV	Varicella zoster virus
WB	Western blot
WT	Wildtype

4. Introduction

4.1 On Viruses – A Matter of Definition

Viruses, not strictly alive, yet ubiquitous, have shaped the evolution of life on our planet[1-3]. Being the perfect parasite, a virus has outsourced the largest parts of its metabolism to its host. As a result, the virus needs the host for reproduction, and when it leaves the host and spreads to new environments, it keeps nothing but its genetic information, a few structural proteins and in some cases essential enzymes.

Viruses are the only organisms known to employ ribonucleic acid (RNA) to store genetic information. Comparatively unstable in nature and error-prone in replication, RNA seems to be an inferior choice for the storage of indispensible information. Some viruses have found a way to not only cope with this problem, but make it their advantage in the "*struggle for life"*.

From 2000 bp of single-stranded DNA that do not encode for more than two genes (Circoviridae) to the recently discovered Pandoraviridae with 2500 kb of double-stranded DNA, more than 2500 proteins and particles that are bigger than some bacteria, viruses have adapted to all kinds of environments[4] and are thought to be the reservoir of the greatest genetic diversity on earth[5].

Lacking some important properties of life, viruses do not easily fit into the binary nomenclature established in the *Systema Naturae* by Carl von Linné drafted almost three centuries ago. Nevertheless, categorizing is a necessity, in virology as much as in botany or zoology. There have been a number of more or less useful proposals for the scientific classification of viruses. Very few, like the remarkable Baltimore Classification[6] have remained important in virology education. Although the species is widely accepted as being the most important category in biological classification, virologists have for a long time failed to provide an at least useful definition of a species in virology. While the biologic species concept is controversial, the situation in virology seems to be worse. Viruses used to be categorized based on their hosts, their morphology and diseases or symptoms that they were able to cause. Although sequencing of genetic information became possible and provides valuable insights in virus phylogeny, there is no simple threshold of genetic identity that justifies a species. Starting in the early 1970s, the International Committee on Taxonomy of Viruses (ICTV) has adopted the Linnaean principle of a hierarchical system of order, family, subfamily, genus and species. Finally, in 1991, the following species definition was adopted by the ICTV:

"A virus species is a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche."[7]

The term polythetic has raised criticism ever since it has been introduced in the virological species definition, partly because it was found to be obscure and not easily understood by a number of virologists and partly because a monothetical species definition, in which all members of a species have to share certain species defining properties (e.g. a genome nucleotide motif) was seen as superior. In 2013, after turning down several attempts of redefining a species, the ICTV has agreed on a new species definition:

"A species is a monophyletic group of viruses whose properties can be distinguished from those of other species by multiple criteria."[8]

This, in turn, has been excoriated by the advocates of the term polythetic as a key feature of species definition[9]. Pairwise Sequence Comparison (PASC) has led to the description of an astonishing number of new virus species based on their genetic divergence[10], without providing biological evidence that those species form entities that are distinguishable by criteria other than genetic divergence. This will likely be the impetus to an ongoing debate about the classification of viruses, especially since the taxonomical unit “species” remains the most important denomination in virus classification.

4.2 On Herpes

Capable of infecting hosts from mollusks to humans, herpesviruses are an extremely diverse group of viruses. Common to this group are a relatively large dsDNA genome ranging from 124–295 kb in length as well as some distinguished morphological features such as an icosahedral capsid of approximately 125 nm in diameter comprised of 161 capsomeres and one capsomeric structure that serves as a “door” for DNA packaging and release[11]. Capsid and core (linear dsDNA and core protein) form the nucleocapsid and are surrounded by a tegument of rather amorphous appearance. The outside of a virion, the envelope, is a bilipid layer of cellular origin which is spiked with viral glycoproteins. Above those phenotypic commonalities, herpesviruses share four important biological properties:

1. Herpesviruses employ an array of characteristic viral enzymes such as the DNA polymerase, the DNA packaging terminase, the thymidine kinase and the thymidylate synthetase.
2. The viral metabolism is concentrated in the nucleus of the cell where the capsid is assembled and from where it has to egress by means that are not completely understood.
3. Production of viral progeny (termed “lytic replication”) is associated with a cytopathic effect.
4. Herpesviruses are able to establish latent infections that cause lifelong persistence in the host and can lead to reactivation of lytic replication. Latent infection is defined as presence of the viral genome maintained by the cell without the formation of infectious progeny virus.

Herpesviruses are omnipresent in nature. Most vertebrae animals that were closely examined for the occurrence of herpesviruses have yielded at least one distinct species. So far, more than 200 hundred species have been described; actual numbers should exceed this by far. Typically, herpesviruses are adapted to infect a single host species or a few closely related species (e.g. equids).

With the availability of sequence information, the former family of herpesviruses has been reclassified as the order of *Herpesvirales* including three families, the *Herpesviridae* (infecting mammals and sauropsids), the *Alloherpesviridae* (infecting fish and amphibians) and the *Malacoherpesviridae* (infecting exclusively bivalves)[12].

Due to its relevance for human medicine as well as farm and companion animals, the family of *Herpesviridae* has received most of the attention in research. Historically, in the absence of DNA or amino acid sequence information, herpesviruses have been classified into three groups according to their biological properties[13]. This classification not only remained useful for the *Herpesviridae* but has received major support from information obtained from DNA sequences[14].

1. Alphaherpesvirinae

Characteristic for this subfamily is the capability to replicate relatively fast in a variety of hosts. Latent infections are typically, although not exclusively, established in sensory ganglia. The family *Alphaherpesvirinae* contains five genera: *Mardivirus*, *Iltovirus*, *Scutavirus*, *Simplexvirus* and *Varicellovirus*. While mardiviruses and iltoviruses infect birds, simplexviruses and varicelloviruses have a mammalian host range. The single virus species of the monotypic genus *Scutavirus* – *Chelonid herpesvirus 5* infects marine turtles. In addition, there are *Alphaherpesviruses* known to infect reptiles that do not belong to any of the genera mentioned above[15].

2. Betaherpesvirinae

This subfamily shares a restricted host range and a comparatively slow replication cycle. Infected cells typically show cytomegalia and latency can be established in multiple tissues, including kidney, secretory glands and lymphoid organs. Four genera, *Cytomegalovirus*, *Muromegalovirus*, *Probiscivirus* and *Roseolovirus* are recognized.

3. Gammaherpesvirinae

Viruses belonging to this subfamily show a tropism for lymphoid cells and establish latency in lymphoid tissues. Gammaherpesviruses are especially known for their ability to modulate cellular pathways and thus influence cellular metabolism and proliferation. Their host range is relatively restricted. To date, four genera are accepted: *Lymphocryptovirus, Macavirus, Percavirus, and Rhadinovirus*.

Recently, research on elephant herpesviruses has led to the proposal of a new subfamily in the Herpesvirales, tentatively named *Deltaherpesvirinae*[16]. This, however, has not been officially approved by the ICTV.

4.3 On Marek's Disease Virus (MDV)

Eponymous for the genus of *Mardivirus*, Marek's disease virus (MDV) is an alphaherpesvirus of chicken and should be addressed as Gallid Herpesvirus 2 (GaHV-2) based on ICTVs nomenclature. When Jozef Marek first described a neurological disorder as polyneuritis in chicken – now already more than hundred years ago[17] – nothing was known about the viral etiology of the disease, nor was the name Marek's disease (MD) adopted for this condition. More importantly, neither Marek nor other early descriptions of very similar conditions mentioned anything about a lymphoproliferative nature of the disease[18,19]. Thus, it became a cornerstone of MD research, when Pappenheimer and colleagues recognized that 10% from 60 cases they studied not only showed the typical neurological lesions that where described earlier, but additionally had lymphoid tumors in the ovaries and a number of other organs. As eminent pathological feature of the disease, they described lymphoid infiltrations of peripheral nerves which sometimes were accompanied by lymphoid tumors in other organs and tissues. On the grounds of their findings, they suggested to name the disease neurolymphomatosis gallinarum[20]. Following the description of MD as a tumor causing disease, confusion arose between MD and lymphoid leukosis, which was described about the same time and is macroscopically not easily distinguishable. With the expansion of the poultry industry in the middle of the last century, both MD and avian leukoses became more common and were, in the United States, eventually summarized as lymphomatosis of domestic chicken. This confusion entailed significant problems in interpreting experimental results and eventually led to the (re-)separation of "fowl paralysis" from avian leukoses. A discussion during the first meeting of the World Veterinary Poultry Association subsequently let to the suggestion of the name MD for "fowl paralysis" by Biggs in 1962[21].

Older publications reflect that MD was commonly believed to be infectious. Transmission experiments started in the early 1920's and continued for about forty years until unambiguous transmission was achieved[22]. During those studies, it became clear that 0.45 µm filtration as well as mechanical cell homogenization or ultrasonication of the inoculum resulted in a dramatic loss of infectivity. Direct cell transplantation, i.e. tumor transmission, was ruled out by studies that found that tumors in chicken, which had been experimentally infected by injection of whole blood or tumor suspension, were nevertheless of host cell origin[23,24]. Since no microorganism isolated from blood or tumors of infected animals was able to cause the disease, it was concluded that the causative agent of MD was a strictly cell-associated virus that lost infectivity when host cells died or were destroyed[24].

With the development of suitable cell culture techniques, it became possible to infect chicken kidney cells (CKC)[25] or duck embryo fibroblasts (DEF)[26] with blood or tumor cells from chickens that showed MD. Infected cells in cell culture showed typical signs of herpes infection (inclusion bodies and herpes-like particles)[27]. Clear evidence for the causal relationship between herpesvirus infection and development of MD was found when the herpesvirus of turkeys (HVT) as well as an attenuated MDV strain provided protection from MD if used as a vaccine[28,29].

Based on serological cross reaction but different biological properties, three herpesviruses capable of infecting chicken used to be classified as serotypes of MDV. With the availability of DNA sequence information, this classification is not accepted anymore. The one and only

MDV is the former MDV-1, MDV-2 is now to be addressed as Gallid Herpesvirus 3 (GaHV-3) and HVT (formerly MDV-3) as Meleagrid Herpesvirus 1 (MeHV-1)[30].

Until today, the life-cycle of MDV has not been entirely elucidated. What seems to be clear is that natural infection occurs via the inhalation of dust derived from infected feather follicle epithelial cells shed by an infected chicken. It is believed that alveolar macrophages or dendritic cells are the primary cells to be infected upon phagocytosis of infected particles. The current model of MDV infection suggests a rather hierarchical chain of events: B-cells become infected through contact with infected phagocytes and subsequently T-cells get infected by contact with B-cells. The CD-4+ T-cell not only serves as a target for lytic replication and latent infection but also seems to be the vehicle that is employed by the virus to reach its final destination, the feather follicle epithelium from where the virus is shed into the environment. While undoubtedly useful to understand the life cycle of the virus, biology may not strictly adhere to the exact sequence of events predicted by this model. Recent observations in our lab for example suggest that the presence of B-cells may not be essential for development of MD in chicken (Luca Bertzbach, pers. communication). While being dependent on living host cells in cell culture or experimental infections of chicken, the virus maintains infectivity in dust for months. The reason for this is enigmatic to MDV research. There is, however, clear evidence for cell-free infectious virus from feather follicles[31], which very likely contributes to the prolonged infectivity of feather derived dust.

Latently infected cells can be found in infected animals from day 7 post infection (p.i.)[32]. Although CD4+ T-cells appear to be the primary target of latent infection, latently infected B-cells as well as other T-cell populations have been reported[33]. Throughout herpesvirus research, the chain of events that eventually leads to the establishment of latent infection is controversially discussed[34,35]. In MD, discrimination between latently infected T-cells and those that are transformed is virtually impossible. Further, it is unknown if there is a true coexistence of latent infection and transformation as the “endpoint” of MDV infection, or if over time latent infection necessarily leads to transformation of infected cells[36]. Latency seems to be regulated at least partially by changes of expression of ICP4, an immediate early protein and major regulator of transcription[37]. In addition, it is regulated by transcripts from the 1.8 kb region present in the internal repeat long (IRL) antisense to the ORF encoding pp38 (a phosphoprotein indicative of lytic replication) and transcripts originating in the MDV *Eco*RI Q Fragment (meq) region of the genome. Unlike other alphaherpesviruses, the members of the *Mardivirus* genus are maintained in latently infected cells through integration of their genomes into host chromosomes. Integration seems to be random but dependent on telomeric repeats at the end of the viral genome[38].

Undoubtedly, the most important clinical feature of MD are lymphomas formed by transformed T-cells. Prerequisite to transformation is a productive lytic replication of MDV which leads to latent infection of a large population of T-cells. One possibility is that from these latently infected cells only a single, or a few cells undergo transformation resulting in the development of solid lymphomas. Alternatively, it seems possible that initially a larger number of T-cells are transformed and selected post transformation resulting in oligo- or even monoclonal origin of lymphomas[39]. Much work has been done to identify the contributors to transformation. This work has led to the conclusion that the *meq* protein, a basic leucine zipper, plays the central role in MDV induced transformation and tumorigenesis. There is much evidence for several key functions of *meq* such as

transcriptional regulation, transactivation and chromatin remodeling. A detailed description of all the implications of malignant transformation would go beyond the scope of this thesis. The interested reader will find an authoritative review of the MDV lifecycle including events related to transformation in the literature[36].

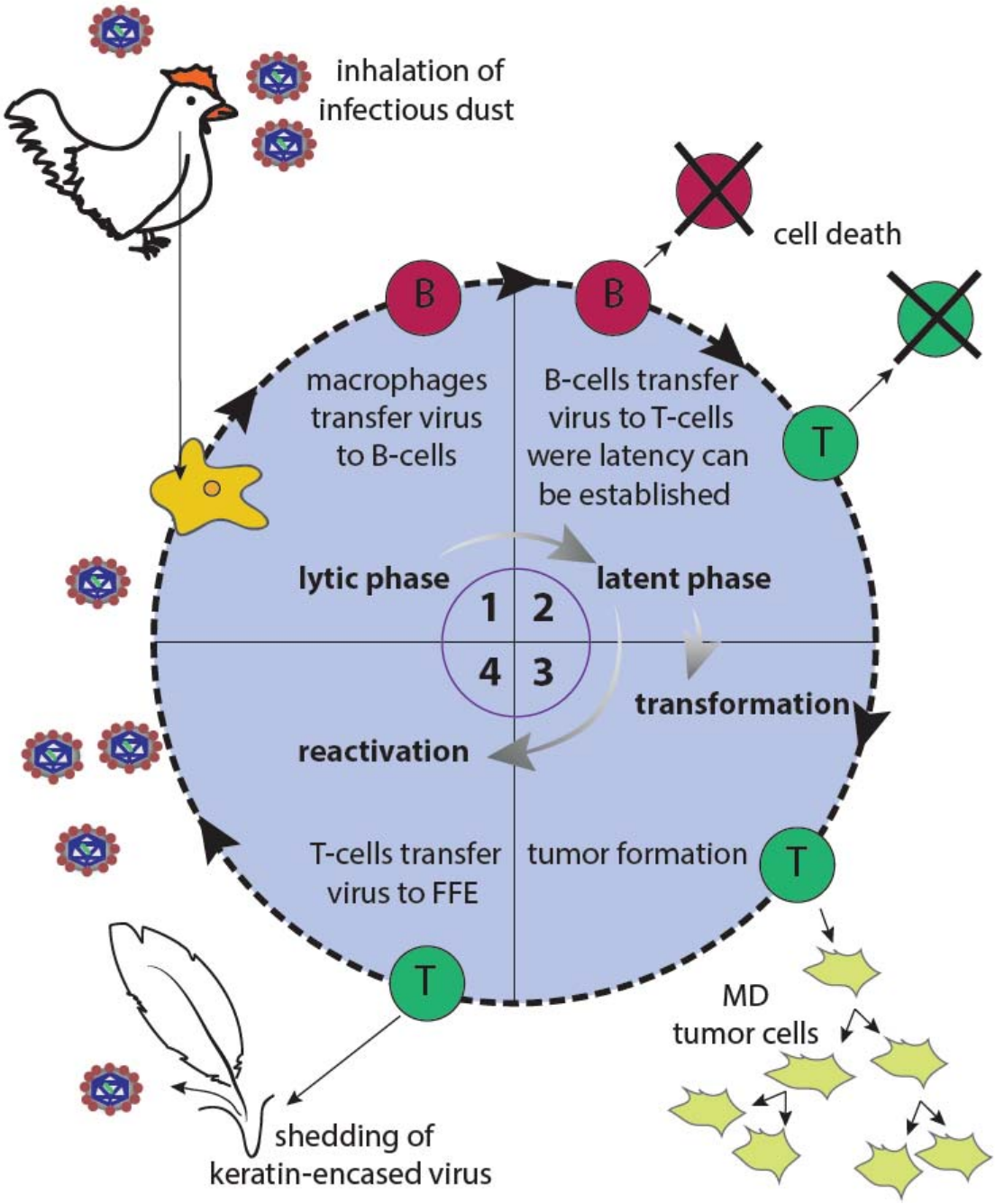

Figure 1 Schematic overview of the MDV life cycle according to the "Cornell Model" of MDV infection. Chicken get infected by inhaling dust or dander that contains infectious MDV. Alveolar macrophages take up virus particles and transfer virus to B-cells. Lytically infected B-cells transfer virus to T-cells, were, in addition to lytic infection, latent infection can be established. Furthermore, T-cells can be transformed which eventually leads to tumor formation. Prerequisite for shedding of infectious virus is the infection of the feather follicle epithelium (FFE) from where virus can be shed inside of keratinized and desquamated cells. This image was kindly provided by Kathrin Eschke, FU Berlin.

Related to the discovery of the herpesviral etiology of MD, the possibility of vaccination with attenuated MDV strains (HPRS-16) and heterologous viruses (HVT) was brought into perspective[28,29]. Following this, vaccination against MD became one of the most successful vaccination campaigns in the history of veterinary medicine, reducing disease incidence by more than 99%[32]. An important feature of vaccines against herpesviruses is that they are typically imperfect in terms of eliminating the pathogenic virus and control disease outbreak by suppressing viral replication rather than averting it[36]. Vaccination against MDV is generally permissive to infection with and shedding of pathogenic strains. This in turn means that field strains, while still circulating, experience a tremendous selection pressure and are selected for maximum fitness in the vaccinated host. In line with these thoughts, we observed a remarkable increase in the virulence of MDV field strains over the past decades[36]. Not even 10 years after the introduction of the first vaccines, it became necessary to change vaccine strain and strategy in response to an alarming occurrence of vaccination failures. A bivalent vaccine comprised of HVT and SB-1 (a strain of GaHV-3) both of which protected against MD outbreaks showed synergistic effects when combined and became the new standard of chicken vaccination[40]. In the beginning of the 1990s, a similar situation occurred and again it was necessary to adjust vaccination strategies[40]. The introduction of CVI-988 (an attenuated strain of MDV) resolved issues with vaccine breakthroughs in the United States and is currently the gold standard of vaccination[41]. However, there is the constant threat of evolution towards higher virulence and the debate is ongoing if CVI-988 will still be able to protect chicken in the near future[42,43]. With the ability of virus strains to break through vaccine protection came increased mortality in unvaccinated chicken and appearance of so far atypical symptoms such as early mortality without lymphoma formation and acute encephalitis[22]. With the emergence of even more virulent strains, it became practical to classify virus strains based on their virulence. The following schema proposed by Witter[44] is widely accepted:

virulent MDV (vMDV) strains – response in HVT vaccinated chicken does not differ from the JM/102W prototype strain

very virulent MDV (vvMDV) strains – induce more lesions in HVT vaccinated chicken than JM/102W but is not different from Md5 in HVT/SB-1 vaccinated chicken

very virulent + MDV (vv+MDV) strains – induce more lesions than Md5 in HVT/SB-1 vaccinated chicken

Apart from vaccination, the genetic makeup of chickens is important to prevent severe cases of MD[45]. Long before MDV was identified as the causative agent of MD, scientists at the Cornell University have attempted to breed chicken with good economical traits and improved resistance to what was known as the “avian leukoses complex” through mass selection in an infected environment[46]. This strategy continued to be successful in generating genetically resistant chicken lines without knowing the genetic basis of resistance[47,48]. Even today, the exact basis for genetic resistance is not completely understood. There is, however, compelling evidence for an important role of the major histocompatibility complex (MHC) B haplotype. It seems clear that the B21 haplotype confers resistance to MD development while other B haplotypes like B19 render chicken highly susceptible to MD. Despite the importance of the MHC haplotypes, other genes are clearly important, as well. Several non-MHC genes are reported to influence genetic resistance to MD, an informative

collection of details can be found in literature[49,50]. As far as the evolutionary trend towards increased virulence is concerned, a selection pressure arising from genetic changes in available hosts cannot be excluded.

Genetic structure of MDV

A typical member of the family *Alphaherpesvirinae*, MDV possesses a linear dsDNA genome of roughly 180 kb. It is classified as an E type genome encompassing two unique regions, the unique long (U_L) and unique short (U_S) which are flanked by inverted repeats, the terminal repeat long (TRL), the internal repeat long (IRL), the terminal repeat short (TRS) and the internal repeat short (IRS).

Figure 2 Genetic structure of MDV. The two unique regions, unique long (UL) and unique short (US) are each flanked by inverted repeats. The terminal repeat long (TRL) and the internal repeat long (IRL) are flanking the UL region, the internal repeat short (IRS) and the terminal repeat short (TRS) are flanking the US region.

4.4 On Evolution

Charles Darwin and his revolutionary concept of evolution through natural selection[51] has, like very few others, influenced our understanding of biology and the development of life on our planet. Darwin, maybe together with Jean Baptiste Lamarck, who first acknowledged the environment as driving force of evolution, have to be credited for leading biology out of the dark age of religious belief in creation of an inalterable life by some obscure higher intelligence and into a prosperous time of reasoning and understanding of evolutionary factors that shape the life on this planet and possibly this universe.

Part of the beauty of Darwin's concept of evolution through natural selection is its simplicity and straightforward applicability. Evolution is driven by natural selection, so the prerequisite for evolution must be genetic variation. If there is no genetic variation and all organisms are not only equally good but just the same, there is nothing to select from. Genetic variation arises from two sources. The first is mutation and the second is recombination. Mutation, however, seems to be even more fundamental than recombination. What would recombination be good for without an available diversity produced by mutation? If the progenitors of life, presumably microorganisms in the primordial soup, would have been able to replicate their genetic information without ever making any mistake, it is quite clear that we would still be just that: some sort of microorganism in some sort of soup. Fortunately, they made mistakes and so life arose from the soup and here we are to understand how it all happened.

What happens is, that out of the pool of individuals comprising a population, not all are equally well suited to the respective environment; there are differences in fitness in every

given situation. Let me use a well-known example to illustrate the action of natural selection. About fifty million years ago, ancestors of current horses used to be small creatures hiding in ancient forests in which they were likely very fit. Being small enabled them to move efficiently through the undergrowth and let them hide in all kinds of places when threatened by predators. Caused by a change in climate, in some parts of the world the forest was declining, and large grass savannas developed where once woods had been. The ancient horse found itself in an environment to which it was not well adapted: no place to hide, different food to eat and an unfamiliar firm ground to move on. It was a pity for the horse and many of them were taken by predators, but there were some who, due to genetic variation, were a little larger than average. Those had the slight advantage of longer legs that enabled them to move a little faster, and so they escaped more frequently when predators took their toll. This meant that larger and faster horses lived longer and thus created more offspring, passing on the genetic information for being larger and faster than average. Among the offspring, again, due to genetic variation, some were slightly larger than their siblings; providing those with the same advantage that their parents had one generation before. Over time, the more efficient reproduction of larger and faster horses led to a transformation in the phenotype of the entire species. Extraordinary in the evolution of horses is that the same features remained to be an advantage for a very long time, which makes the horse an ideal example for a transforming selection process. Before we switch back to viruses, let me explain one of the fundamental molecular reasons that leads to genetic variation.

4.5 On Nucleic Acid Replication

Genetic information is stored as the sequence of four organic bases in a nucleic acid. Today, on our planet, deoxyribonucleic acid (DNA) is the substance of choice for all organisms except for some viruses that store their genetic information in the form of ribonucleic acid (RNA), a remarkable exception to a general rule that will be of interest in this and the following paragraph. Whenever replication is to be achieved, the entire genetic information must be copied and made available to the progeny. For this, enzymes known as either DNA or RNA polymerases are responsible. These enzymes use an existing single stranded nucleic acid polymer as template for the polymerization of a reverse copy, from monomers known as nucleotides. Nucleic acid polymerization is an error prone process, every now and then a monomer containing the wrong base is incorporated. This is called a mutation. The following section will provide an overview of viral replicative nucleic acid polymerases and their function with special respect to the fidelity of DNA/RNA replication.

The primary enzymatic function of a nucleic acid polymerase is to catalyze the addition of a nucleotidyl moiety, complementary to the template, from an incoming nucleoside triphosphate (NTP) to the 3’-hydroxyl (3’-OH) end of the nascent daughter strand. The catalytic reaction comprises four stages: (1) binding of template-primer and NTP, (2) incorporation of the correct nucleoside phosphate into the nascent strand via Watson-Crick base pairing, (3) release of pyrophosphate, and (4) translocation of the replication complex along the template. Indispensable for the catalytic reaction is the presence of divalent cations, Mg^{2+} or Mn^{2+}, in the reaction. A long standing hypothesis, known as two-metal-ion catalysis[52], requires two divalent cations to be coordinated by conserved Asp residues. The metal ion in position A is approaching the 3’-OH on the primer terminus lowering the pKa of

the 3'-OH with leads to a nucleophilic attack of the electron rich 3'-O- on the α-phosphate of an incoming nucleotide. Meanwhile, the metal ion in position B binds the phosphates of the incoming nucleotide, stabilizing its position for nucleophilic attack and facilitating the formation of a pyrophosphate leaving group after the formation of a new phosphodiester bound. The reaction ends with the release of the pyrophosphate and the translocation of the complex by one nucleotide. Hence, polymerases are not only catalytic enzymes, but also molecular motors that use the energy released from nucleotide transfer to move along the template, exhibiting a force 5-6 times higher than what is known from myosin or kinesin molecular motors[53]. Polymerization then proceeds until a termination point is reached (end of the template or termination signal).

Fairly recently, this model has been challenged by the proposal of a three-metal-ion catalysis[54]. The authors of this study were able to demonstrate the necessity of a third metal ion for product formation through *in crystallo* metal ion titration. They showed the occurrence of a third metal ion that is coordinated from the nucleic acid polymer and the pyrophosphate leaving group. This "C" position has a lower binding affinity than A and B, its saturation thereby defines the minimum metal ion concentration of the reaction. Moreover, the authors propose that binding of the third metal ion is the rate limiting step of the reaction and thus that nucleic acid polymerization is rate limited by the chemistry of the reaction rather than by conformational changes of the polymerization complex.

General structure of nucleic acid polymerases

The 3-dimensional structure of all known nucleic acid polymerases can be described in analogy to the description of the Klenow fragment of *E. coli* DNA polymerase I as palm, thumb and fingers of a "cupped" right hand[55]. Among these domains, the palm is the most conserved between polymerases. Both the A and B motif for coordinating the metal ions are located in the palm, the chain elongation takes place here. The finger domain is contacting the incoming nucleotide and can vary considerably among polymerases, meeting requirements for different templates (ds- or ss-nucleic acid) and ribonucleoside triphosphate (rNTP) or deoxyribonucleoside triphosphate (dNTP) substrates. Finger domain mutations are known to increase substrate specificity, leading to resistance to nucleotide analogues in herpesviruses[56,57]. The finger domain is involved in the selection of the right nucleotide and undergoes considerable conformational change orienting the incoming NTP in the catalytic site. Impacts on the overall replication fidelity will be examined in this report. The thumb domain interacts with the template upstream of the active site and together with the inner surface of the finger forms the template binding channel of the complex. In family A and B polymerases, the thumb seems to influence the switch between editing and polymerization and thus influences the fidelity of replication[58,59].

Depending on the extent of their functions, viral polymerases can have several additional domains including methyltransferases, RNAse H domains and, for this work most importantly 3'→5' exonuclease domains.

Replication fidelity

Overall, data regarding the replication fidelity of organisms varies dramatically. Some of the variation may be due to significant biases in methods used to assay fidelity. Another important fact may be that in vitro measurements of polymerase fidelity can differ

dramatically from mutation rates observed in vivo. This makes it quite obvious that other members of the replication complex as well as cellular mechanisms contribute to overall replication fidelity in viruses. For this thesis, the fidelity conferred by the polymerase is crucial.

Nucleic acid polymerases are generally capable of two major types of errors: base substitution and single base insertion or deletion errors (indel mutations). The majority of errors are base substitutions[60]. However, single base deletion errors can occur nearly as frequently as base substitution errors, single base insertion errors are comparatively rare[61]. Nonetheless, nucleic acid replication is typically remarkably accurate. There is, however, a considerable difference between RNA viruses and organisms that use DNA as storage for genetic information. While some RNA viruses introduce more than one mutation per genome per replication, DNA organisms generally replicate their genomes with less than 0.01 mutations per replication cycle[62]. How replication fidelity is achieved and why there is such a difference between organisms, is one of the most intriguing questions of enzyme research. The following two factors contribute to replication fidelity.

1. *Nucleotide selectivity*

Finding the nucleotide that forms the appropriate Watson-Crick base pair is imperative for reliable genome replication. The correct hydrogen bonding presents a major barrier for base substitutions. The thermodynamic difference between correct versus incorrect base pairing should account for error rates of about 1 in 100[63]. Since even some of the worst RNA polymerases do better than that, there must be another reason for nucleotide selectivity. One factor may be the exclusion of water from the catalytic site. Removal of hydrogen bonds between NTP and water reduces the entropy of the system, the removal of water would thereby increase the enthalpy differences between correct and incorrect bonding and contribute to replication fidelity[64]. Furthermore, there is evidence for steric hindrances to incorrect nucleotide incorporation as incorrect base pairing is predicted to cause steric clashes and effect subsequent conformational changes[65].

2. *Exonucleolytic proofreading*

Various replicative DNA polymerases, not limited to viruses, contain a conserved 3'→5' exonuclease domain that contributes to replication fidelity through excision of erroneously incorporated bases[66]. This process is known as proofreading and lowers replication errors by 10- to 100-fold. This is undoubtedly evidenced by exonuclease deficient mutants of proofreading polymerases that show a respective loss of fidelity[67,68]. A reasonable balance between exonucleolysis and polymerization is essential for efficient proofreading. The literature[65,69] favors the following explanation: if correct base pairs are being formed, polymerization is a smooth and rapid process. The celerity of this process leaves little time for transition of the newly incorporated base to the exonuclease site where it would be excised. In contrast, misinsertion generates a mismatched primer terminus that is more difficult to extend, slowing down polymerization drastically and thus allowing the primer terminus to fray and move to the exonuclease site where the wrongly incorporated base is excised. Proofreading is able to correct indel mutations by excising misaligned intermediates near the primer terminus. Proofreading is a unique feature of DNA dependent DNA

polymerases and not observed in RNA or RT polymerases. There is, however, evidence for exonucleolytic proofreading by a separate 3'→5' exonuclease in coronaviruses[70].

Viral nucleic acid polymerases

Reflecting their unique diversity of nucleic acid usage, viruses encode for many different replicative nucleic acid polymerases. The major subgroups are:

RNA-dependent RNA Polymerases (RdRp)

RdRps are essential enzymes encoded in the genetic information of RNA viruses that replicate without a DNA intermediate. RdRps were discovered in the early 1960s based on the actinomycin D resistance of poliovirus replication. Replication fidelity of RdRps is relatively low. Estimates for several viruses, including poliovirus and vesicular stomatitis virus (VSV), show error frequencies in the range of 1 per 10,000 bp [71].

RNA-dependent DNA Polymerases (Reverse Transcriptase, RT)

Independently described by David Baltimore[72] and Howard Temin[73] in 1970, the existence of RT broke the central dogma of molecular biology by identification of an enzyme that could use a RNA template to polymerize DNA. RTs are encoded by retroviruses to reverse transcribe their genome enabling integration and subsequent propagation by the host cell machinery. Exceptionally, DNA viruses like the *Hepadnaviridae* encode a unique polyprotein that comprises functional domains for RT, RNAse H and DNA-dependent DNA polymerization[74]. More recently, *Metaviridae*, *Pseudoviridae* and *Caulimoviridae* were shown to utilize RTs for replication[75]. In the absence of proofreading, RTs are believed to display high intrinsic error rates. *In vitro* measurements of the *Human immunodeficiency virus* (HIV) RT have shown error rates as high as 1 per 1,700 polymerized nucleotides[76], the biological relevance of this measurement has been challenged by later estimates of an overall mutation rate of only 1 per 100,000 polymerized nucleotides[77,78]. In contrast to this, more recent findings suggest that the error rate of HIV is as high as 4×10^{-3} mutations per copied nucleotide, but only 2% of the errors are inflicted by RT, the rest being the result of host cell RNA editing enzymes[79]. The ongoing discussion about HIV replication fidelity is an excellent example for the very problematic assessment of the true replication fidelity in viruses. Another evolutionary important feature of RT is its ability to switch from one RNA to another during minus-strand DNA synthesis giving rise to chimeric DNA recombined from two different genomic RNAs.

DNA dependent DNA polymerases (DdDp)

DNA polymerases are categorized based on their sequence homology and similarity in crystal structure. There are 7 families known today[80].

Family	Role	Organisms	Examples
A	Replication + Repair	Eukaryotes, Prokaryotes, Viruses	Pol I, T7 DNA polymerase, Pol γ
B	Replication + Repair	Eukaryotes, Prokaryotes, Viruses	Pol II, Pol B, Pol ζ, Pol α, polymerases of herpesviruses
C	Replication	Prokaryotes	Pol III
D	Replication	Archaea	Pyrococcus furiosus DNA polymerase II
X	Replication + Repair	Eukaryotes	Pol β, Pol σ, Pol λ, Pol μ
Y	Replication + Repair	Eukaryotes, Prokaryotes	Pol ι, Pol κ, Pol η, Pol IV, Pol V
RT	Replication	Viruses, Eukaryotes	Polymerases of retroviruses, Polymerase of HBV, Telomerase

Table 1 Overview of different types of DNA Polymerases and their classification.

All known replicative viral DdDps belong to families A and B[81]. Although structurally different, viral family A and B polymerases share a conserved 3'→5' exonuclease domain composed of the three motifs Exo I, Exo II, and Exo III. This common feature qualifies viral DdDps for proofreading leading to relatively low replication error rates which are estimated to be between 10^{-7} and 10^{-8} [(60)]. In organisms with larger genomes, additional DNA repair pathways employing several DNA polymerases lower mutation rates further, achieving overall rates as low as 10^{-9} to 10^{-11} per base per replication cycle[82]. However, African Swine Fever Virus (ASFV) presents an exception to this rule as it displays unusually high mutation rates[83]. The characterization of an error prone viral repair DNA polymerase similar to mammalian Pol β has resulted in the hypothesis that error prone DNA repair may lead to an increased mutation rate in DNA viruses[84].

The polymerases of herpesviruses belong to the B family of polymerases, sometimes referred to as α-like polymerases due to their similarity to mammalian Pol α[85]. Typical for this family of polymerases is the intrinsic proofreading activity and the association with a processivity factor for highly accurate and processive polymerization. In the case of *Alphaherpesvirinae*, the processivity factor is encoded by the U_L42 gene. Besides the processivity factor, other proteins usually associate with family B polymerases to form the replication complex, sometimes called replisome[86]. In alphaherpesviruses these are:

U_L9 – origin binding protein, binds to the CGTTCGCACTT motif in the origin of replication, unwinds DNA and initiates replication

ICP8 (U_L29) – ssDNA binding protein, binds to U_L9/DNA and enhances helicase activity

U_L5 and U_L52 – form the helicase-primase complex that is recruited by U_L9/ICP8

U_L8 – binds at the replication fork and recruits U_L30/UL42

U_L30 – DNA polymerase

U_L42 – polymerase accessory subunit, processivity factor

The replication of viral DNA starts out as a U_L9 dependent theta replication and becomes a U_L9 independent rolling-circle amplification. The mechanisms by which the switch is achieved remain elusive. For one of the sequencing strategies employed in this project, the fact that circular viral DNA is produced at some point during replication will be of great importance. A more detailed description of the herpesviral DNA replication can be found in a recent review[85].

Recombination

Although not central to the objective of this thesis, the role of recombination as major driver of genetic diversity should not be discarded. Viruses have powerful ways to approach recombination. Be it the reassortment of segmented genomes[87], the template switch conducted by RT[75] or the rapid recombination observed in herpes[88]- and poxviruses[89], recombining genetic elements is a key factor in viral evolution[90]. It must be appreciated that recombination is the mechanism that unlinks beneficial and deleterious mutations and enables accumulation of beneficial mutations over time. Especially with a background of high mutation rates, efficient recombination is essential for viral evolution.

4.6 On Quasispecies

As mentioned above, the term species is controversial in virology. Yet there is another biological entity of interest to this thesis:

“A quasispecies is a large group or cloud of related genotypes that exist in an environment of high mutation rate, where a large fraction of offspring are expected to contain one or more mutations relative to the parent.” *(Wikipedia 2017)*

Originally invented by Manfred Eigen to describe the molecular evolution in an RNA world at the beginning of life[92], the term quasispecies has been adopted by Esteban Domingo and colleagues to describe the unprecedented genetic diversity observed in some RNA virus populations[93].

Quasispecies theory predicts that a population of viruses that cover a large sequence space but is comprised of individuals with a comparatively low fitness will outcompete a population with high individual fitness covering a small sequence space. Here, the “mean fitness” of the population outweighs the fitness of individuals, the quasispecies *per se* is the target of natural selection. This is sometimes referred to as the “survival of the flattest”[91].

I have described at length how mutations occur and that some organisms, in particular RNA viruses, exhibit comparatively high mutation rates. Here I will explain the biologic relevance of this observation employing the quasispecies concept introduced above.

Upon infection, a virus particle enters a susceptible cell to initiate its own replication. It brings along a single copy of its genome for replication. In the face of a high mutation rate, the resulting progeny is expected to have not the exact same sequence as the parent but maybe one or two mutations. Cycles of replication and infection of new cells will amplify this genetic divergence, leading to a viral population that does not share the exact same genetic makeup but is rather diverse. Error prone replication gives rise to a cloud of highly related but not

identical genomes that originated in a single (or few) initial sequence a few generations before. It is important to note that a very fit mutant which outcompetes others will not become absolutely predominant because high mutation rate will yield an offspring that again has acquired mutations resulting in different fitness levels. On the whole, the genetic disintegration has significant advantages for viral infection. Due to its high diversity, the viral population is preadapted to all kinds of possible selection pressures. Be it the hosts immune response or some antiviral drug, wherever selection pressure strikes, the virus already has the answer, likely there will be phenotypes in the cloud that can cope with the situation. Quasispecies also cooperate on a population level[94], and some variants may be favored in specific cell types or tissues but will be unable to execute their advantage in absence of a diverse quasispecies. This was conclusively demonstrated by experiments with polio where a neuro-virulent variant was unable to cause disease in absence of a natural distribution of other variants[95]. In the light of these observations, it seems logical that increasing replication fidelity in quasispecies viruses should attenuate their phenotype. There is evidence for this from work with poliovirus, where polymerase mutants that exhibited a higher fidelity than WT resulted in severely attenuated phenotypes[95,96].

Is low fidelity replication the sole key to evolutionary success? Would a higher genetic variability always be better? Of course not. Low fidelity replication that leads to quasispecies formation can be advantageous for organisms with a comparatively small genome, a large population, a high reproduction and efficient recombination. But even under these circumstances there is a barrier, an error threshold beyond which the genetic consensus information is lost and the population extinct. Even low-fidelity replicators must not make too many mistakes. In fact, it is a hallmark of quasispecies theory, that populations are extremely sensitive to a further increase of mutation rates. Viruses forming quasispecies are evolutionary selected to replicate at the brink of extinction, a less than ten-fold increase in mutation frequency causes the collapse of the population[97]. On the contrary, large DNA based organisms like bacteria and yeasts are extremely robust to an increase of mutation frequency and survive beyond 100-fold or even 1000-fold increases[98,99].

In summary, I would like to point out five important implications of the quasispecies concept.

1) High genetic variation is a strong selection advantage if it does not go beyond a certain “error threshold”

2) Highly diverse and mutational robust populations will, even at the cost of lower individual fitness, outcompete phenotypes of high individual fitness, “survival of the flattest”

3) RNA viruses replicate close to their error threshold, both increased and decreased genetic variation will attenuate viral populations

4) The target of natural selection is a genetically highly diverse population rather than an individual phenotype

5) Quasispecies cooperate on a population level to achieve highest “mean fitness”

There is evidence for coherence with these points in a number of RNA and DNA RT viruses, including poliovirus[95], HIV[100], hepatitis B virus (HBV)[74], hepatitis C virus (HCV)[101] and Influenza viruses[102].

Information on quasispecies behavior in DNA viruses is scarce, there is, however, a debate about the genetic variation seen in some large dsDNA viruses including herpesviruses like the human cytomegalovirus (HCMV)[103].

4.7 Introduction to the Project

The dramatic increase in the virulence of MDV is truly exceptional in the history of virology and vaccination. In the light of ever increasing virulence, it has been argued that this somewhat accelerated evolution of the pathogen may be driven by imperfect vaccination that imposes a fierce selection pressure on the virus, permitting only the fittest phenotypes to replicate and be transmitted. And indeed, there is experimental evidence for the increased selection of particularly virulent strains that efficiently circulate in vaccinated chicken flocks[104]. It is not altogether unrealistic that selecting for resistance against vaccination is an important driver of viral evolution. But how does immunoescape happen? Could high genetic variation in an infected host play a role in the emergence of ever more virulent virus strains? As mentioned above, there is not much known about the genetic diversity of herpesviruses inside a host. In the case of HCMV, where some data suggests that genetic diversity is as high as in quasispecies RNA viruses, the debate is ongoing if coinfection with several strains is the reason for this diversity rather than diversity achieved through high mutation rates[105].

The primary hypothesis is that herpesviruses show little genetic variation during replication *in vitro* and *in vivo,* and that vaccination decreases variability further but can result in the emergence of progeny viruses with altered genotypes and increased virulence.

The three specific goals of this project were:

1) Establish a system that enables high-throughput sequencing of the strictly cell-associated MDV to assess genome variability

2) Determine virus genome variability as a function of viral DNA polymerase fidelity using viruses with altered replication fidelity in cultured cells

3) Examine pathogenicity of polymerase mutants in the natural host and determine sequence space in different compartments of the host in vivo

4.7.1 Specific goal 1 - *Establish a system that enables high-throughput sequencing of the strictly cell-associated MDV to assess genome variability.*

The avidly cell-associated nature of MDV provides a challenge to any direct sequencing approach. Avoiding PCR and Sanger sequencing, which would narrow our view to specific loci and, more importantly, could disguise sequence variability and introduce bias, was one

of the major challenges of this project. When this project started, little was known about sequencing techniques for MDV and cell-associated viruses in general. Nevertheless, there are reports about the harvest of nucleocapsids[106] and more feasible, about the extraction of DNA from capsids directly from infected cells and without prior purification[107]. The protocol of Volkening and Spatz was slightly modified and performed as stated in the materials and methods section. The method makes use of the extraordinary stability of herpesvirus capsids. Once packaged, viral DNA is extremely well protected against enzymatic digestion. The principle of this method is to remove all cellular and otherwise not encapsidated DNA by digestion with an unspecific endo-exonuclease from *Staphylococcus aureus* (micrococcal nuclease) and subsequently release viral DNA from nucleocapsids through an extensive proteinase K treatment.

Besides the low DNA yield and sometimes poor quality in terms of purity and integrity of viral sequences, it seemed unsatisfactory to us to only get access to DNA that was previously selected for packaging. Since likely only the smaller fraction of viral DNA gets packaged into the capsid, the loss of non-encapsidated sequences is unfavorable end eliminates the possibility for potentially interesting comparisons between packaged and total viral DNA in a cell. Finally, the method did not seem useful for the extraction of viral DNA from infected animal tissues.

To enable enrichment all viral sequences from infected cell and tissue lysates, we employed a tiling array designed to capture the sequence of MDV strain RB-1B. Our tiling array works through complementary base pairing between RNA baits and the target DNA sequence. The RNA baits are 80 bp in size and contain a biotin tag that enables purification from solution using the interaction with streptavidin coated paramagnetic beads (Dynabeads MyOne, Invitrogen). The strong interaction between biotin and streptavidin enables vigorous washing and purification of specifically hybridized DNA with minimal carryover of non-target sequences. The method is reported to be robust to single nucleotide polymorphisms (SNP) and used for SNP detection in evolution biology[108,109]. The manufacturer reports unrestricted capture efficiency in targets with 95% bp identity (Alison M. Devault, personal communication). We have observed good capture efficiency using the above baitset to capture the sequence of GaHV-3 strain SB-1 that shares only about 80% sequence homology (unpublished data).

4.7.2 Specific goal 2 - *Determine virus genome variability as a function of viral DNA polymerase fidelity using viruses with altered replication fidelity in cultured cells.*

Given the operating mode of herpes DNA polymerases, a reduction in replication fidelity should be achievable if the activity of the proof reading exonuclease could be reduced. In MDV, as in other alphaherpesviruses, the ORF U_L30 codes for the DNA polymerase, a protein of approximately 138 kD. Although the crystal structure of the MDV polymerase is unknown, a structure similar to the structure of other alphaherpesvirus polymerases seems likely based on amino acid sequence comparison[110,111].

As mentioned earlier, all three exonuclease domains are known to be conserved among viral proofreading polymerases. A mutational analysis of the 3'→5' exonuclease domain of HSV-1 was conducted in the 1990s[112]. Kühn and Knopf describe mutations in the conserved Exo

domains, that reduced exonuclease activity of HSV Pol to a variable degree, leaving polymerase activity unaffected or slightly reduced. Certain mutations in the Exo I domain completely abolish exonuclease activity while causing no negative impact on polymerase processivity. Highly conserved residues in Exo I seem to coordinate an Mg^{2+} ion essential for nuclease activity[113], so that mutations in this region are lethal to exonuclease function. Mutations targeting Exo II and Exo III regions seem to leave a residual exonuclease function but in the same time interfere with the polymerase activity to a variable degree.

	Domain	Polymerase %	Exonuclease %
WT POL		100	100
D368A	Exo I	103,3	0
E370A	Exo I	111,2	0
D471A	Exo II	31,5	20,1
Y538S	Exo III	1,3	0
Y557S	Exo III	72,5	42,5
Y577F	Exo III	70,6	16,8
D581A	Exo III	81,6	6

Table 2 Point mutations in the Pol gene (U_L30) of HSV-1 and their influence on enzymatic activities. Data according to Kühn et al. 1996[112].

Based on this report and on its confirmation by crystallographic analysis[114], we found conserved regions within the amino acid sequence of MDV Pol that share the conserved regions I-VII of family B polymerases as well as all three exonuclease domains. A schematic view of a herpes DNA polymerase with the position of conserved amino acids in MDV and HSV-1 is provided in figure 3. The exact position of conserved residues from Table 2 can be found in figure 4.

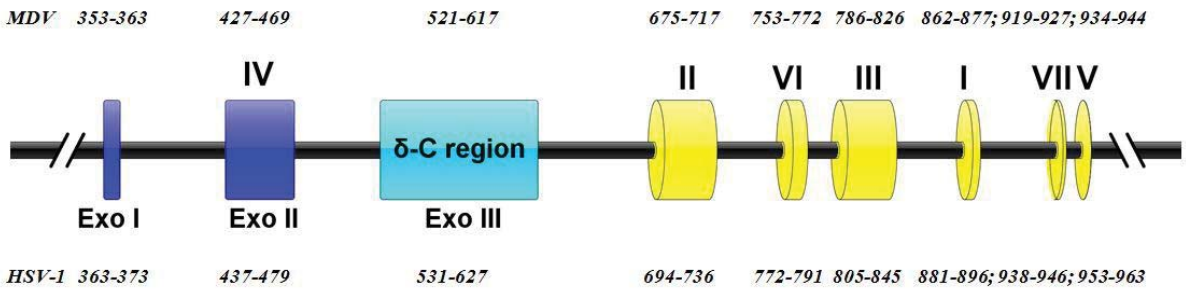

Figure 3 Schematic representation of a herpesvirus DNA polymerase with the conserved regions I-VII as well as the δ-C region and exonuclease domains I-III. The exact amino acid position for conserved regions in MDV and HSV-1 are provided as numbers above and below the illustration.

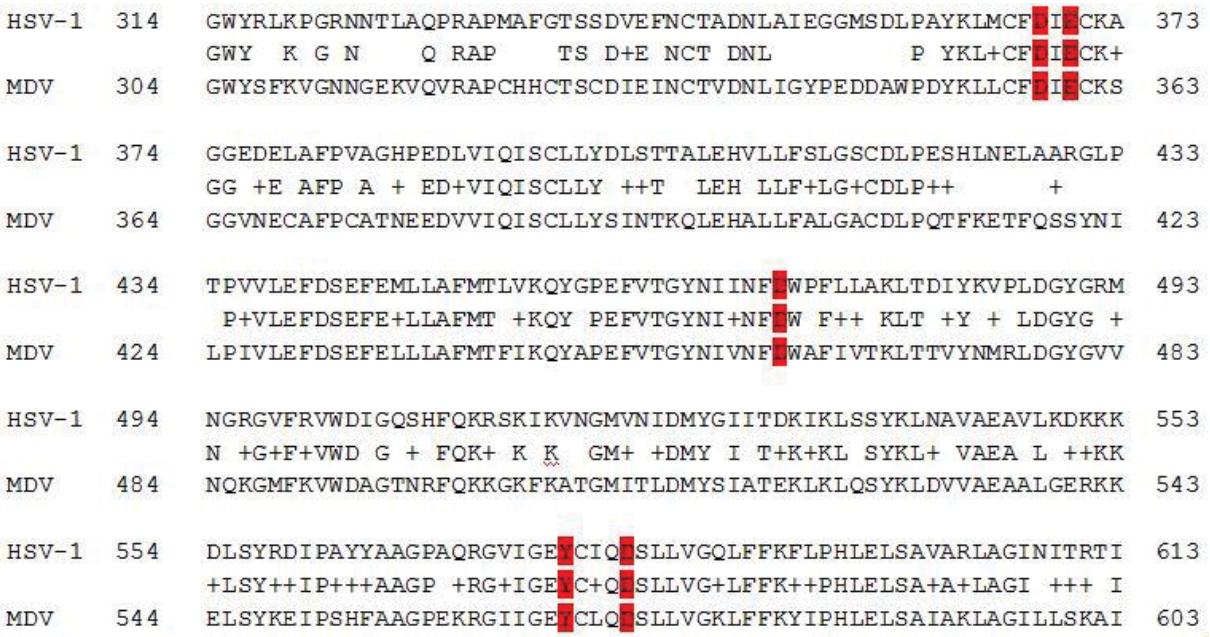

Figure 4 Comparison of the amino acid composition of viral DNA Polymerases (HSV-1 and MDV). Conserved amino acids in Exo I-III targeted by Kühn et al. 1996 are highlighted in red.

It added confidence to our approach that several other studies that targeted the 3'→5' exonuclease activity of family B DNA polymerases in organisms like the bacteriophage T4, *Escherichia coli* or *Saccharomyces cervisiae* identified homologous mutations that caused a loss of exonuclease activity[115-117].

Following the identification of conserved residues within each of the three exonuclease domains, primers were designed to introduce the mutations D358A, D358A/E360A, D461A, Y547S, Y567F and D571A via red mediated mutagenesis[118]. To minimize the chance for reversion, the maximum possible change in nucleotides per codon was conducted. For the exact changes in nucleotides please refer to Table 3.

Change (one letter)	Change (three letter)	Codon changed
D358A	Asp → Ala	GAT → GCG
E360A	Glu → Ala	GAA → GCT
D461A	Asp → Ala	GAT → GCG
Y547S	Tyr → Ser	TAT → AGC
Y567F	Tyr → Phe	TAT → TTC
D571A	Asp → Ala	GAT → GCG
L755F	Lys → Phe	TTG → TTC

Table 3 Amino acids and codons changed in the Pol gene (U_L30) in this study.

Considering the lack of information from literature, mutations in any of the exonuclease domains did not seem to be a viable option to generate a MDV polymerase with increased fidelity. Exonucleolytic proofreading has likely reached an evolutionary maximum, beyond which excision of correctly paired bases would disturb DNA replication[65]. Given the contribution of nucleotide selectivity to the fidelity of DNA replication, generating a mutant with increased nucleotide selectivity seemed the most viable option.

Through the characterization of an acyclovir resistant Pol mutant in HSV-1, the mutation L774F in the finger domain of HSV-1 Pol was held responsible for improved nucleotide selectivity and consequently enhanced replication fidelity[57,119]. We found this residue to be conserved in the sequence of MDV Pol and constructed a MDV mutant carrying the homologous mutation L755F (Fig. 5).

```
HSV-1   734   STLSLRADAVAHLEAGKDYLEIEVGGRRLFFVKAHVRESLLSILLRDWLAMRKQIRSRIP  793
              +TL      +++L    DYLEI V G+ L FVK H+RESLL+ILL+DWLAMRK IR++IP
MDV     715   TTLVHDDTNLSNLRPQDDYLEINVQGKLLRFVKPHIRESLLAILLKDWLAMRKAIRAKIP  774
```

Figure 5 Comparison of the amino acid composition in the finger domain of viral DNA Polymerases (HSV-1 and MDV). The conserved amino acid examined by Hwang et al. 2004 is highlighted in red.

Acyclovir (ACV) is a guanosine analog that has been the first line treatment option for human alphaherpesviruses since its discovery more than 40 years ago[120]. The specificity with which this drug targets viral replication is remarkable and results from a two-step mode of action. ACV is a prodrug that requires phosphorylation to compete with deoxyguanosine triphosphate (dGTP) for incorporation into a growing DNA strand. Phosphorylation of ACV is a three-step process; the first of these steps is virus-specific. Viral thymidine kinase (TK) accepts ACV as template and phosphorylates it with sufficient kinetics to provide ACV-monophosphate as template for cellular kinases to produce first ACV-diphosphate and eventually ACV-triphosphate (ACV-TP). ACV-TP can now act as inhibitor of DNA polymerization through competition with dGTP for incorporation opposite of a cytosine base. Once ACV gets incorporated, DNA synthesis is terminated for ACV lacks the 3'-OH group required for addition of subsequent nucleotides. The DNA polymerase stalls at this step and becomes inactivated being stuck on the terminated DNA strand[121].

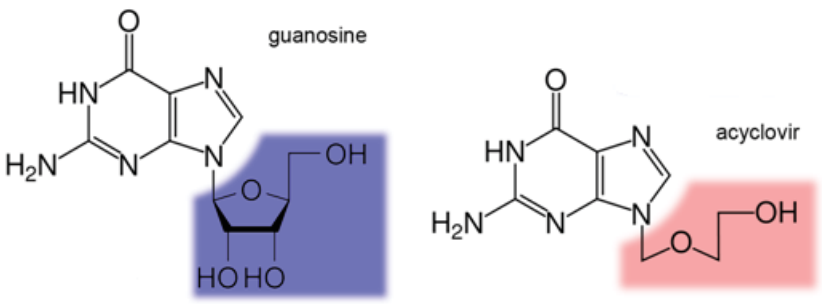

Figure 6 Comparison of Guanosine and Acyclovir

https://en.wikipedia.org/wiki/Aciclovir#/media/File:Guanosine-acyclovir-comparison.png

The selectivity for herpesviral DNA replication arises from two facts: first, viral TK is much better in phosphorylating ACV than any cellular enzyme, second, viral DNA polymerase is much more permissive to the incorporation of ACV-TP than cellular polymerases[122]. Although ACV principally inhibits the DNA polymerases of all herpesviruses, it is less efficacious in treatment of beta- and gammaherpesviruses due to less efficient phosphorylation through viral kinases (betaherpesviruses lack TK)[123,124]. Now, resistance to ACV is a phenomenon commonly encountered in laboratories as well as in clinical settings. Generally, there are two ways of acquiring ACV resistance in alphaherpesviruses. The first way requires mutations in the TK gene. TK mutations can have a variable magnitude, everything from the loss of functional TK to a less active TK to a TK more specific for deoxythymidine (dT) can be encountered. The second option is a DNA polymerase that is not as susceptible to inhibition mediated by ACV. Resistance conferred through changes in the DNA polymerase is attributed to an increased nucleotide selectivity[57]. TK mutations are much more common than Pol mutations[122,125]. Typically, ACV resistance leads to a more or less attenuated phenotype, but it appears that there are exceptions to this rule[126]. Given the fact that increased nucleotide selectivity of the DNA polymerase is a mechanism by which ACV resistance is achieved, an ACV resistant Pol mutant that was found to increase replication fidelity in HSV-1 seemed an ideal choice for our experiments.

4.7.3 Specific goal 3 - *Examine pathogenicity of polymerase mutants in the natural host and determine sequence space in different compartments of the host in vivo.*

Following the characterization of Pol mutants in vitro, three mutants, Y567F, Y547S (Exo mutants, low fidelity) and L755F (finger domain, high fidelity), were selected for the infection of chicken. The goal was to identify any effect of an altered DNA replication fidelity on MDV pathogenesis. This experiment attempted to answer the question if lower or higher replication fidelity could represent a selection advantage in a host infection scenario. During a 90 days trial chicken were monitored for MD symptoms and sampled for different tissues.

5. Material and Methods

5.1 Material

5.1.1 Chemicals, consumables and equipment

5.1.1.1 Chemicals

Name [Identifier]	Manufacturer
Acetic acid [A3686, 2500]	VWR, Darmstadt
Acyclovir (Aciclovir) [PZN 7311950]	Ratiopharm, Ulm
Acrylamide, 30% [A124.2]	Carl-Roth, Karlsruhe
Activated calf thymus DNA, type XV [D4522]	Sigma-Aldrich, St. Louis
Agar (agar bacteriological) [2266.2]	Carl-Roth, Karlsruhe
Agarose Standard Roti® grade [3810.4]	Carl-Roth, Karlsruhe
Ammoniumacetat [131114]	Applichem, Darmstadt
Ammoniumpersulfate [K38297601]	Merck, Darmstadt
AMPure XP paramagnetic beads [A63881]	Beckman Coulter, Brae
Anti-FLAG® M2 magnetic beads [M8823]	Sigma-Aldrich, St. Louis
Arabinose L (+) [A11921]	Alfa Aesar, Karlsruhe
Bromophenolblue, sodium salt [A512]	Carl-Roth, Karlsruhe
Buffer SuperB® [520112]	Covaris, Woburn
BSA (Bovine serum albumin fraction V) [A6588.0100]	Applichem, Darmstadt
Calcium chloride hexahydrate [121214]	Applichem, Darmstadt
CDP-Star® Ready-to-use [12041677001]	Roche, Mannheim
Chloramphenicol [3886.1]	Carl-Roth, Karlsruhe
Chloroform [411 K3944831]	Merck, Darmstadt
Complete®, protease inhibitor cocktail [11697498001]	Roche, Mannheim
Deoxyribonucleic acid from fish sperm [74782]	Sigma-Aldrich, St. Louis
Dexamethasone, 4 mg/ml [6933074]	Bela-Pharm, Vetchta
Dextran Sulphate Sodium salt [17-0340-01]	Pharmacia Biotech, Uppsala
Dimethyl sulfoxide (DMSO) [1.02952.2500]	Merck, Darmstadt
Dithiothreitol (*DTT*) [6908]	Carl-Roth, Karlsruhe
dNTP Mix (10mM total) [BIO-39053]	Bioline, Luckenwalde
dTTP, ^{3}H labeled, 1 mCu/ml [NET221H250UC]	Perkin Elmer, Waltham
dNTP set of dATP ,dCTP ,dGTP, dTTP 100 mM each [K461-KIT]	VWR Darmstadt
EDTA (ethylendiamine tetraacetic acid, disodium salt) [A2937, 1000]	Applichem, Darmstadt
Ethidium bromide 1% [2218.2]	Carl-Roth, Karlsruhe
Ethanol, absolute, Reag. Ph.Eur. [20821.330]	VWR, Darmstadt
Ethanol 99%, denatured with 1% MEK [A5007]	Applichem, Darmstadt
Formaldehyde 37%, stabilized with 10% MeOH [A0823]	Applichem, Darmstadt
Formamide deionized Molecular biology grade [A2156]	Applichem, Darmstadt
Fugene HD® [E2311]	Promega, Mannheim
Glucose, anhydrous [A1422]	Applichem, Darmstadt

Glycerol 99%, p.a. [A2926,2500]	Applichem, Darmstadt
Glycine [631340]	Applichem, Darmstadt
2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonsäure [9105]	Carl-Roth, Karlsruhe
Hydrochloric acid, 37%, p.a. [4625.2]	Carl-Roth, Karlsruhe
Isopropyl alcohol (2-propanol) [A0892]	Applichem, Darmstadt
Kanamycin sulphate [T832.2]	Carl-Roth, Karlsruhe
Lipofectamine 2000® [11668027]	Life Tech., Carlsbad
Lithium chloride, p.a. [131392]	Applichem, Darmstadt
β-mercaptoethanol (2-mercaptoethanol) [Cat. No.28625]	Serva, Heidelberg
Magnesium chloride hexahydrate [5833.025]	Merck, Darmstadt
Magnesium sulfate heptahydrate [M2773]	Sigma-Aldrich, St. Louis
Methanol, Reag. Ph.Eur. [20847.320]	Carl-Roth, Karlsruhe
Methylcellulose [8421]	VWR, Darmstadt
Methionin, ^{35}S-labeled [NEG009A500UC]	Sigma-Aldrich, St. Louis
N-Lauroylsarcosine [L9150]	Merck, Darmstadt
Orange G [1.15925.0025]	VWR, Darmstadt
Paraformaldehyde [P6148]	Sigma-Aldrich, St. Louis
Penicillin G potassium salt [A1837]	Applichem, Darmstadt
Pepsine from porcine gastric mucosa [P7012]	Sigma-Aldrich, St. Louis
Piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) [9156]	Carl-Roth, Karlsruhe
Polyethylenimine, linear (PEI) [764582]	Sigma-Aldrich, St. Louis
Polyethylenglycole 8000 [0263]	Carl-Roth, Karlsruhe
Potassium acetate, KCH_3CO_2 [A4279,0100]	Applichem, Darmstadt
Potassium chloride [131494]	Applichem, Darmstadt
Potassium dihydrogenphosphate [3904]	Carl-Roth, Karlsruhe
Phenol, water saturated [A0447]	Applichem, Darmstad
Phenol/Chloroform/Isoamylalcohol 25:24:1 [A156]	Carl-Roth, Karlsruhe
Phenylmethylsulfonylfluoride (PMSF) [52332]	Sigma-Aldrich, St. Louis
Rotiszint® eco plus [0016.3]	Carl Roth, Karlsruhe
Sodium chloride, NaCl [A3597,5000]	Applichem, Darmstadt
Sodium dodecyl sulfate (SDS) [75746]	Sigma-Aldrich, St Louis
Sodium hydroxide, NaOH [1.06462]	Merck, Darmstadt
Sodium phosphate, monobasic, monohydrate [S9638]	Sigma-Aldrich, St Louis
di-Sodium hydrogenophsohate dodecahydrate [A3906]	Applichem, Darmstadt
tri-Sodium citrate dehydrate [Cat. No A1357]	Applichem, Darmstadt
Streptomycin sulfate [HP66]	Carl Roth, Karlsruhe
Sucrose [131621]	Applichem, Darmstadt
Temed [2367.3]	Carl Roth, Karlsruhe
Tetradecanoylphorbol-acetat (TPA) [P8139)	Sigma-Aldrich, St. Louis
Trichloroacetic acid (TCA) [131067]	Applichem, Darmstadt
Tryptone [A2210,0250]	Applichem, Darmstadt
Tris(hydroxymethyl)-aminomethane (Tris) [A1086,5000]	Applichem, Darmstadt
Triton X-100 [8603]	Merck, Darmstadt
Tween-20 [9127.2]	Carl Roth, Karlsruhe
Water Molecular biology grade [A7398]	Applichem, Darmstadt
Water, ultrapure, DEPC treated [T143]	Carl Roth, Karlsruhe
Yeast extract granulated [212750]	Becton, Heidelberg

5.1.1.2 Consumables

Name	Feature/Cat.No.	Company
Cell culture dishes	6-well, 24-well, 100 mm 150 mm	Sartsedt, Nümbrecht
Cell culture flasks	25 ml, 75 ml	Sartsedt, Nümbrecht
Conical test tubes 17x120	15 ml	Sartsedt, Nümbrecht
Conical test tubes 30x115	with and without feet, 50 ml	Sartsedt, Nümbrecht
Cryotubes	1.8 ml	Nunc, Kamstrupvej
Covaris, AFA tubes	50 µl, 130 µl, 1 ml	Covaris, Brighton
BD Falcon Cell Strainers	40 µm	BD Falcon, San Jose
Eppendorf tubes	1.5 and 2 ml	Sarstedt, Nümbrecht
Electroporation cuvettes	90 µl	VWR Darmstadt
Kimtech Science, Precision Wipes	[Cat. No 05511]	Kimberly-Clark, Roswell
Microscope cover glasses	[Cat. No. ECN631-1569]	VWR, Darmstadt
Nitrile gloves	Size L	Hansa-Medical 24, Hamburg
Parafilm® M	-	Bems, Neenah
Pipettes	5, 10, 25 ml	Sarstedt, Nümbrecht
Pipette tips	P1000, 200, 100 and 10	VWR, Darmstadt
Petri dishes for bacteria	100 mm	Sarstedt, Nümbrecht
PVDF 0.45	[T830.1]	Roth, Karlsruhe
SuperFrost® Plus	[J1800AMNZ]	Menzel Glaser, Braunschweig
Transfection polypropylene tubes	-	TPP, Trasadingen
Whatmann blotting paper	(WM Whatmann 3MM)	GE Healthcare, Freiburg
Sterile syringe filters PVDF	0.45 µm, 0.2 µm	VWR, Darmstadt

5.1.1.3 General Equipment

Name	Feature/Cat.No.	Company
Bacterial incubator	07-26860	Binder, Turtlingen
Bacterial incubator	shaker Innova 44	New Brunswick Scientific, New Jersey
Bunsen burner	Type 1020	Usbeck, Radevormwald
Cell incubators	Excella ECO-1	New Brunswick Scientific, New Jersey
Centrifuge 5424,	Rotor FA-45-24-11	Eppendorf, Hamburg
Centrifuge 5804R,	Rotors A-4-44 and F45-30-11	Eppendorf, Hamburg
Chemismart imaging system	5100	Peqlab, Erlangen
Covaris M220		Covaris, Brighton

Electroporator	Genepulser Xcell	Bio-Rad, Munich
Electrophoresis power supply Power Source 250 V		VWR, Darmstadt
FACScalibur flow cytometer	FACScalibur	BD Bioscience, San Jose
Freezer -20°C	-	Liebherr, Bulle
Freezer -80°C	-	GFL, Burgwedel
Galaxy mini centrifuge	-	VWR, Darmstadt
Gel electrophoresis chamber Mini Electrophorese System	-	VWR, Darmstadt
Gel electrophoresis chamber	SUB-Cell GT	Bio-Rad, München
Ice machine	AF100	Scotsman, Vernon Hills
INTEGRA Pipetboy	-	IBS Integrated Biosciences, Fernwald
Magnetic stirrer RH basic KT/C	-	IKA, Staufen
Mini Protean 2D gel chambers	Protean	Biorad, München
Protean Tetra Cell chambers	Protean	Biorad, München
Photospectrometer	Nanodrop 1000	Peqlab, Erlangen
Newbauer counting chamber	-	Assistant, Sondheim/Rhön
Nitrogen tank	ARPEGE70	Air liquide, Düsseldorf
Orbital shaker	0S-10	PeqLab, Erlangen
Pipetman	P1000, P100, P10	VWR, Darmstadt
Perfect Blue™ Horizontal Maxi-Gel System	Perfect Blue	PeqLab, Erlangen
pH-meter	RHBKT/C WTW pH level 1	Inolab, Weilheim
Sterile laminar flow chambers	-	Bleymehl, Inden
Thermocycler Flexcycler	ThermoFlex	Analytik Jena, Jena
Thermocycler	GeneAmp PCR System 2400	PerkinElmer, Waltham
Thermocycler	T-Gradient	Biometra, Göttingen
UV Transiluminator	Bio-Vision-3026	PeqLab, Erlangen
Transiluminator printer	P93D	Mitsubishi, Rüsselsheim
Transiluminator	VL-4C, 1x4W-254 nm	Vilber-Lourmat, Eberhardzell
Vortex	Genie 2™	Bender&Hobein AG, Zurich
Water baths	TW2 and TW12	Julabo, Seelbach
Water bath shaker	C76	New Brunswick Scientific, New Jersey

5.1.1.4 NGS Sequencing Platform and Consumables

Illumina MiSeq Sequencer		Illumina Inc., San Diego
MiSeq Reagent Kit v3	MS-102-3003	Illumina Inc., San Diego
NEB Ultra II Library prep for Illumina	E7645L	New England Biolabs, Ipswich
Library Quant Kit	E7630L	New England Biolabs, Ipswich
Multiplex Oligos for Illumina	E7600S	New England Biolabs, Ipswich

5.1.1.5 Microscopes

Fluorescence microscope	Axiovert S 100	Carl Zeiss MicroImaging GmbH, Jena
Fluorescence microscope	Axio-Observer.Z1	Carl Zeiss MicroImaging GmbH, Jena
Microscope AE20	AE20	Motic, Wetzlar

5.1.1.6 Software

Axiovision - software for Zeiss microscopes	Axiovision 4.8	Carl Zeiss MicroImaging GmbH, Jena
Chemi-Capt	-	Vilber-Lourmat, Eberhardzell
Graphpad Prism 5	Version 5	Graphpad Software Inc, La Jolla
Illumina experiment manager	1.14	Illumina Inc., San Diego
Image J 1.41	Version 1.41	NIH, Bethesda
Miseq software updater	2.6	Illumina Inc., San Diego
ND-1000	V.3.0.7	PeqLab, Erlangen
Vector NTI 9	Version 9	Invitrogen Life Technologies, Grand Island
Vision-Capt	-	Vilber-Lourmat, Eberhardzel
Genome Sequencer software suite	Version 3	Roche, Penzberg
R	Version 3.1	R Development Core Team, Wien

5.1.2 Enzymes and Markers

Name	**Cat.No.**	**Company**
ApaLI	[R0507L]	New England Biolabs, Ipswich
AvrII	[R0174S]	New England Biolabs, Ipswich

BamHI	[R0136]	New England Biolabs, Ipswich
BamHI HF	[R3136]	New England Biolabs, Ipswich
Calf intestinal phosphatase	[M0290S]	New England Biolabs, Ipswich
DpnI	[ER1701]	New England Biolabs, Ipswich
EcoRI	[R0101	New England Biolabs, Ipswich
EcoRI HF	[R3101]	New England Biolabs, Ipswich
EcoRV	[R0195]	New England Biolabs, Ipswich
GeneRuler 1kb Plus DNA Ladder	[SM1331]	Thermo Scientific, Darmstadt
LongAmp DNA Polymerase	[M0323S]	New England Biolabs, Ipswich
HindIII	[R0104]	New England Biolabs, Ipswich
Klenow Enzyme, exo-	[M0212S]	New England Biolabs, Ipswich
KpnI	[R0142L]	New England Biolabs, Ipswich
Lamba protein phosphatase	[P07535]	New England Biolabs, Ipswich
Micrococcal Nuclease	[M0247S]	New England Biolabs, Ipswich
NotI	[R0189]	New England Biolabs, Ipswich
PageRuler Prestained plus marker	[26619]	Thermo Scientific, Darmstadt
Phusion DNA Polymerase	[M0530S]	New England Biolabs, Ipswich
Primestar GXL DNA Polymerase	[R050A]	Takara Bio, Kusatsu
Proteinase K	[7528.2]	Thermo Scientific, Darmstadt
Q5 DNA Polymerase	[M0491L]	New England Biolabs, Ipswich
RNase A	[7528.2]	Carl-Roth, Karlsruhe
RNase free DNAse	[19253]	Qiagen, Hilden
SacI	[R0156L]	New England Biolabs, Ipswich
sensiFast Master Mix	[BIO-94020]	Bioline, Luckenwalde
SmaI	[R0441S]	New England Biolabs, Ipswich
T4 ligase	[M02025]	New England Biolabs, Ipswich
Taq DNA-Polymerase	[01-1020]	PeqLab, Erlangen
XbaI	[R0145S]	New England Biolabs, Ipswich
XhoI	[R0146S]	New England Biolabs, Ipswich
XmaI	[R0180S]	New England Biolabs, Ipswich

5.1.3 Plasmids

Name	**Cat.No.**	**Company**
pcDNA3.1 (-)	[V795-20]	Invitrogen, Carlsbad
pEGFP-C1	[632470]	Clontech, Mount View
pTM1	[pvitro-mcs]	Invivogen, San Diego
pBlueScript SK+	[212205]	Agilent, La Jolla
pEP	[41017]	[118] available from Addgene

5.1.4 Antibodies

Name	Dilution	Company
Alexa goat anti-chicken IgG (H+L) 488	1:3,000	Invitrogen Life Technologies, Grand Island
Alexa goat anti-chicken IgG (H+L) 546	1:3,000	Invitrogen Life Technologies, Grand Island
Alexa goat anti-rabbit IgG (H+L) 568	1:3,000	Invitrogen Life Technologies, Grand Island
Alexa goat anti-mouse IgG (H+L) 647	1:3,000	Invitrogen Life Technologies, Grand Island
Chicken anti MDV, IgY polyclonal	1:1,000	Produced on site[127]
Goat anti-rabbit HRP	1:5,000	Cell Signaling, Boston
Mouse anti Flag-FITC labeled	1:1,000	Sigma-Aldrich, St Louis
Rabbit anti- 6xHis epitope	1:5,000	Rockland, Limerick
Rabbit-anti Flag epitope	1:1,000	Sigma-Aldrich, St Louis

5.1.5 Kits for Molecular Biology

Name	Cat.No.	Company
BCA Protein Assay Kit	[23227]	Pierce, Rockford
TNT coupled reticulocyte lysate	[L4610]	Promega, Mannheim
GF-1 AmbiClean PCR/Gel Purification Kit	[GF-GC-200]	Vivantis, USA
Hi Yield Gel/PCR DNA Fragments Extraction Kit	[HYDF100-1]	SLG, Gauting
innuSpeed tissue DNA	[845-KS-1540050]	Analytik Jena, Jena
Omniscript RT Kit	[205110]	Qiagen, Hilden
PeqGold Plasmid Mini Kit	[12-6942-02]	Peqlab, Erlangen
RTP® DNA/RNA Virus Mini Kit	[1040100300]	STRATEC Molecular GmbH, Berlin
QuikChange Site-directed Mutagenesis Kit	[200523]	Agilent, Santa Clara
Qiagen Plasmid Midi Kit	[12145]	Qiagen, Hilden
QiaQuick Gel Extraction Kit	[28704]	Qiagen, Hilden
NEB Monarch Gel Extraction Kit	[T1020S]	New England Biolabs, Ipswich
EZ 96 blood DNA Kit	[D1192-01]	Omega Biotek, Norcross

5.1.6 Buffers, Media and Antibiotics

5.1.6.1 Buffers

1. General

Phosphate buffered saline (PBS): 2 mM KH_2PO_4, 10 mM Na2HPO4, 137 mM NaCl, 2.7 mM KCl, pH 7.4
Phosphate buffered saline with Tween 20 (PBST): PBS, 0.05% Tween 20
Tris buffered saline (TBS): 50 mM Tris-HCl, pH 7.5. 150 mM NaCl, pH 7.4
Tris acetate EDTA (TAE): 40 mM Tris, 1 mM EDTA, 20 mM Acetic acid, pH 8.0
Tris boric acid EDTA (TBE): 89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0

2. DNA Extraction and related procedures

Tris-EDTA (TE): 10 mM Tris-HCl, 1 mM EDTA, pH 8.0
Elution buffer (EB): 10 mM Tris-HCl, pH 8.0

Hirt lysis buffer: 10 mM Tris-HCl, 20 mM EDTA, 1,2% (w/v) SDS, pH 8.0

TEN buffer: 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0
Sarcosine lysis buffer: 75 mM Tris-HCl, 25 mM EDTA, 3% (w/v) N-Lauroylsarcosine, pH 8.0

Micrococcal nuclease, **permabilization buffer**: 320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl, 1% (w/v) Triton X-100, pH 7.5
Micrococcal nuclease, **nuclei buffer**: 10 mM Tris-HCl, 2 mM $MgCl_2$, 10% (w/v) sucrose, pH 7.5
Micrococcal nuclease, **2x nuclease buffer**: 40 mM PIPES,7% (w/v) sucrose, 20 mM NaCl, 2 mM $CaCl_2$, 10 mM ß-Mercaptoethanol, 200 µM PMSF
Micrococcal nuclease, **digestion buffer**: 100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0,5% (w/v) SDS, ph 8.0

PEG bead binding buffer: 7.5-15% (w/v) PEG 8000, 1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 0.05% (v/v) Tween 20

Buffer **P1**: 50 mM Tris-HCl, 10 mM EDTA, 100 µg/ml RNAse A, pH 8.0
Buffer **P2**: 200 mM NaOH, 1% (w/v) SDS
Buffer **P3**: 3 M Potassium acetate, pH 5.0

3. Western Blotting (WB)

Laemmli buffer: 0.1% ß-Mercaptoethanol, 0.0005% Bromophenol blue, 10% Glycerol, 2%SDS, 63 mM Tris-HCl, pH 6.8
SDS-Page running buffer: 25 mM Tris HCl, 190 mM Glycine, 0,1% SDS

4. Protein extraction and related procedures

Native lysis buffer[128]: 150 mM NaCl, 50 mM Tris HCl, 0,5% (w/v) Pluronic F-127, 0,5% (w/v) ß-D-Glucopyranoside, pH 7.4
BC-100[129]: 20 mM Tris HCl, 20% (w/v) Glycerol, 0.2 mM EDTA, 100 mM KCl, 1 mM DTT, 0.5 mM PMSF, pH 8.0
BC-300: The same as BC-100 but with 300 mM KCl
Native elution buffer: BC-300, 200 µM FLAG peptide
pH elution buffer: 100 mM Glycine, pH 3

5. Polymerase and exonuclease assays

Polymerase buffer (1x): 50 mM Tris HCl, 0.05% (w/v) BSA, 0.5 mM DTT, 7.5 mM $MgCl_2$, 100 µM dCTP, dATP, dGTP, 20 µM dTTP, 1 µCi ^{3}H-dTTP
Exonuclease buffer (1x): 50 mM Tris HCl, 0.05% (w/v) BSA, 0.5 mM DTT, 7.5 mM $MgCl_2$

5.1.6.2 Media for bacterial cultures

LB medium: 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl
LB Agar: 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 15 g/l Agar
SOC medium: 20 g/l tryptone, 5 g/l yeast extract, 0.6 g/l NaCl, 0.2 g/l KCl, 2 g/l $MgCl_2$*$6H_2O$, 2.5 g/l $MgSO_4$*$7H_2O$, 3.6 g/l glucose

5.1.6.3 Antibiotics

Antibiotic	Working concentration	Supplier [Cat. No.]
Ampicillin	100 µg/ml	Carl Roth, Karlsruhe [K029.2]
Chloramphenicol	30 µg/ml	Carl Roth, Karlsruhe [3886.3]
Kanamycin	50 µg/ml	Carl Roth, Karlsruhe [T832.4]
Gentamicin	30 µg/ml	Carl Roth, Karlsruhe [0233.3]
Penicillin G	100 IU/ml	Carl Roth, Karlsruhe [HP48.3]
Streptomycin	100 µg/ml	Carl Roth, Karlsruhe [0236.3]

5.1.6.4 Cell culture media

Minimal Essential Medium (MEM) Earle's	Biochrom, Berlin [F 0315]
Dulbecos MEM	Biochrom, Berlin [FG 0415]
RPMI 1640	Biochrom, Berlin [F 1215]
OptiMEM	Thermo Fisher, Darmstadt [31985070]
Fetal Bovine Serum (FBS)	Biochrom, Berlin [S 0415]
Trypsin (0.05% in PBS)	Biochrom, Berlin [L 2103-20G]

5.1.7 Cells, Viruses, Bacteria and Animals

5.1.7.1 Cells

Name	Features	Reference
CEC	Chicken embryo fibroblasts/cells, primary cells, VALO SPF strain	Primary cells
DF-1	Spontaneously transformed chicken embryo fibroblasts	ATCC CRL-12203
RK13	Rabbit epithelial kidney cell line	ATCC CCL-37
293T	Human epithelial kidney cell line, SV-40 T-antigen	ATCC CRL-11268
BTI-TN-5B1-4	*Trichoplusia ni* ovary cell line	ThermoFisher [B85502]

5.1.7.2 Viruses

RB-1B U_L47-eGFP	vvMDV strain derived from a BAC clone, labeled with eGFP fused to a tegument protein[130].
HVT 90SE	Meleagrid Herpesvirus 1 (MeHV-1) also known as Herpesvirus of Turkeys (HVT). BAC originally derived from vaccine strain FC126[131].

5.1.7.3 Bacteria

Name	Genotype	Source
DH10β	F- endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu) 7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ	Invitrogen
Top10	F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
MegaX	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ$^-$ *rps*L *nup*G *ton*A	Invitrogen
Top10 F’	F+ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
GS1783	DH10B λcI857 △(cro-bioA)<>araC-PBAD, I-SceI	[132]

5.1.7.4 Animals

Specific pathogen free (SPF) chicken eggs were obtained from Valo Biomedia (Osterholz-Scharmbeck). Eggs were incubated in house until hatch and 1 day old chicks were used for animal experiments.

5.1.8 Primers used in this project

1. Primers used for en passant mutagenesis

1.1 Primers used to generate Pol mutant viruses

Description	Sequence
D461A Fwd	TGCTCCAGAATTTGTAACTGGGTATAACATTGTCAATTTTGCGTGGGCATTCATTGTTACTAGGGATAACAGGGTAATCG
D461A Rev	TGTATACTGTTGTTAATTTGGTAACAATGAATGCCCACGCAAAATTGACAATGTTATACCGCCAGTGTTACAACCAATTA
D571A Fwd	AGGTCCAGAAAAACGGGGAATTATAGGAGAATATTGTCTTCAGGCGTCATTGTTGGTGGGTAGGGATAACAGGGTAATCG
D571A Rev	TGTATTTAAAAAATAATTTTCCCACCAACAATGACGCCTGAAGACAATATTCTCCTATAAGCCAGTGTTACAACCAATTA
Y567F Fwd	CCCAGCCATTTTGCAGCAGGTCCAGAAAAACGGGGAATTATAGGAGAATTCTGTCTTCAGTAGGGATAACAGGGTAATCG
Y567F Rev	TTTCCCACCAACAATGAATCCTGAAGACAGAATTCTCCTATAATTCCCCGTTTTTCTGGAGCCAGTGTTACAACCAATTA
E360A Fwd	ATGCATGGCCAGATTACAAACTTCTATGCTTTGATATTGCTTGTAAATCCGGAGGAGTAATAGGGATAACAGGGTAATCGATTT
E360A Rev	GCATGGAAATGCACATTCGTTTACTCCTCCGGATTTACATGCAATATCAAAGCATAGAAGGCCAGTGTTACAACCAATTAACC
D358A/E360A Fwd	ATGATGCATGGCCAGATTACAAACTTCTATGCTTTGCGATTGCTTGTAAATCCGGAGGAGTAGGGATAACAGGGTAATCGATTT
D358A/E360A Rev	AAATGCACATTCGTTTACTCCTCCTCCGGATTTACAAGCAATCGCAAAGCATAGAAGTTTGCCAGTGTTACAACCAATTAACC
L755F Fwd	TTTGTGAAACCGCATATTCGCGAAAGTTTATTCGCGATCTTATTAAAAGATAGGGATAACAGGGTAATCG
L755F Rev	GGCCAGCCAATCTTTTAATAAGATCGCGAATAAACTTTCGCGAATATGCGGCCAGTGTTACAACCAATTA
Y547S Fwd	GGCCGAGGCTGCATTAGGAGAACGGAAAAAAGAATTGTCTAGCAAGGAAATACCCAGCCTAGGGATAACAGGGTAATCG
Y547S Rev	GGACCTGCTGCAAAATGGCTGGGTATTTCCTTTCGAGACAATTCTTTTTTCCGGCCAGTGTTACAACCAATTA

1.2 Primers used to generate revertants of Pol mutant viruses for animal experiments

Description	Sequence
Rev Y547S Fwd	GAGGCTGCATTAGGAGAACGGAAAAAAGAATTGTCTTATAAGGAAATACCCAGCCATTTTAGGGATAACAGGGTAATCG
Rev Y547S Rev	GTTTTTCTGGACCTGCTGCAAAATGGCTGGGTATTTCCTTATAAGACAATTCTTTTTTCCGTTTGTTACAACCAATTAACC
Rev L755F Fwd	GCTATTGCGCTTTGTGAAACCGCATATTCGCGAAAGTTTATTGGCGATCTTATTAAAAGATAGGGATAACAGGGTAATCG
Rev L755F Rev	ATTGCTTTTCTCATGGCCAGCCAATCTTTTAATAAGATCGCCAATAAACTTTCGCGAATATGCGTGTTACAACCAATTAACC
Rev Y567F Fwd	TTGCAGCAGGTCCAGAAAAACGGGGAATTATAGGAGAATATTGTCTTCAGGATTCATTGTTTAGGGATAACAGGGTAATCG
Rev Y567F Rev	TAAAAAATAATTTTCCCACCAACAATGAATCCTGAAGACAATATTCTCCTATAATTCCCCGTTTGTTACAACCAATTAACC

2. Primers for qPCR quantification

Description	Sequence	Modification
MDV ICP4 qPCR Fwd	CGTGTTTTCCGGCATGTG	
MDV ICP4 qPCR Rev	TCCCATACCAATCCTCATCCA	
MDV ICP4 PROBE	CCCCCACCAGGTGCAGGCA	Fam-Tamra
Chicken iNOS qPCR Fwd	GAGTGGTTTAAGGAGTTGGATCTGA	
Chicken iNOS qPCR Rev	TTCCAGACCTCCCACCTCAA	
Chicken iNOS PROBE	CTCTGCCTGCTGTTGCCAACATGC	Fam-Tamra

3. Primers for Sanger sequencing

Description	Sequence
UL30 PolSeq 01	GGTATCAGTATCGACTTTGC
UL30 PolSeq 02	GGAGCTGCTTCGCCCGATGG
UL30 PolSeq 03	AGTGTTAGAATTTGACAGCG
UL30 PolSeq 04	GTGATGGTCTTTGACTTTGC
UL30 PolSeq 05	AAGGGCTTAATTCTTTTGGC
UL42 Seq 01	CCTCCTTCAGCTTTCAAAGC
UL42 Seq 02	ATGAATTATGGAGCGCATCC

4. Primers intended for cloning

Description	Sequence
UL30 pBlue Fwd	ATATAGCGGCCGCCACCATGTCAGTAGACGGAACT
UL30 pBlue Rev	ATATACCATGGAGATTCTTGTGATGTGCC
UL30 pTM1 Fwd	ATATAGGATCCCTGCATTGGCTACTATGTCAG
UL30 pTM1 Rev	ATATACTCGAGAGATTCTTGTGATGTGCC
UL42 pTM1 Fwd	ATATAGGATCCGTTGTCAATGGCAGGAAT
UL42 pTM1 Rev	ATATACTCGAGCTCCATGACATACTTACAGG

UL30 RsrII Fwd	TATATACGGTCCGCATCACCATCACCATCACATGTCAGTAGACGGAACTAAAAC
UL30 RsrII Rev	CTATTACGGACCGTTAATATCGATGGGGAGTTGC
UL30 NotI long Fwd	ATATAGCGGCCGCAATGTCAGTAGACGGAACTAAAACATTTTT
UL30 KpnI long Rev	ATATACCATGGGACAAACAGATTCTTGTGATGTGCC
UL30 Blunt Fwd	ATGTCAGTAGACGGAACTAAAAC
UL30 Blunt Rev	GCGATCTTAATATCGATGGGG
UL42 Blunt Fwd	GTTGTCAATGGCAGGAAT
UL42 Blunt Rev	CGAAAGTCTCATTGGTTG
pBlue_RBS Fwd	ATATGCGGCCGCTAAGGAGGATATATATGTCAGTAGACGGAACTAAAAC
pBlue_RBS Rev	CCATTTTTGTATAGTACCGGT
UL30 N-Terminal FLAG Fwd	TTGGGGCCCAATTCTGACGATATCTGCATTGGCTACTATGGACTACAAAGACGATGACGACAAGGGATCAGTAGACGGAACTTAGG GATAACAGGGTAAT
UL30 N-Terminal FLAG Rev	CGATGTACGGATTGAAAAATGTTTTAGTTCCGTCTACTGATCCCTTGTCGTCATCGTCTTTGTAGTCGCCAGTGTTACAACCAATTA
BamHI-CMV-UL30-FLAG-BGH fwd	TATATGGATCCATGTACGGGCCAGATATACG
KpnI-CMV-UL30-FLAG-BGH rev	TATATGGTACCTCAGAAGCCATAGAGCCC
3xFLAG Fwd	TTGGGGCCCAATTCTGACGATATCTGCATTGGCTACTATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGATATCGACT ACAAAGACGATGACGACAAGGGATAGGGATAACAGGGTAAT
3xFLAG Rev	TTCCGTCTACTGATCCCTTGTCGTCATCGTCTTTGTAGTCGATATCATGATCTTTATAATCAGCCAGTGTTACAACCAATTA
UL30-AgeI Fwd	ATACGTTGAAAAGATCACCGGTAC
UL30-BglII Rev	ATATAAGATCTAGATTCTTGTGATGTGCC
NheI-FLAG	ATATGCTAGCCACCATGGACTACAAAGACGATGAC
DK-112 miniF-F	TTGTAATTCATTAAGCATTCTGCC
DK-113 miniF-R	ATAAGTGGATAACCCCAAGGG

5. Primers for NGS library amplification

Description	**Sequence**
P5 Primer Illumina	AATGATACGGCGACCACCGA
P7 Primer Illumina	CAAGCAGAAGACGGCATACGA

5.2 Methods

5.2.1 Cell culture methods

Chicken Embryonic Cells (CEC)

Chicken embryonic cells (CEC) were prepared weekly from 11 day old chicken embryos as previously described[133]. Egg shells where disinfected with ethanol and cracked open to extract the embryo. The embryo was transferred to sterile PBS to remove extremities and internal organs. Using scissors, the remainder was cut to small pieces and washed with PBS on a magnetic stirrer (medium agitation) for 10 minutes. Pieces of tissue were digested in 100 ml of 0,05% trypsin for 10 minutes, the suspension was filtered through a sterile gauze and trypsinization was stopped with the addition of MEM containing 10% FBS. The suspension was centrifuged at 800 g for 10 minutes and the digestion procedure was repeated twice to obtain a suspension of fully detached cells. After the last centrifugation, the pellet was washed with PBS and resuspended in 10% FBS MEM. Cells were counted, seeded at the desired confluence and maintained in 0.5 to 10% FBS MEM, 100 IU/ml penicillin G and 100 µg/ml streptomycin at 37 °C in a 5% CO_2 incubator.

Transfection

CEC were transfected using a standard Polyethylenimine (PEI) transfection procedure adapted from the literature[134]. For the reconstitution of viruses, 10 µl PEI (1 mg/ml) was added to 1-2 µg of BAC DNA in 100 µl Optimem. The DNA:PEI complex was allowed to form during a 20 minutes incubation at room temperature. Then, the suspension was added to one well in a 6-well dish with a confluent monolayer of CEC (approximately 10^6 cells) and 2 ml of 10% FBS MEM. Cells and transfection mixture were incubated at 37 °C for 3-4 hours before the medium was removed and changed back to 10% FBS MEM. The next day, density and viability of the cells was assessed and medium was changed, using 5-10% FBS MEM if cells appeared to be hurt by transfection, or with 1% FBS MEM if cells were confluent. After 2 days, medium was changed again to 0.5-1% FBS MEM, afterwards medium was changed every 2-3 days using 0.5% FBS MEM.

The same protocol was used for the transfection of plasmids into CEC. The amount of PEI and DNA was scaled up for transfections on 100 mm plates, typically 50-100 µl PEI and 10-20 µg DNA were used on 100 mm plates.

Infection of cells and propagation of virus

Cells were infected by co-seeding uninfected and infected cells or by addition of infected cells to an already adherent monolayer of uninfected cells. Virus was propagated in cell culture through serial passaging. When cytopathic effects became conspicuous, infected cells were trypsinized and 1/10 of the detached cells were seeded with fresh cells onto a new cell culture plate. For the purpose of this study, one passage is defined as 5 days of infection.
Viruses that grew poorly in cell culture were propagated by using 1/3 of infected cell suspension for co-seeding with uninfected cells.

For virus reconstitution from BAC DNA, the entire amount of infected cells from one or several wells of a 6-well dish was harvested 7 days post transfection and co-seeded with uninfected cells onto a 100 mm dish.

Trypsinization

For trypsinization of adherent CEC monolayers, medium was completely aspirated, cells were washed with PBS and 2 ml of 0.05% trypsin/2 mM EDTA (in PBS) were added. Cells were allowed to detach for 1 to 5 minutes at room temperature and resuspendend in 10% FBS MEM. If a significant amount of cell clumps remained, a 0.70 µm cell strainer was used to obtain a suspension of fully detached cells.

Fixation and staining procedures

Infected CEC were fixed using 3.7% formalin freshly prepared from the 37% stock solution. Only in rare cases, when preservation of GFP fluorescence was crucial, cells were fixed using a 4% formaldehyde solution freshly prepared from paraformaldehyde powder. In both cases, the following procedure was applied: Medium was aspirated and cells were washed with PBS once before formalin was added. Fixation took place during 5 minutes at room temperature, then the formalin was removed and replaced by PBS. If immediate immunoflourescent staining was desired, cells were permeabilized with 0.01% triton X 100 (in PBS) during 10 minutes incubation at room temperature. Triton X was removed, cells were washed with PBS and 3% BSA (in PBST) was added and left for 30 minutes under gentle agitation on a horizontal shaker at room temperature to block any non-specific protein binding. The BSA was removed and an anti MDV IgY[127] (kindly provided by Luca Bertzbach) was added as primary antibody at a 1:1000 dilution in 3% BSA (in PBST). Antibody binding was allowed to occur during 2 hours incubation at room temperature again applying gentle horizontal agitation. The primary antibody was removed and cells washed three times for 5 minutes with PBST before the secondary antibody was added at a 1:5000 dilution in 3% BSA (in PBST). Typically goat anti chicken IgG coupled to Alexa 488 or Alexa 546 was used as secondary antibodies. Binding of the secondary antibody was allowed to occur during a 45 minutes incubation under gentle horizontal agitation at room temperature in the dark. Afterwards the secondary antibody was removed and the cells washed again three times for 5 minutes with PBST. Following the final wash, cells were covered with PBS and stored in the dark at 4 °C or used immediately under the microscope.

Plaque size assays

To determine plaque sizes, CEC were transfected or infected with 50-100 pfu per well in a 6-well cell culture plate. On day 7 post transfection or day 5 post infection, cells were formalin-fixed and stained with the appropriate combination of antibodies. Pictures were taken on an Axiovert-Observer Z1 inverted fluorescent microscope using an AxioCam with the Axiovert software and 50x magnification. Plaque size areas were measured with the NIH Image J 1.49 software and converted to plaque diameters with were expressed relative to WT virus in box plots constructed with GraphPad Prism 5.

Cryoconservation of cells and virus

To generate cryoconserved virus stocks, cell-associated virus was harvested with the cells when the virus began to induce cythopathic effects (depending on the virus, 4 to 7 days post infection (p.i.)). Following trypsinization, cells were centrifuged at 600 g and the pellet was resuspended in 30% FBS MEM. From this suspension, 800 µl aliquots were transferred to cryo-tubes and 20% DMSO MEM was added dropwise. Cryotubes were immediately transferred into cryo containers filled with isopropanol and moved to a -80 °C freezer for slow freezing (temperature decreased by approximately 1 K per minute). The following day, samples were moved to liquid nitrogen containers for long-term storage.

Plaque reduction assay, Acyclovir (ACV) response

To test the sensitivity of MDV to ACV, a plaque reduction assay was performed. ACV was obtained as standard pharmaceutical powder for intravenous use (Aciclovir ratiopharm, 250 mg) and dissolved in sterile water to obtain a 100 mM stock solution. The stock was aliquoted and frozen at -20 °C. To avoid freeze thaw cycles, one aliquot at a time was removed from the freezer, diluted to 1 mM and used immediately. For plaque reduction assays, 100 pfu of either WT or L755F was used per well in a 6-well plate to infect CEC. Exactly 2 ml of medium was used in each well, so that the addition of 2 µl 1 mM ACV would mean a total concentration of 1 µM in the well. Following this procedure, concentrations between 10 µM and 100 µM of ACV were tested. The drug was added at the time of infection and medium was changed every second day, adding fresh ACV each time until the plate was formalin-fixated at day 7 post infection.

Dexamethasone treatment of cell cultures

To promote viral growth in cell culture, infected cells were maintained in medium containing 50 µM dexamethasone. This treatment was well tolerated by CEC and prolonged cell survival for several days. Dexamethasone was added to the medium at the time of infection or one day post transfection. To avoid pharmacological effects of dexamethasone in the animal experiment, dexamethasone was withdrawn 24 hours prior to stock production. No dexamethasone was added for cryoconservation. Prior to the infection of animals, stocks were thawed and centrifuged at 500 g and 4 °C for 5 minutes. The supernatant was discarded and cells were resuspended in the appropriate volume of 10% FBS MEM for the infection of animals.

Dexamethasone was exclusively used in the form of its water soluble sodium phosphate (CAS No. 2392-39-4) marketed as injectable solution for animals (Bela Pharm, Vechta).

Titration of viruses

Titrations were performed in duplicates on 6-well plates using serial dilutions of a previously frozen virus stock. Virus stock was diluted in medium from 1:10 to $1:10^5$ and incubated at 37°C until visible plaques formed (typically 5d post infection). Subsequently, cells were fixed and stained using the described procedures. Plaques were counted in wells with 10-100 plaques and plaque forming units (pfu) for the respective stock were calculated based on dilution and plaque count.

Preparation of viral stocks for animal experiments

To avoid attenuation from prolonged passaging in cell culture, virus for animal experiment was prepared from low passage virus. WT and mutants as well as revertants were used for stock preparation at passage 2 or 3 post transfection. Stocks were titrated three times independently to calculate the inoculum for animal experiments.

Titration of virus as fitness marker

To compare the fitness of different viral BAC clones obtained from extrachromosomal DNA extracted from a diverse population of viruses in cell culture, titrations were performed as follows. The original virus stock was generated and titrated as described above. From these stocks, 10000 pfu were used to infect CECs on a 15 cm cell culture dish. On day 4 post infection, stocks were generated from each of the infected plates by trypsinization of infected cells and resuspension in a final volume of 8 ml cryo-medium. The resulting cryopreserved stocks were titrated again to obtain comparable titers for each of the clones as well as WT and the intact diverse population at passage XII.

5.2.2 BAC mutagenesis

Generation of electrocompetent *E. coli*

To obtain electrocompetent GS1783 bacteria harboring the desired BAC, the following procedure was adapted from literature[135].

A single clone of GS1783 harboring the desired BAC was inoculated in 5 ml LB containing Cam and let grow o/n. From the overnight culture, 4 ml were inoculated into 200 ml of LB Cam and let grow at 32 °C until on OD 600 of 0.5-0.7 was measured. The culture was then transferred to a 42 °C shaking water bath and incubated for 15 minutes. Following this, the culture was immediately chilled on ice under gentle agitation for 20 minutes. In a pre-cooled centrifuge, bacteria were pelleted at 4500 g for 10 minutes at 4 °C. The pellet was washed thrice with 50 ml of ice-cold 10% glycerol (in water). After the third wash, the pellet was resuspendend in 500 µl of 10% glycerol, aliquoted to 50 µl in pre-chilled tubes and snap frozen in liquid nitrogen.

Electroporation

50 µl competent cells and 100-200 ng of purified desalted PCR product were mixed and pipetted into a 1mm gap electroporation cuvette. Electroporation was performed using the following conditions: 1.8 kV, 25 µF and 200 Ω. Bacteria were washed out of the cuvette with 1 ml of SOC and transferred into a 32 °C shaker for recovery before plating on LB Agar with appropriate antibiotics.

"En passant" mutagenesis

The entire genome of MDV is available as a single bacterial artificial chromosome (BAC) based on a single copy F- plasmid of *E. coli*[136]. Mutations were introduced into BAC sequences using a two-step Red mediated markerless recombination system known as *en passant* mutagenesis[118]. Originally found in the bacterial phage λ, the Red recombination system has three components that together tackle DNA double strand breaks[137].

Gam prevents both the endogenous *E. coli* nucleases RecBCD and SbcCD from digesting free ends of double stranded DNA

Exo is a 5'-->3' exonuclease that degrades linear dsDNA by recession of the 5'-prime end, leaving either a negative ssDNA or a dsDNA with single stranded overhangs.

Beta binds to ssDNA, protects it from degradation and promotes strand invasion to eventually achieve recombination by annealing to a complementary DNA sequence.

To achieve introduction of point mutations via markerless recombination PCR products containing a selection marker, the desired change in sequence and homologous sequences flanking the region of interest are required. In this case selection is achieved through introduction of *aphA*I, a gene of about 1 kb, conferring resistance to kanamycin. To generate a suitable PCR product, primers with the following features were designed:

1. Annealing site (20 bp) to the 3' or 5' terminus of the kanamycin cassette from plasmid pEPKanS[118] (*aphA*I + *Sce*I restriction site)
2. A sequence homologous to the site where the mutation is to be introduced, containing the desired change in nucleotides (typically 1-3 bp)
3. A "recombination arm", homologous to the sequence of the integration site

The respective primers were used to amplify the kanamycin cassette from pEPKanS (purified from a dam+ strain of *E. coli*) using LongAmp® Polymerase in a two-step PCR protocol (see table 3).

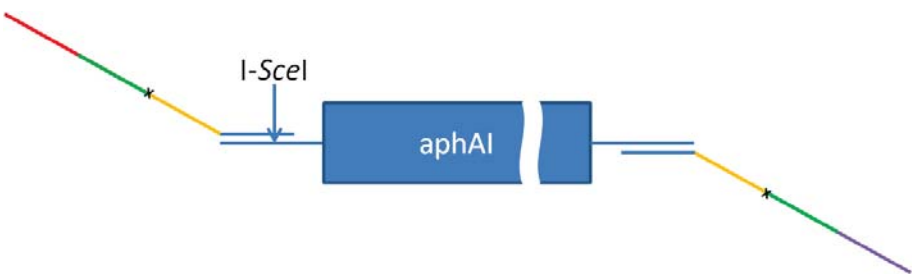

Figure 7 Design for *en passant* primers. Primers contain an annealing site (20 bp) to the *aph*AI Knm resistance gene from plasmid pEPKanS[118] (blue), a sequence homologous to the target site (yellow) including the desired nucleotide change (black cross) and a recombination arm homologous to the region flanking the target site (green, red, violet).

Temperature	Time	Purpose	No. of cycles
95 °C	**120 s**	Initial denaturation	
98 °C	**30 s**	Denaturation	} **10**
55 °C	**20 s**	Primer annealing	
65 °C	**60 s** per kb	Elongation	
98 °C	**30 s**	Denaturation	} **32**
60 °C	**20 s**	Primer annealing	
65 °C	**60 s** per kb	Elongation	
68 °C	**300 s**	Final elongation	
8 °C	**∞**	Hold	

Table 4 PCR programme for *en passant* mutagenesis

The PCR product was digested with *Dpn*I to remove intact plasmids before gel purification. The purified fragment was electroporated into electrocompetent bacteria of the *E. coli* strain GS1783 harboring the respective BAC. After recovery in SOC medium, the entire reaction was plated on LB-Agar containing chloramphenicol (Cam) and kanamycin (Knm) and incubated for 48 hours at 32 °C. The recombinant Knm resistant intermediates were picked and grown in 5 ml LB with Cam and Knm o/n. BAC DNA was prepared using a standard "MiniPrep" procedure and digested with the restriction enzyme of choice for restriction fragment length polymorphism (RFLP) analysis. Clones that showed the correct RFLP pattern were selected for resolution, i.e. the removal of *aphA*I by I-*Sce*I restriction followed by homologous recombination.
For resolution, confirmed clones were grown o/n in 5 ml of LB with Cam/Knm, the next morning, 200 µL of culture were diluted with 1.6 ml of LB containing only Cam and grown for 1-2 hours to reach logarithmic growth. I-*Sce*I expression was induced by the addition of 200 µL 10% arabinose (in LB) and incubation at 32 °C for 1 hour. During this step, I-*Sce*I a mitochondrial homing endonuclease of *Saccharomyces cerevisiae* is expressed from an arabinose inducible promoter (araBp) and cuts at its unique recognition site in front of *aphA*I, producing a DNA double strand break. Now, bacteria were moved to a 42 °C shaking water bath to induce expression of the Red system from a temperature sensitive promoter. During another incubation of 2-4 hours at 32 °C, proteins of the Red system recognize the double strand break and facilitate homologous recombination between loci flanking *aphA*I. Eventually, the Knm resistance gene is removed, and the viral locus seamlessly restored while retaining the desired mutation. Bacteria were than diluted 1:1000 and 1:100000 in LB and 50 µL were streaked out on LB plates containing Cam. Following 48 hours of incubation, clones were picked and grown in 5 ml LB o/n for BAC DNA preparation, RFLP and sanger sequencing of the mutated region.

En passant mutagenesis can be used in a similar manner to insert or delete larger sequences from viral BACs[132]. For insertion of longer stretches of DNA sequence or entire genes, a transfer plasmid containing the gene of interest containing the *aphA*I insertion must be generated and amplified with primers containing overhangs homologous to the region were insertion is intended.

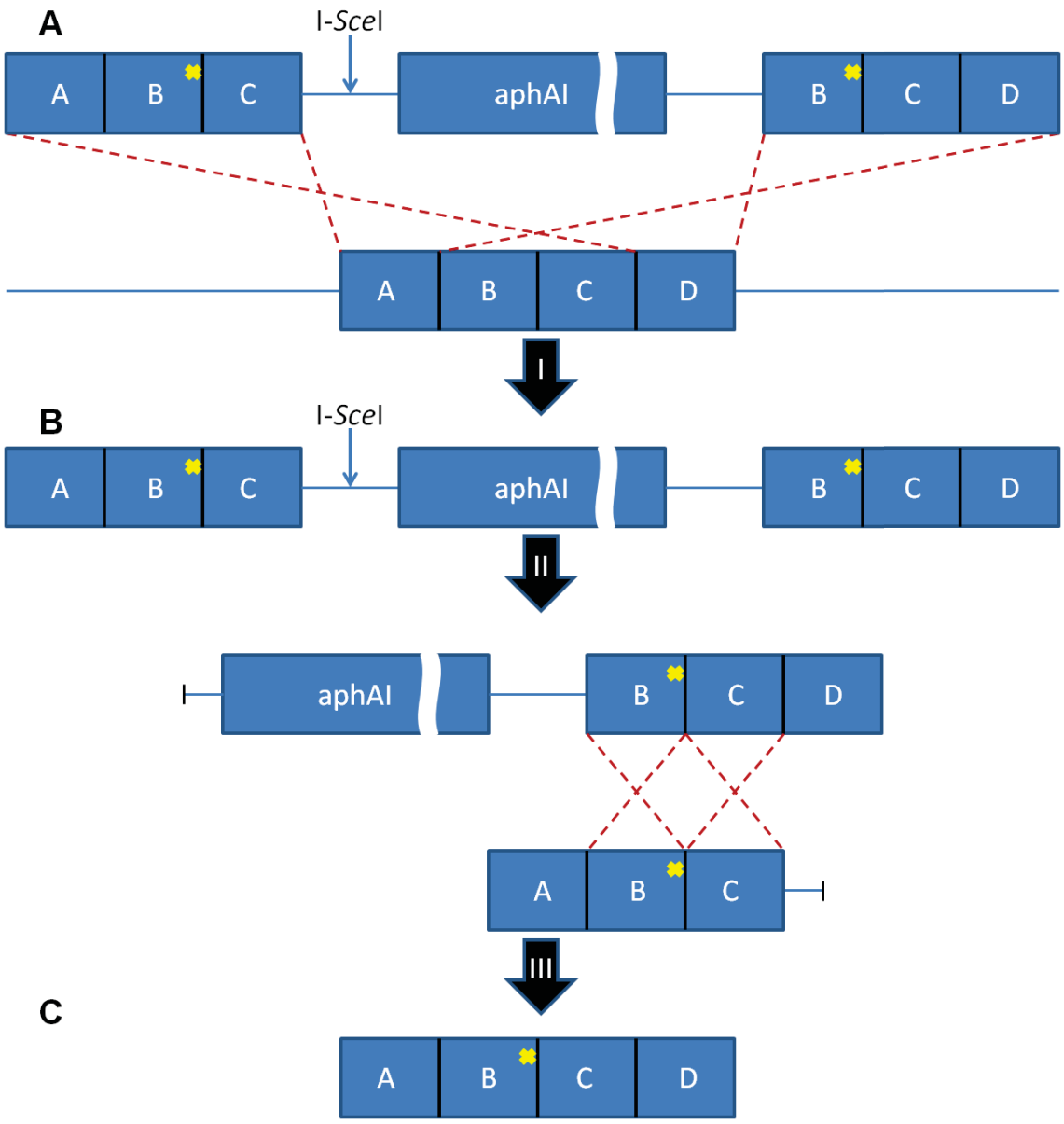

Figure 8 Two step Red mediated recombination (*en passant* mutagenesis). **(A)** GS1783 electrocompetent *E. coli* that harbor the BAC of interest are transformed with a PCR product containing a kanamycin resistance gene (*aph*AI) flanked by sequences homologous to the recombination site and including the desired change in nucleotides (yellow cross) as well as a single I-*Sce*I recognition site. The first recombination event occurs between the homologous sites and yields the insertion of the kanamycin resistance in the target region. **(B)** Upon induction, the I-*Sce*I endonuclease is produced and induces a double strand break at its unique 18 bp recognition site. This double strand break is repaired through a second homologous recombination that takes place in the duplicated region at the target site. This recombination event leads to the exclusion of the kanamycin resistance and leaves **(C)** the desired change within the target site.

5.2.3 DNA extraction procedures

Isolation of total DNA from infected cell monolayers (sarcosyl extraction)[138]

For extraction of total DNA from cell culture samples, the monolayer was washed with PBS, cells were trypsinized, resuspended in growth medium and pelleted by a 5 minute centrifugation at 4°C and 800 g. The pellet was washed with 10 ml of ice cold PBS and centrifuged again under the same conditions. Cells from a 100 mm dish were resuspended in 500 µL TEN buffer and lysed by the addition of 250 µL sarcosine lysis followed by a 15 minute incubation at 65 °C. RNA was removed by the addition 10 µL RNAse A (10 mg/mL) and 30 minutes incubation at 37 °C. Protein was digested during a 16 hour incubation after the addition of 10 µL proteinase K (20 mg/mL). DNA was extracted using a standard phenol/chloroform procedure followed by alcohol precipitation. After centrifugation, DNA was resuspended in 50 µL TE or EB buffer and DNA concentration determined by spectrophotometry.

Extraction of extrachromosomal DNA (Hirt extraction)[139]

The extraction of extrachromosomal DNA enables recovery of circular viral genomes from infected cells. DNA obtained by this method can be used for RFLP analysis or transformation of electrical competent E. coli (MegaX, Invitrogen). To extract extrachromosomal DNA, infected cells where trypsinzed, pelleted by centrifugation at 4 °C and 2000 g for 5 minutes. The pellet was washed once with 10 ml of ice cold PBS, centrifuged as mentioned above and resuspended in 200 µl PBS per 100 mm dish. 200 µL Hirt lysis buffer was added mixed gently by inverting the tube 10 times and left for 20 minutes at RT. Then, 100 µL of a 5 M NaCl solution was added to obtain 1 M NaCl final. The tube was incubated at 4 °C for >16 hours (o/n). Following this, the mixture was centrifuged at 4 °C and 15000 g for 30 minutes to pellet the precipitated protein/chromosomal DNA. The supernatant was taken off and used for a regular phenol/chloroform extraction followed by ethanol precipitation. For desalting, the pellet was incubated with 2 ml of 70% ethanol for 30 minutes, the ethanol was removed and the procedure repeated four times in total. Finally, the pellet was resuspended in 25 µL of DEPC treated ultrapure water and frozen at -20 °C or used directly for electroporation of *E. coli*. The procedure was scaled up and buffer volumes were multiplied by 2.5, when 150 mm cell culture plates were used as starting material.

Phenol/chloroform extraction of nucleic acids[140]

To extract nucleic acids from precleared cell lysates, the following procedure was performed. The cell extract was mixed with an equal volume of phenol/chloroform/isoamylalcohol (25:25:1) and mixed gently by inverting the tube 10 times. For phase separation the mixture was centrifuged at 11000 g for 10 minutes at RT. The aqueous phase containing the nucleic acids was taken off carefully to avoid contamination with denatured proteins at the interphase and gently mixed with an equal volume of chloroform. Centrifugation was carried out as mentioned above and again, the aqueous phase was taken off carefully.

If absolute purity of the nucleic acid was crucial (e.g. use in NGS), an additional first step was added to the protocol where an equal volume of pure phenol saturated with TE was added to the precleared cellular extract.

Alcohol precipitation of nucleic acids[140]

To precipitate DNA/RNA from aqueous suspensions, 2 volumes of ethanol or 0.7 volumes of isopropanol were used. If the DNA/RNA extract was not rich in salt an additional 0.1 volumes of 3M sodium acetate or 8M lithium chloride was added to facilitate precipitation. Precipitation was allowed to occur during a 30 minute incubation at RT, then the mixture was centrifuged at >15000 g for 30 minutes. To prevent overheating of the sample, a cooling centrifuge set to 4 °C was preferred. The obtained pellet was washed with 70% ethanol and resuspended in the desired amount of EB, TE or water.

Micrococcal nuclease treatment[107]

To obtain DNA from viral nucleocapsids, infected cells were harvested from a 15 cm cell culture dish, pelleted at 4 °C and 2000 g for 5 minutes. The pellet was washed once with ice cold PBS, centrifuged again and afterwards resuspended in 10 ml permeabilization buffer. To pellet the nuclei, 15 minute centrifugation at 1300 g and 4 °C was carried out; the obtained pellet was again resuspended in 10 ml permeabilization buffer and centrifuged using the same conditions. The nuclei pellet was then resuspended in 100 µl nuclei buffer and 100 µl 2x nuclease buffer with 2,5 µl micrococcal nuclease (200 Kunitz Units per µl) and 5 µl RNAse A (10 mg/ml) was added. Digestion of unprotected nucleic acids took place during 1 hour incubation at 37°C. The reaction was stopped by the addition of 2,5 µl 0.5 M EDTA. To release DNA from viral nucleocapsids, 400 µl digestion buffer and 5 µl proteinase K were added and incubated at 55°C >16h (o/n). The obtained extract was used for phenol/chloroform extraction of DNA.

When used directly for NGS library preparation, it was necessary to remove small DNA fragments in the extracts. To achieve this, AMPure XP magnetic beads (Beckman Coulter) were employed for DNA size selection. To avoid binding of fragments <500 bp, AMPure beads were removed from the buffer and resuspended in an equal volume of 7.5% PEG bead binding buffer[141].

Extraction of DNA from animal tissues

To extract DNA from animal tissues, the innuSpeed tissue DNA kit from Analytic Jena was used according to the manufacturer's instructions. In brief, 50 mg of tissue was homogenized using a bead mill procedure. The homogenate underwent RNAse A and proteinase K digestion as suggested by the manufacturer with the exception that proteinase K treatment was extended to 90 minutes to ensure release of viral DNA from nucleocapsids. The resulting lysate was cleared by addition of a protein denaturing buffer and high speed centrifugation. The DNA contained in the supernatant was captured on DNA binding columns. After several washes, DNA was eluted in 200 µL EB and used for further analysis (mainly qPCR and NGS).

Extraction of plasmid/BAC DNA from E. coli cultures[140]: “MiniPrep”

To obtain plasmid or BAC DNA for methods such as PCR, cloning and RFLP analysis, 5 ml LB were inoculated with the bacterial clone of interest and incubated at 37° (32° for recombination competent strains) o/n. Bacteria were pelleted at 5000 g for 10 minutes and the pellet was resuspended in 250 µL buffer P1. An equal amount of P2 was added and tubes were inverted 10 times for gentle mixture of the contents. After 5 minutes at RT, 250 µL buffer P3 was added and the mixture was incubated for 5 minutes on ice to facilitate removal of proteins. To remove denatured protein,5 minutes of centrifugation at >15000 g was carried out. The supernatant was used for phenol/chloroform extraction.

Extraction of plasmid/BAC DNA from E. coli cultures: “MidiPrep”

To obtain plasmid or BAC DNA for transfection of cells, Qiagen’s column based MidiPrep kit was used according to the manufacturer’s instructions. In brief, 50 ml (high copy number plasmids) to 200 ml (BACs) of LB were inoculated with the bacterial clone of interest. Bacteria were pelleted and resuspended in 4 or 10 ml of buffer P1, cells were lysed with an equal amount of buffer P2 and protein was precipitated following addition of 4 or 10 ml buffer P3. For removal of precipitated protein, the mixture was centrifuged at 230000 g at 4°C for 30 minutes and filtered through a 40 nm cell strainer. DNA was captured during gravity flow through on DNA binding columns, washed twice and eluted in 5 ml of a proprietary isopropanol containing elution buffer. DNA was precipitated using isopropanol. The pellet was resuspended in 100-500 µL EB or TE.

Isolation of DNA from chicken blood

Isolation of total DNA from 10 µl chicken EDTA blood was performed in a 96-well plate format using the E-Z® 96 blood DNA isolation kit from Omega Biotek according to the manufacturer’s instructions with two important modifications. (1) The volume of blood used for DNA extraction was reduced to 1/25th of the amount suggested by the manufacturer. The kit is designed for use with mammalian blood where the vast majority of cells are erythrocytes and do not contain a nucleus. The red blood cells of all other vertebrates including the *Sauropsida* contain a nucleus[142] which implies a considerably higher DNA content of chicken whole blood when compared to mammalian whole blood. To avoid overloading of the DNA binding columns, 10 µl of chicken EDTA blood was diluted with PBS to a total volume of 250 µl used in the extraction procedure. (2) To ensure release of viral DNA from nucleocapsids, the proteinase K digestion was extended from 10 minutes to 90 minutes.

5.2.4 PCR and qPCR protocols

Polymerase chain reactions (PCR) were carried out for multiple purposes which will briefly be described here. Generally, PCR reactions were performed in a total volume of 50 µl. Reactions were scaled down to 25 µl if a large number of clones had to be screened; otherwise, the reaction volume was scaled up to 100 µl if larger amounts of PCR product were required. All polymerases were used in the respective reaction buffers provided by the manufacturer. The final reaction typically contained 1x reaction buffer, 0.2-1 µM of each primer, 200-400 µM dNTPs, 1 ng- 1 µg of template DNA and the recommended amount of DNA polymerase. If primer specific annealing temperatures were applied, the NEB Tm Calculator (http://tmcalculator.neb.com) was used to identify annealing temperatures, suitable for both the respective primers and polymerase.

1. Sanger sequencing and cloning

   To obtain DNA for sanger sequencing and DNA cloning procedures, PCR reactions were conducted using primers specific for the region of interest. To avoid erroneous sequencing results, high fidelity DNA polymerases such as Phusion® or Q5® were used in the following PCR program.

Temperature	Time	Purpose	No. of cycles
98 °C	**60 s**	Initial denaturation	
98 °C	**20 s**	Denaturation	**25-40**
50-68 °C (primer specific)	**15 s**	Primer annealing	
72 °C	**30 s** per kb	Elongation	
72 °C	**300 s**	Final elongation	
8 °C	**∞**	Hold	

Table 5 High fidelity PCR programme

2. PCR reactions to determine presence and orientation of specific sequences

   PCR reactions were conducted with taq Polymerase if the PCR was not indented for use in downstream procedures such as sequencing or cloning. To screen for successful cloning and the correct orientation of inserts, PCR was conducted with isolated plasmid DNA or directly with material obtained from bacterial colonies on agar plates (colony PCR). The following reaction conditions were applied.

Temperature	Time	Purpose	No. of cycles
95 °C	**120-300 s**	Initial denaturation	
95 °C	**30 s**	Denaturation	**30-42**
48-65 °C (primer specific)	**30 s**	Primer annealing	
68 °C	**60 s** per kb	Elongation	
68 °C	**300 s**	Final elongation	
8 °C	**∞**	Hold	

Table 6 Taq PCR programme

3. PCR reactions with overhanging primers

To achieve the introduction of desired sequences into cloning vectors or through homologous recombination, PCR reactions with primers containing specific overhangs (e.g. restriction sites, protein tags, recombination flanks) were performed. LongAmp® and PrimeStar® DNA polymerases showed superior efficiency in incorporating overhangs into PCR products. If fidelity was only a minor concern (e.g. for co-integration of a selection marker) the error prone LongAmp® polymerase was used in a two step PCR regimen, details are provided in the BAC mutagenesis section. For reactions requiring higher DNA copying fidelity, PrimeStar® DNA polymerase was used under the following conditions.

Temperature	Time	Purpose	No. of cycles
98 °C	**30 s**	Initial denaturation	
98 °C	**10 s**	Denaturation	} **10**
55 °C	**15 s**	Primer annealing	
68 °C	**60 s** per kb	Elongation	
98 °C	**10 s**	Denaturation	} **32**
60 °C	**15 s**	Primer annealing	
68 °C	**60 s** per kb	Elongation	
68 °C	**180 s**	Final elongation	
8 °C	**∞**	Hold	

Table 7 Mutagenesis PCR programme

Quantitative PCR (qPCR) analysis

Throughout the study, a number of qPCR reactions were performed to quantify the amount of viral DNA in relation to host DNA. To achieve highest specificity, a TaqMan protocol was carried out, using primers and probe specific to the target region. To determine the copy number of cellular DNA, primers and probe for the inducible nitric oxide synthase (iNOS) were constructed. For the detection of viral DNA, primers and probe were designed to match a region in ICP4, a gene located in the IRS and TRS of the viral genome. Since avian cells carry a diploid set of chromosomes and ICP4 occurs twice in the viral genome, this design enables direct comparison of copy numbers without mathematical correction.
DNA was extracted from cell cultures or animal tissues according to one of the procedures mentioned above. Standard curves were generated using a MDV BAC dilution for ICP4. For iNOS, a plasmid carrying part of the chicken iNOS gene was used. Dilutions were calculated based on initial spectrophotometry measurements and carried out in 10 copy steps from 0 to 10^7 copies.
Reactions were performed on a ABI 7500 fast real time PCR machine (Thermo Fischer) using the sensiFast® master mix (Bioline, Luckenwalde), 100 nM of each primer and probe and 2 µL sample DNA in a 20 µL reaction. Reactions were generally performed in duplicates using the following conditions (see table 8).

Temperature	Time	Purpose	No. of cycles
95 °C	**180 s**	Initial denaturation	
95 °C	**15 s**	Denaturation	} **35**
55 °C	**15 s**	Primer annealing	
68 °C	**30 s**	Elongation	

Table 8 qPCR programme

The gene copy numbers measured in this procedure were typically normalized and results formulated in viral copy numbers/chicken copy numbers.

5.2.5 Expression and purification of MDV DNA polymerase

Purification of FLAG tagged MDV Pol

For purification of functional MDV DNA polymerase, CEC were transfected with an HVT clone harboring an insert of CMV-MDV_U_L30-FLAG-PolyA in its MiniF region. This gave rise to virus with WT-like growth that overexpressed U_L30 as determined by WB analysis. The virus was propagated for two passages and frozen at a titer of $>10^5$ pfu/ml for future experiments. Frozen virus was used to infect CEC on a 150 mm plate, infected cells were either harvested when large plaques became apparent (typically 3 days p.i.) or expanded in one more passage to harvest a larger number of infected cells. For harvest, cells were trypsinized, centrifuged at 1,200 g (4 °C) and the resulting pellet was washed once with ice-cold PBS. A pellet resulting from one 150 mm plate (about 2×10^7 cells) was resuspended in 500 µl buffer SuperB® containing protease inhibitor H and EDTA or 900 µl native protein extraction buffer[128] containing Complete® protease inhibitor. Volumes were scaled up as necessary. The sample was transferred to AFA glass tubes, microTubes (130 µl) were used for smale scale purification and WB analysis, milliTubes (1,000 µl) for preparative purification. Lysis was performed using a Covaris M220 focused ultra-sonicator applying the following conditions: peak incident power: 75 W, duty cycle: 10%, cycles per burst: 200, duration: 240 s, temperature: 6 °C. The crude lysate was cleared during a 10 minute centrifugation at 15,000 g and 4 °C. The supernatant was collected for purification and the pellet containing cellular debris was discarded after first WB analyses had indicated that no significant amount of FLAG tagged protein was retained within the pellet.

For purification, the supernatant was mixed with an equal volume of each TBS and BC-300[129] (or only BC-300 if native protein extraction buffer was used) and transferred to a LoBind® microcentrifuge tube (1.5 or 2 ml as needed). To this mixture a 50% slurry of Anti-FLAG® M2 magnetic beads corresponding to 1/5 of the original lysate volume was added and incubated for 6 h to o/n at 4 °C under gentle end-over-end rotation. The next day, magnetic beads were collected with a neodymium bar magnet, the supernatant was removed and the bead pellet washed with five times with 1 ml TBS, each time the pellet was resuspended and collected again magnetically. Bound protein was eluted with 200 µM FLAG peptide in BC-300, the volume corresponding to 1/10 of the original lysate volume during 2 minutes at room temperature. To ensure that the majority of protein was eluted in this step, a pH elution was performed with the same volume of 0.1 M Glycine pH 3. Protein was used either directly (for functional assays) or stored at -20 °C after the addition of 1/3 (v/v) 99% Glycerol for WB analysis.

Normalization of protein input

To accurately determine the enzyme activity of purified proteins, normalization of the functional assay input is crucial. To achieve good relative quantification of protein input, two quantification steps were employed. First, the total protein concentration in purified extracts was determined using a commercial BCA assay kit (BCA Protein Assay Kit, Pierce) according to the manufacturer's instructions. Based on total protein concentration, the input volume for WB analysis was normalized to equal protein levels. On the resulting WB, target protein was detected using a rabbit polyclonal anti FLAG antibody together with an anti-rabbit-HRP conjugated secondary antibody. Final protein input normalization was based on

the intensity of the bands detected using the above procedure and a Vilber-Lourmat Imaging System with the Bio 1D software (Bioprofil, Vilber-Lourmat). Please note that absolute quantification of protein input was not attempted.

In vitro transcription and translation

The in vitro T'N'T kit (Promega L4610) was used according to the manufacturer's instructions. Briefly, 0.5-1 µg of circular plasmid DNA was incubated for 90 minutes with the rabbit reticulocyte lysates under the addition of T7 RNA polymerase and ^{35}S-Methionin in a total volume of 50 µl at 30 °C. Following this incubation, 5 µL aliquots were removed, mixed with an equal volume of 2x Laemmli buffer and loaded onto a 7.5 % polyacrylamide gel for SDS gel electrophoresis. The sample was allowed to pass through the stacking gel at 75 V, electrophoresis was continued at 120 V once the sample had reached the running gel. The SDS PAGE gel was dried for 3 hours in a "Model 583" gel dryer (Biorad) and subsequently exposed to a radiation sensitive film for 24, 48 or 96 hours. The film was developed and dried for visual examination.

5.2.6 Functional assays using recombinant MDV DNA polymerase

Polymerase and exonuclease assays

Polymerase activity was measured for each Pol mutant by adding a normalized amount of purified Protein to 50 µl of 2× polymerase buffer and 10 µl activated calf thymus DNA (2 µg/µl), water was added to 100 µl. The reaction was incubated at 37 °C for 30 minutes and at 42 °C for 30 minutes and cooled to 4 °C when incubation time was over. DNA was precipitated by the addition of 50 µg fish sperm DNA (4 µg/µl) and 100 µl of 10% trichloroacetic acid (TCA) and spotted on a Whatman glass filter. The filter was washed with 10 ml 10% TCA, 20 ml 70% Ethanol and 10 ml 99% Ethanol and dried at room temperature o/n.

Exonuclease activity was measured for each Pol mutant by adding a normalized amount of purified Protein to 50 µl of 2x exonuclease buffer and 8 µl activated calf thymus DNA (2 µg/µl) as well as 1 µCi of radioactive exonuclease template, water was added to 100 µl. The reaction was incubated at 37 °C for 30 min and at 42 °C for 30 min and cooled to 4 °C when incubation time was over. The reaction was terminated by the addition of 10 µl 0.5 M EDTA and DNA was precipitated by adding 50 µg fish sperm DNA (4 µg/µl) and 100 µl of 10% trichloroacetic acid (TCA). The mixture was centrifuged at 15,000 g for 30 min at 4 °C. The supernatant was carefully removed from the pellet and another 50 µg of fish sperm DNA was added and thoroughly mixed in to facilitate the removal of carryover acid insoluble radioactivity. Following another 30 minutes centrifugation at 15,000 g and 4 °C, the top 200 µl of supernatant was used for liquid scintillation counting.

As a negative control for both reaction types, WT enzyme was heat inactivated during 10 minutes at 98°C and used under the conditions stated above.

Liquid scintillation counting

Dried glass filters from polymerization reactions were placed into 20 ml scintillation vials and 10 ml scintillation fluid was added. The vials were thoroughly agitated in an end-over-end shaker for 10 min and scintillation was counted in a TRI-Carb liquid scintillation counter using an appropriate protocol.

The supernatant of exonuclease reactions (200 µl) was neutralized with 50 µl of 1 M NaOH and buffered with 100 µl 1 M Tris-HCl pH 8,0. The resulting solution was mixed with 10 ml scintillation fluid and filled into 20 ml scintillation vials for counting in a TRI-Carb liquid scintillation counter using an appropriate protocol.

Preparation of radioactive exonuclease templates

Exonuclease substrate was prepared similar to a procedure described previously[112], by the incorporation of ^{3}H-dTTP into activated calf thymus DNA using exo^{-} Klenow Enzyme. In 100 µl reaction volume 1× NEB buffer 2 with 20 units of exo^{-} Klenow Enzyme was supplemented with 50 µg activated calf thymus DNA, 100 µM dCTP, dATP, dGTP, 10 µM dTTP and 25 µCi ^{3}H-dTTP. Incorporation of radioactivity was allowed to occur during 30 minute incubation at 37 °C. The reaction product was extracted with phenol/chloroform and precipitated with ethanol. The resulting pellet was washed extensively with 70% ethanol. Trace amounts of unincorporated radioactivity were removed by gel filtration through Illustra microspin g-25 columns (GE Healthcare) according to the manufacturer's instructions. Successful incorporation of radioactivity was determined by liquid scintillation counting. The exonuclease template was adjusted to 0,2 µCi per µl and stored at -20 °C until further use.

5.2.7 NGS Sequencing and target enrichment

DNA sequencing procedures

DNA sequencing was performed on an Illumina MiSeq machine. Sequencing libraries were prepared from DNA extracted from infected chicken cells, tissues of infected animals or E. coli transformed with viral BAC sequences.

One to five micrograms of total DNA was diluted in 130 µl TE and fragmented to a peak fragment size of 500-700 bp using the Covaris M220 focused-ultrasonicator. The typical settings were: peak incident power: 75W, duty cycle: 5%, cycles per burst: 200, duration: 52s, the settings, however, were adjusted if the procedure failed to produce fragments of the desired size. Size distribution of DNA fragments was confirmed via agarose gel electrophoresis (1% Agarose gels) or using a Bioanalyzer (Agilent Technologies). Fragmented DNA (100 ng to 1 µg) was used for NGS library preparation using the NEBNext Ultra II Library Prep Kit for Illumina platforms (New England Biolabs). The kit was used according to the manufacturer's instructions, the bead based size selection step was performed using AMPure XP magnetic beads (Beckman Coulter) selecting for inserts of 500-700 bp. To achieve a library yield >500 ng and complete the adaptor sequences, 3-5 PCR steps were performed at the end of the protocol.

Enrichment of viral sequences

To specifically enrich viral sequences from DNA extracts that contained mainly sequences of chicken origin, a tiling array was employed. This array consisted 6292 biotinylated RNA 80-mers and was designed against the sequence of RB-1B (GenBank EF523390.1) using the bioinformatic tools and expertise of Mycroarray, Ann Arbor, MI. The process was the following:

The sequence of RB-1B (GenBank EF523390.1) was run through RepeatMasker.org to soft-mask simple repeats and low complexity DNA. 0.48% of the sequence was masked, containing 0.45% simple repeats and 0.03% low-complexity DNA.
Following this, 80-mer baits with ~3x flexible tiling density, or ~27bp probe spacing were designed. In flexible tiling, bait position is allowed to shift slightly up or downstream, so that the baits are evenly distributed along the entire locus, since most loci are not perfect multiples of the spacing interval. This process returned 6,365 unfiltered baits.
These baits were screened against the chicken (*Gallus gallus*) reference genome using proprietary software of Mycroarray. Briefly, all the baits were BLASTed against the genome, so that specific/unique baits will have no hits, and non-specific/non-unique baits will have one or more hits. Then the melting temperatures (Tm's) of all the hits were tallied and a judgement was made about whether or not to drop that individual bait from the baitset, depending on how many, the Tm composition of the hits, and the performance of the neighboring baits. There were very few probes that proved non-specific and had many hits in the chicken genome, those baits were dropped from the baitset design. The process described above was repeated for the human hg38 reference genome. The same baits were flagged as unspecific, thus it was not necessary to drop any more baits to avoid contamination with human sequences.
Taking the 6,354 baits that passed the filter, all baits containing any soft-masked sequence

were removed. This left 6,299 baits that did not produce any chicken or human hits and did not contain simple repeats. Since herpes virus genomes contain large inverted repeats, a self-BLAST (keeping only opposite-strand hits) was applied in order to assess the self-complementary of the baitset; i.e., are there baits that could anneal to each other? This returned approximately 4,440 instances of bait-to-bait hybridization. In order to mitigate these issues Mycroarrays software automatically reversed and complemented problematic baits to achieve an optimal baitset. Applying this process, only 6 instances of bait-to-bait hybridization were found, a number small enough to be disregarded. The resulting baitset of 6299 biotinylated RNA 80mers was used for target enrichment of viral DNA sequences.

Since the original design produced excellent target enriched sequencing libraries, a second baitset was ordered. The design was the same as mentioned above with one modification: To the final design, the MiniF sequence, containing the bacterial origin of replication and antibiotic resistance of the virus BAC was added. This meant the addition of another 298 RNA baits, leaving the final baitset with 6597 probes.

The outstanding ability of this method to enrich viral target sequences without introducing a measurable bias was confirmed by enrichment and re-sequencing of MDV DNA from a recent study conducted by colleagues from the Pennsylvania State University[143]. Crude DNA extracts from feather dust and chicken feather were provided by M. Szpara and underwent the enrichment and sequencing procedure described above. The resulting raw data was bioinformatically analyzed at the Pennsylvania State University. Analysis revealed that with the tiling array a great increase in coverage was achieved without any loss of accuracy in SNP detection by sequencing (M. Szpara, personal communication). This makes target enrichment a superior way of NGS sequencing of MDV DNA for SNP detection.

Sequence data analysis

To identified mutations the sequence data was mapped against the reference sequence using the Genome Sequencer software suite (v. 3.0; Roche). Mutations with a frequency above 10 % and a coverage of at least 5 reads were extracted from the 454HCDiffs.txt file. To compare positions of mutations over multiple samples the data was merged by position and mutation using R[144]. For final counts of mutations, original Pol mutations were subtracted from the total count; consequently reversions of original Pol mutations were counted as mutations. For analysis of BAC clones, only mutations with a presence of more than 90% in NGS reads were accepted for final counts.

5.2.8 Animal experiments

For the *in vivo* experiments, one day old Valo specific pathogen free (SPF) chicken (Valo Biomedia) were randomly assigned to one of the groups and infected with a Pol mutant virus or one of the respective control viruses. For the exact number of birds in each group see table 9. Infection was performed intraabdominally using 4000 pfu of each virus in 500 µl cell culture medium. The animals were housed in four groups A-D, keeping the mutants apart and housing animals infected with WT and revertant viruses together. Although housed together, animals in group D received different inocula (group D1-D5), despite this, all viruses used in group D were confirmed to share the original WT sequence. Within each group 10 uninfected birds (9 in group A) were placed to monitor for horizontal transmission of the virus. Animals were kept under a 12 hours light regimen in stainless steel cages with wood litter and straw. Enrichment was provided by perches, sand baths and picking stones. Rooms were air-conditioned and temperature was regulated starting from an air temperature of 28 °C on day 1 going down to 20 °C on day 21. In the first ten days, heat lamps were provided additionally. Food and water were available *ad libidum*. Blood samples (20-60 µl) were taken on days 4-7-14-21-28 post infection (p.i.) for infected animals and on days 21-28-35-42 for uninfected contact animals. Blood was mixed with 10 µl of 200 µM EDTA (pH 8) and stored at -80 °C until DNA extraction. During 90 days, the animals were monitored twice daily and terminated at the first clinical sign of MD. Each animal that died or was euthanized during the experiment underwent a thorough post mortem examination for gross pathological signs of MD. These signs included: Solid lymphomas in any organ, inflammation of peripheral nerves evidenced by marked swelling and ocular lymphomatosis as evidenced by characteristic changes in the iris. Samples were taken from each animal; typically at least samples from spleen, liver, kidney and skin with feathers were frozen from each animal for DNA extraction. On day 91 p.i. the remaining animals were terminated and samples were taken accordingly. All animal experiments were performed in accordance with relevant institutional and legislative regulations. Animal experiments were approved by the Landesamt für Gesundheit und Soziales in Berlin, Germany (approval G0218-12).

Group		Virus	No. of infected birds (day 1)	No. of uninfected contact birds
A		Y547S	25	9
B		Y567F	25	10
C		L755F	25	10
D	D1	WT Dex	12	10
	D2	Y547S Rev	13	
	D3	Y567F Rev	13	
	D4	WT	13	
	D5	L755F Rev	13	

Table 9 Numbers of birds infected with the different Pol mutant (Y547S, Y567F and L755F), Revertant (Rev) WT (WT) and dexamethasone treated (WT Dex) viruses.

5.2.6 Statistical analyses

MDV Plaque Size: Plaque sizes were analyzed using Kruskal–Wallis one-way analysis of variance with Dunn's multiple comparison. Always, all groups are compared to WT.

MD incidence during the 90 day animal experiment is plotted as survival curve and statistically analyzed using the Log-rank (Mantel-Cox) Test.

Virus titers were analyzed using one-way ANOVA with Bonferroni's multiple comparison. Always, all groups are compared to WT.

A difference is considered statistically significant if $P \leq 0.05$. Different levels of significance are marked in figures by the following number of asterisk:

* $P \leq 0.05$

** $P \leq 0.01$

*** $P \leq 0.001$

All statistical analyses were performed using GraphPad Prism v5.01.

6. Results

6.1 Generation and *in vitro* characterization of Pol mutant MDV

Based on available information from literature as discussed above, eight Pol mutants of MDV were generated through en passant mutagenesis. Seven of these had point mutations in one of the exonuclease domains and one carried a point mutation in the finger domain of Pol. Successful BAC mutagenesis was confirmed by RFLP analysis using two different restriction enzymes and subsequent Sanger sequencing of promising clones (see fig. 9).

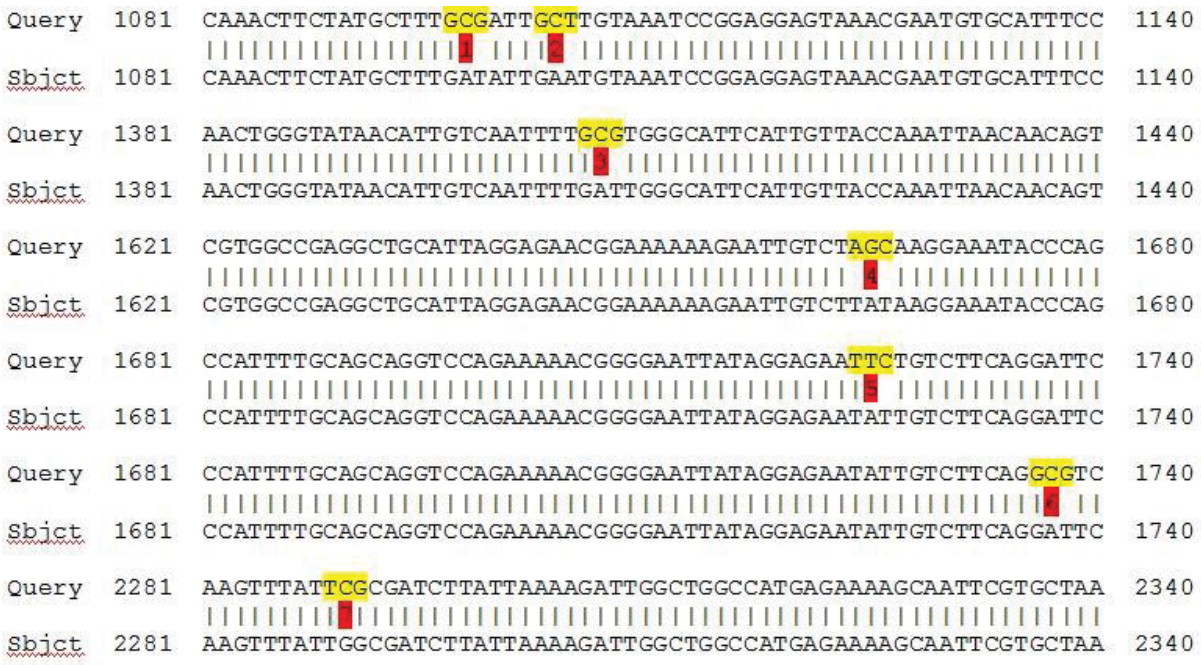

Figure 9 Results of Sanger sequencing of the mutated UL30 gene aligned to the WT sequence. Codon changes are marked in yellow. These mutations are coding for the following amino acid changes: (1) D358A, (2) E360A, (3) D461A, (4) Y547S, (5) Y567F, (6) D571A, (7) L755F.

All of the mutant viruses could be reconstituted upon transfection in CEC cell culture. In vitro growth post transfection was achieved for all viruses, visible plaques started to appear 5-7 days post transfection. All plaque sizes were compared to WT using Kruskal-Wallis nonparametric test with Dunn's multiple comparison. Viruses could be classified into three groups according to their plaque sizes:

1. Plaques caused by mutants D358A/E360A, Y547S and L755F were not significantly different in size when compared to WT
2. Plaques caused by mutants D358A, E360A and Y567F were slightly smaller than those caused by WT virus ($P<0.05$)
3. Plaques caused by mutants D461A and D571A were markedly smaller than those caused by WT ($P<0.001$)

The biological significance of plaque size differences post transfection as it is observed between groups 1 and 2 is questionable since the double mutant D358A/E360A falls into group 1 whereas mutants D358A and E360A are in group 2. Notably, Dunn's multiple comparison did not reveal any significant differences between WT and viruses from group 2 when all pairs of columns were compared with one another.

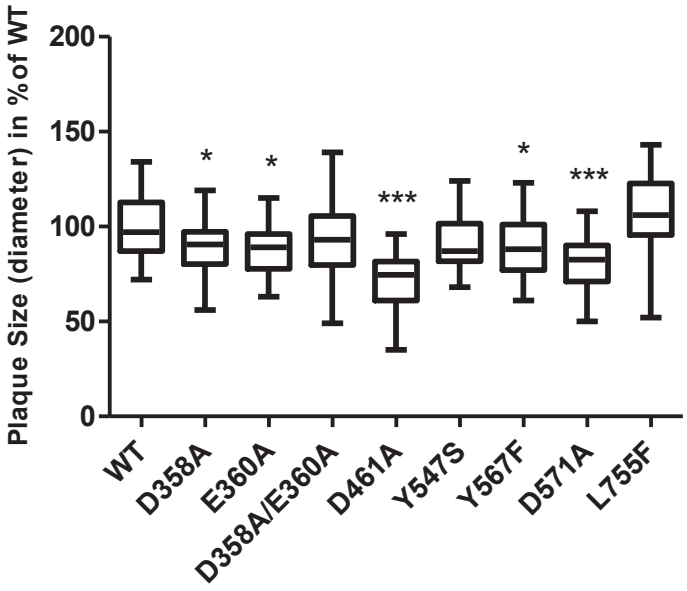

Figure 10 Plaque Size (diameter) of the different Pol mutants in percent of WT, 7 days post transfection, n= 50 (2x 25) plaques per virus, Kruskal-Wallis with Dunn's multiple comparison, all plaque sizes were compared to WT.

This picture changed dramatically upon passaging of viruses in cell culture. Several mutants lost their ability to grow after 2-5 passages in CEC. This is illustrated by a continual decrease of plaque size, as well as by the comparison of viral genome copy number ratios in cell culture. WT and some of the Pol mutants showed a vivid growth. In contrast, other mutants barely grew, as the number of plaques formed after the first passage remained almost unchanged. To illustrate the difference in *in vitro* genome replication, qPCR was performed on total DNA extracts from CEC cell cultures. Excluded from this analysis were mutants that did not maintain visible growth beyond passage II.

From passage II onwards, viruses could be grouped into two classes:

1. Viruses with WT-like growth potential in CEC. The average plaque sizes and genome copy numbers remained stable during passaging and were comparable to WT. Pol mutants Y547S and L755F can be assigned to this group.
2. Viruses with progressively diminished growth potential in CEC. The average plaque sizes and viral genome copy numbers of these viruses progressively and rapidly decreased after each passage.

Viruses that belong to the second group appear to be “suicidal”, as they loose the ability to maintain replication over time.

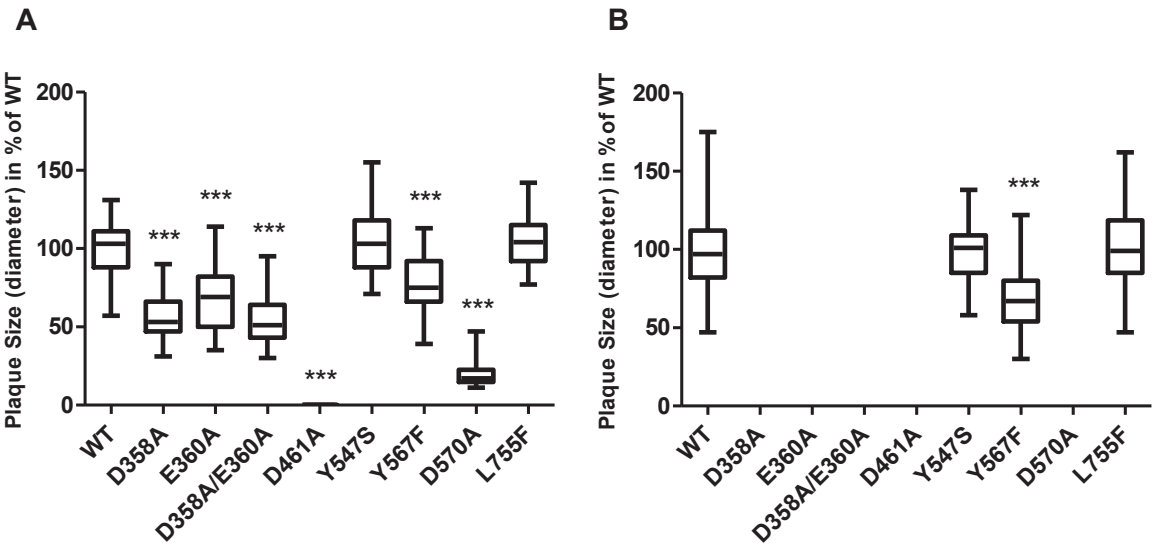

Figure 11 (A) Plaque sizes of the different Pol mutants in percent of WT at passage II. Plaques were measured 5 days post infection, n = 75 (3 ×25) plaques per virus, Kruskal-Wallis nonparametric test with Dunn’s multiple comparison, all plaque sizes were compared to WT. **(B)** Plaque sizes of the different Pol mutants in percent of WT at passage V. Plaques were measured 5 days post infection, n = 150 (3 × 50) plaques per virus, Kruskal-Wallis nonparametric test with Dunn’s multiple comparison, all plaque sizes were compared to WT.

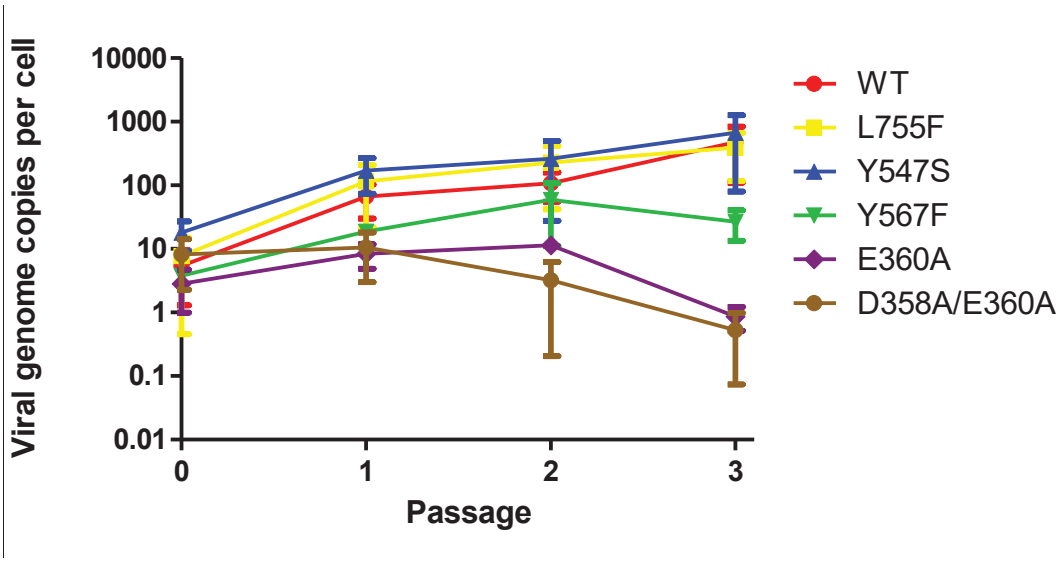

Figure 12 Efficiency of viral genome replication depicted as development of viral genome copy numbers per cellular genome over passaging in CEC. Three independent experiments are shown, the copy numbers were determined in duplicates in each experiment.

Taken together, these results show that some of the Pol mutants exhibit a severely impaired phenotype. Even in cell culture, in the absence of strong selection pressure, viral populations collapse if certain conserved amino acids related to exonuclease activity of the DNA polymerase are mutated. Importantly, the problem is not the reconstitution of viable virus from BAC DNA as this was possible for all of the mutants. Only upon passaging, some viruses experience a dramatic loss of viability.

6.2 *In vitro* analysis of polymerase function

To better understanding the role of DNA Pol in formation of genetic diversity of viral populations it was necessary to determine polymerase and exonuclease activities of different Pol variants. To obtain reliable results, it was vital to purify larger quantities of these proteins and study them in isolation from other DNA polymerases, which are ubiquitous in living systems, because these would confound the measurements.

Under the native promoter of U_L30, little protein is produced in HSV-1 infection, and the amount of protein produced is much lower than what would be expected from a weak promoter[145,146]. There is no information on the amount of Pol that is produced during MDV lytic infection in cell culture. However, the situation might be similar, because we were unable to detect production of His- or FLAG-tagged DNA Pol from its native locus by Western Blotting (WB). To our knowledge, there is no published report on overexpression and purification of MDV Pol. There is, however, a number of papers on the expression and purification of HSV-1 and other herpesvirus DNA polymerases. Literature indicates that it is difficult to purify a sufficient amount of native herpesvirus Pol for functional analysis. The crystal structure of HCMV Pol for example remains unknown due to difficulties in expressing sufficient amounts of protein[85]. There is one report about successful usage of *in vitro* transcribed and translated EHV-1 Pol for *in vitro* functional assays[147]. Generating sufficient amounts of *in vitro* transcribed and translated MDV Pol has been attempted in this study using an *in vitro* T'N'T kit according to the manufacturer's instructions with the addition of ^{35}S-Methionin as radioactive label. After MDV Pol failed to be expressed under the T7 promoter from the pBlueScript vector, the pTM1 vector was used for further trials. This vector contains the 5'UTR of the encephalomyocarditis virus (EMCV) upstream of a multiple cloning site. This 5'UTR serves as internal ribosome entry site (IRES) and was shown to substantially increase the yield in *in vitro* transcription/translation of HCMV Pol[148]. To obtain a pTM1 plasmid with an U_L30 insert, the gene was amplified using primers with overhangs for *BamH*I and *Xho*I. Subsequently the gene was cloned into the MCS of pTM1 using both of these enzymes. This resulted in the following construct: T7_ECMV-5'UTR_U_L30_PolyA. Using this plasmid in an in vitro T'N'T reaction with ^{35}S-Methionine resulted in a discrete band of about 140 kD after 96 hours of exposure to a radiation sensitive film. Comparison to radioactively labeled luciferase transcribed and translated from a control plasmid confirmed, that only very little Pol protein was generated in this procedure. Regardless of the presence of small amounts of protein, the T'N'T reaction mixture had no detectable polymerase activity.

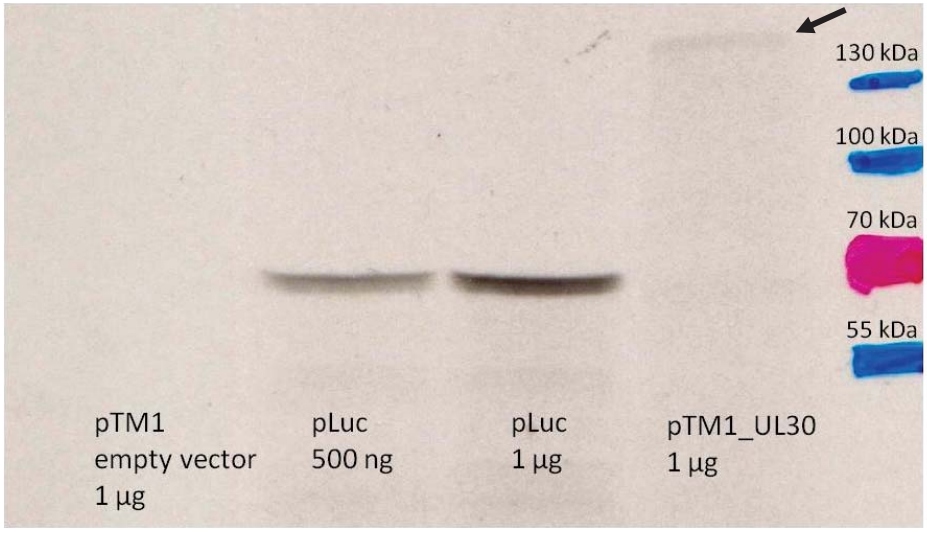

Figure 13 Radiation sensitive film exposed for 96 h to a SDS PAGE gel containing [35]S-Methionine labeled proteins. The black arrow points to a faint band resulting from the in vitro transcription and translation of the pTM1 plasmid containing an MDV UL_{30} WT insert. Positive controls are two different amounts of a proprietary control plasmid (Promega) containing a firefly luciferase insert. Negative control is the empty pTM1 vector.

Herpesvirus Pol has been expressed in different cell culture systems (mainly insect cells) and purified without a tag through phosphocellulose chromatography[112,149] as well as through N-terminal tagging with GST[150] or tagging with 6×His (position not specified)[151]. Since neither 6×His nor FLAG tagging of the DNA Pol in its native locus (both N-terminal, the C-terminus of U_L30 overlaps with U_L31) resulted in a visible band in WB, overexpression of the protein was attempted. Considering the need of a fully functional protein for further experiments, the optimal position for the tag was revisited. Both the extreme N- and C-terminus of the protein are dispensable for polymerase and exonuclease activity in vitro[152,153]. However, it has been conclusively shown, that 35 amino acids at the extreme C-terminus are essential for the interaction with the processivity factor U_L42[11], an interaction that is required for viral DNA replication *in vivo*[153,154]. To avoid interference with the interaction with U_L42, N-terminal tagging of U_L30 seemed to be the superior choice.

Plasmids containing U_L30 under a CMV promoter with an N-terminal 1x or 3×FLAG tag were generated via insertion of U_L30 truncated at the *Age*I restriction site (N-terminal ~ 200 bp missing) cloned through *Age*I and *Bgl*II into the pEP plasmid. Subsequently the missing N-terminal bases of U_L30 were amplified with primers containing overhangs for 1× or 3×FLAG tags as well as an *Age*I restriction site. The PCR product was cut with *Age*I and cloned into the pEP plasmid containing the N-terminally truncated U_L30, resulting in a construct that contained the following order of sequences: CMV_FLAG_U_L30_PolyA. Primers DK112 and DK113 were used to generate a fragment containing long homologous sequences for *en passant* insertion into the MiniF of an MDV (RB-1B) or HVT (90SE) BAC.

A pcDNA3.1+ plasmid containing a C-terminally 1×FLAG tagged U_L30 was kindly provided by Dr. Timo Schippers from our Institute. The U_L30 construct was amplified with primers containing overhangs for *BamH*I and *Kpn*I, cut and cloned into the pEP plasmid from where it was amplified and transferred into MDV and HVT BACs as described above.

Interestingly, I was unable to reconstitute virus from MDV BAC harboring U_L30 under CMV control in the MiniF region. This, however, is consistent with the observation in our lab that some genes under the control of the strong IE CMV promoter interfere with the replication of MDV in cell culture. For unknown reasons, HVT is much more tolerant to overexpression of foreign genetic elements under CMV inside the MiniF (Dr. Dusan Kunec, personal communication). Therefore, it did not surprise that I was able to obtain viable virus from both N-terminally and C-terminally tagged U_L30 constructs. This made it only more surprising to find a difference in the phenotype of the virus depending on the position of the tag in U_L30. The C-terminally tagged virus grew without obvious differences when compared to HVT WT, the N-terminally tagged virus, however, showed a severe defect in growth during the post transfection phase and in first passage and did not adopt a WT-like growth before passage two. To examine this phenomenon, extensive WB and sequence analyses were performed, leading to the following results:

In WB analysis, no band could be detected from the N-terminally tagged constructs when using a polyclonal anti FLAG antibody. In samples from cells infected with HVT harboring the C-terminally tagged construct, a band of approximately 140 kD was prominent. This band however faded with passaging of the virus and could not reliably be detected in virus beyond passage 4 (figure 14).

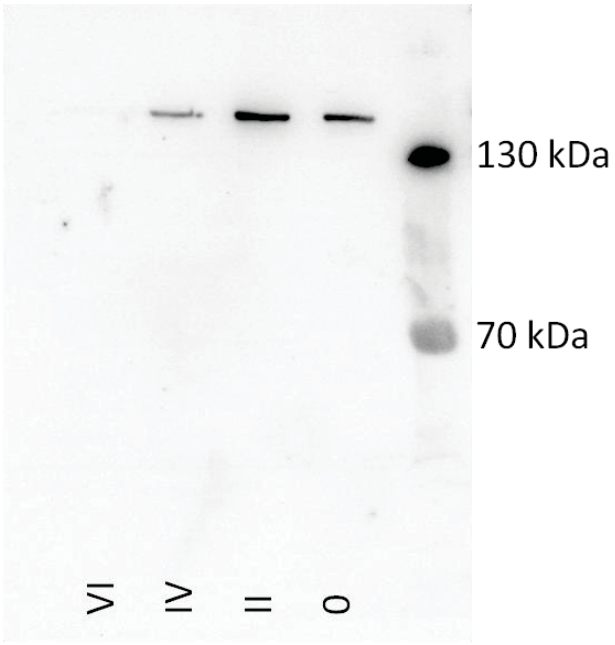

Figure 14 Western Blot of purified C-terminally FLAG tagged MDV DNA polymerase expressed in HVT under the IE CMV promoter. Purified extracts from infected cells at passage 0, I, II, IV and VI of the virus are presented. It is evident, that polymerase expression in this construct decreases with increasing passage number.

Sequencing of the N-terminally tagged constructs revealed that in some cases, there had been recombination events within the U_L30 ORF which likely destroyed the function of the enzyme. In most cases, however, the U_L30 ORF remained intact while the CMV promoter carried mutations that likely resulted in silencing of gene expression. Yet there were cases

were no apparent change in sequence was discovered in the region sequenced and still the WB result remained negative for MDV Pol expression.

Since expression seemed to only work with C-terminally tagged constructs, purification of MDV Pol was attempted from lysates obtained from cells infected with HVT carrying the C-terminally tagged U_L30 construct. Using this strategy, expression of detectable amounts of all mutant MDV DNA polymerases was achieved.

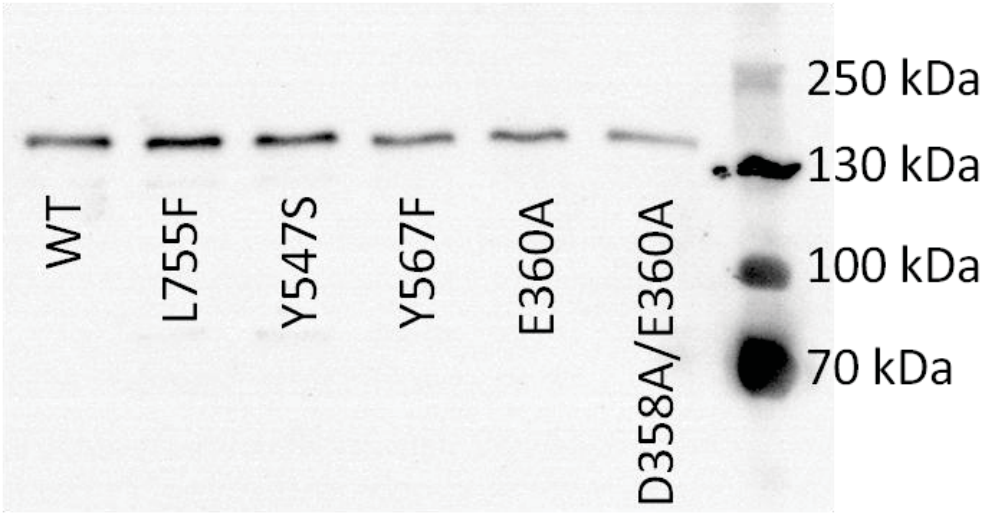

Figure 15 Western Blot of purified C-terminally FLAG tagged MDV DNA polymerases expressed in HVT and harvested from infected cells at passage II post transfection of the viral construct.

Functional analysis of purified protein

Purified MDV DNA polymerases were tested for in vitro polymerase and exonuclease activity adapting procedures from literature[112,147]. Polymerase activity was measured by the ability of the enzyme to incorporate radioactively labeled nucleotides into acid insoluble substance. Contrary, exonuclease activity was measured by excorporation of radioactivity from a radioactively labeled template into acid soluble substance in the absence of dNTPs.

A

MDV Pol	Polymerase activity cpm	Exonuclease activity cpm
WT	9438	2802
D358A/E360A	9406	597
E360A	9331	622
Y547S	9008	2450
Y567F	8445	941
L755F	9001	2913
inactive	1772	481

B

MDV Pol	Polymerase activity % of WT	+/- %	Exonuclease activity % of WT	+/- %
WT	100.0	0.00†	100.00	0.00†
D358A/E360A	99.58	18.77	4.98	3.89
E360A	98.60	3.32	6.06	5.24
Y547S	94.39	25.41	84.84	16.92
Y567F	87.05	16.04	19.80	14.38
L755F	94.30	12.89	104.80	10.88

C

HSV-1	Polymerase %	Exonuclease %	MDV	Polymerase %	Exonuclease %
WT	100	100	WT	100	100
D368A	103.3	0	D358A/ E360A	99.6	5
E370A	111.2	0	E360A	98.6	6.1
Y557S	72.5	42.5	Y547S	94.4	84.8
Y577F	70.6	16.8	Y567F	87.1	19.8
L774F	n.d.	n.d.	L755F	94.3	104.8

Table 10 Enzymatic properties of purified C-terminally FLAG tagged MDV DNA polymerases containing mutations in the exonuclease domain of the protein. The enzymatic activity was measured as the ability to incorporate radioactively labeled nucleotides into acid insoluble substance (polymerase) or the ability to form acid soluble radioactivity from a radioactively labeled DNA template (exonuclease). **(A)** Radioactive counts per minute (cpm) obtained for polymerase and exonuclease activity of the respective mutants and WT. Shown are the means of three independent experiments. **(B)** Enzymatic activity of MDV DNA polymerase mutants in percent of WT activity. Means +/- standard deviation of three independent experiments. **(C)** Comparison of enzymatic activity of HSV-1 and MDV mutant DNA polymerases. Data for HSV-1 from Kühn and Knopf 1996. † Please note that the activity of the WT enzyme was set to 100% to allow comparison in all of the three independend measurements. This does not implicate, that all measurements of WT enzyme activity were exactly the same.

6.3 A case of reversion in Y567F and the rise of a diverse virus population

During many months of cell culture experiments with at least five independent transfections for each virus only one case of reversion from a growth deficient virus to a WT-like phenotype was observed. Typically, one or two wells in a six well dish were transfected to reconstitute virus from BAC DNA as described in the methods section. Since the hypermutators quickly lost fitness upon passaging, we wanted to determine if these viruses could be rescued from their suicidal phenotype in a population size depended manner. The rationale behind this was quite simple, in the background of high mutation rates, a larger population size stochastically increases the chances not only for reversion of the deleterious mutation, but also for the rise of viruses with a combination of beneficial mutations. Tentative trials with transfections of all wells in a six well dish yielded promising results for Pol mutant Y567F but not for the other hypermutators. The virus retained larger plaques for an increased period and thus Pol mutant Y567F was selected for a massive transfection approach. A total of 90 µg BAC DNA was transfected into 6 complete six well dishes and passaged by pooling the entire virus population from the six well plates into one 150 mm dish for the first passage. This population was subsequently split 1:2 for further passages. Around passage V, when typically plaques started to vanish, the number of plaques began to increase again. Sanger sequencing of the questionable part of U_L30 was conducted at this point and surprisingly showed the intact mutation at amino acid 567. The virus continued to grow with improved kinetics when isolation of extrachromosomal DNA was attempted at passage 6. Despite an adequate amount of plaques, DNA from this extraction failed to transform *E. coli* and did not yield any bacterial clones. To resolve this ambiguity, total DNA was extracted from infected cells in passage 6 and subjected to target enrichment and subsequent NGS sequencing. The result was the following: 49% of viral sequences had reverted to an A at position 71,716 of the viral genome, while only 38% had reverted to a T at position 71,717. The mutation I implemented was TAT → TTC at position 71,715- 71,717 of the genome leading to the exchange of tyrosine (Y) to phenylalanine (F) at amino acid position 567 of the viral DNA polymerase. This effect can be undone by reverting to A at position 71,716 since TAC would also code for tyrosine. The single reversion to a T at position 71,717 would leave the mutation intact. This means that 49% of the viral population reverted to WT, while 51% were left with Pol mutation Y567F. It was expected that this would lead to the disappearance of the Pol mutation Y567F within the next couple of passages, because it has detrimental effects on viral fitness. The virus continued to grow in CEC with WT-like kinetics and another Sanger sequencing was performed at passage X. Surprisingly, sequencing results showed that mutation Y567F was still intact. However, the chromatogram showed, that a portion of viral sequences did carry an A at position 71,716. From what the chromatogram suggests, this portion was not larger than 20%.

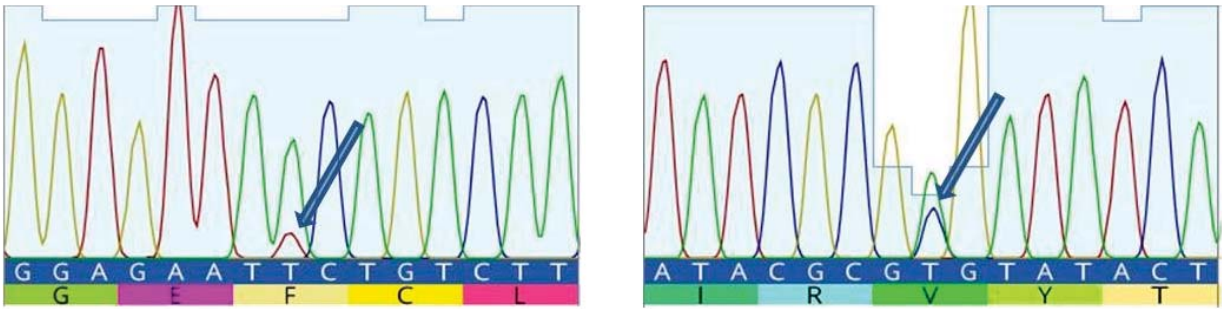

Figure 16 Chromatogram obtained from Sanger sequencing of the mutated region of Pol mutant Y567F in passage X in cell culture. Arrows are pointing to overlapping color peaks indicative of a mixed genotype at this position. A part of the viral population appears to have reverted the Pol mutation Y567F to WT by changing the codon at position 567 from TTC to TAC. At an even higher frequency the mutation V611A occurs, caused by the change of GTG to GCG at the respective position. Color code for base call peaks: Red= A, green= T, yellow= G and blue= C.

The chromatogram also suggested the presence of a considerable proportion (about 35%) of viruses that carried another mutation in the Pol gene, V611A. This mutation did appear in the NGS reads from passage VI with about 15% of the reads and continued to be present at a frequency of about 30% in passage VII. Amino acid residue 611 resides within the Exo III domain of MDV Pol, making it possibly influential for proofreading activity. It may be that the change V611A is at least partially restoring exonuclease function of Pol mutant Y567F. To our knowledge, mutations homologous to MDV Pol V611A have so far not been found in proofreading polymerases. Since sequencing of viral DNA extracts suggested genotypic diversification of the viral population, Hirt extractions were performed from passages XI to XVI. Upon transformation of *E. coli*, more than 30 individual clones were obtained from different passages and subjected to RFLP analysis. The viral BAC seemed to be present in almost all of the clones, but RFLP patterns were often slightly different from WT. Based on this finding, DNA was isolated from 5 of the clones (YF XIV Clone 1-5) and subjected to whole genome sequencing. The result revealed a surprising diversity among these clones. Each clone showed between 50 and 130 SNPs compared to the WT genome. From the 398 mutations observed among the five clones only 18 were shared between any of the 5 individual genomes. Of the five clones which genomes were fully sequenced, three had reverted and two had kept the Pol mutation Y567F. Additional Sanger sequencing of the Pol region in additional 6 clones showed a 50% reversion rate. Both clones that had kept Y567F mutation showed secondary mutations in the Pol gene. This could be indicative of compensation for proofreading deficiency through secondary mutations. To determine phenotypic variability of the clones, DNA of high purity was isolated from 25 *E. coli* BAC clones and transfected into CEC to recover infection progeny. From 25 clones, only 15 (60%) caused a visible cytopathic effect in cell culture. Plaque size assays were performed on 4 of the clones, 12 clones that produced plaques in cell culture were titrated (figure 17). Taken together, these experiments show a high phenotypic diversity in virus clones obtained from the diverse population of partially reverted Y567F between passages XI and XIV. Remarkably, the individual fitness of single clones, as determined by our assays, appears to be relatively variable and often lower not only than WT fitness but also lower than the fitness of the intact diverse population. It is noteworthy that plaque size and titrations are not necessarily in line with each other. Clone YF_XVI_15 for example produces relatively small plaques while achieving titers that reach, if not exceed, WT levels.

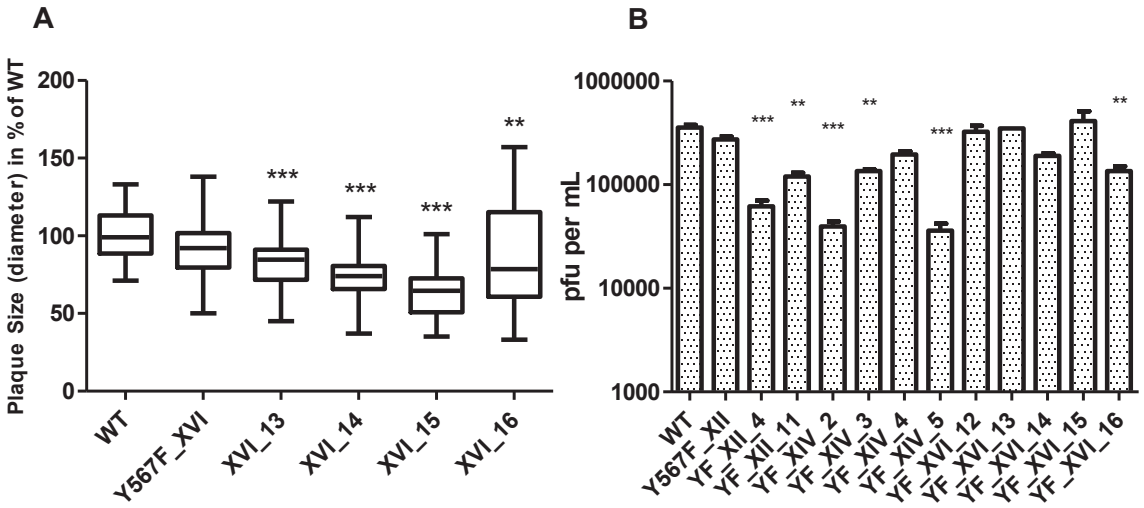

Figure 17 Phenotypes of individual viral clones obtained from the diverse parental population of Pol mutant Y567F from passage XII, XIV and XVI. **(A)** Plaque sizes of MDV WT, the intact diverse population derived from Pol mutant Y567F at passage XVI (Y567F_XVI) and individual viral clones obtained from this population at this passage (XVI_clone). Kruskal-Wallis nonparametric test with Dunn's multiple comparison, compared to WT. **(B)** Titers obtained from MDV WT, the intact diverse population derived from Pol mutant Y567F at passage XVI (Y567F_XVI) and individual viral clones (YF_passage from which the clone was obtained_clone) obtained from this population, one-way ANOVA with Bonferroni's multiple comparison, compared to WT.

6.4 Acyclovir (ACV) resistance of L755F

To validate the phenotype of finger domain mutant L755F, acyclovir resistance was measured in a plaque reduction assay and compared to WT. Unfortunately, the exact nature of the plaque reduction assay in the publication originally describing the phenotype of L774F in HSV-1[57] remains elusive since the reference given therein[155] further refers to another paper where no description of the experimental procedure can be found[56]. At concentrations used by Hwang and colleagues[57], the MDV WT was not inhibited by ACV and thus no difference to the mutant could be observed. Likely due to the fact that chicken are not usually treated with ACV, there is limited data on ACV susceptibility of MDV available. One report claims to have used concentrations of 0.25 mg/ml (roughly 1000 µM) to achieve a less than 100-fold reduction in gB mRNA expression after 96 h[156].

To obtain a viable answer to the question if L755F is more resistant to ACV treatment than WT, a dose response for WT was ascertained. Differences in plaque count could be observed in doses of >10 µM ACV and became obvious at a dose of 30 µM. Thus, L755F was compared to WT at concentrations between 10 µM and 100 µM ACV. At this concentration range, the relative ACV resistance of Pol mutant L755F was confirmed.

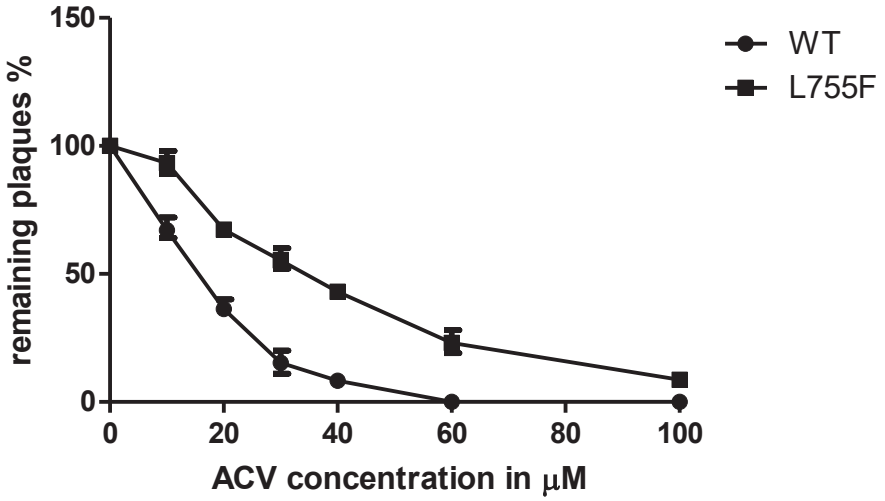

Figure 18 Reduction of plaques in response to increasing concentrations of acyclovir. Plotted are means and range (whiskers) for three independent experiments including Pol mutant L755F and WT control.

6.5 Genetic variability of viruses in vitro

Given the phenotype of Pol mutant viruses, the question whether there is any link between increased genetic variability and a loss of function in mutant viruses had to be addressed. To assess genetic variability of viruses in CEC culture, two independent strategies were designed.

1. Extraction of extrachromosomal DNA and generation of bacterial clones

A solid insight into the nature and distribution of mutations inside a single viral genome cannot be obtained by sequencing of cellular DNA extracts but requires singling of viral genomes prior to sequencing. Since the viruses used in this project are derived from BAC clones, bacterial clones can be generated if the bacterial origin of replication as well as the antibiotic resistance gene remains intact and inside a circular viral genome. Although viral DNA is linear inside the capsid, it circularizes upon entry into the nucleus of a host cell[157], and thus during lytic viral replication, there will always be some amount of circular DNA inside the nucleus. This fact is exploited in the process of generating virus BAC clones[158] and opens an opportunity to generate single bacterial BAC clones from passaged virus. Extrachromosomal DNA was extracted as described in the materials and methods section (Hirt extraction) and electroporated into MegaX electrocompetent cells (Invitrogen). Depending on the time of infection, the condition of CEC and the amount of virus plaques, the success of this method varied considerably. Best results were achieved when plaques were clearly visible but before the cell monolayer was destroyed by convergent plaques. Generally, several hundred plaques were necessary to obtain enough viral DNA for transformation of *E. coli*. Viruses that grew poorly already in the first passages post transfection did not yield enough DNA in this procedure. Another important problem is the selection for intact MiniF sequences, especially the chloramphenicol resistance is likely susceptible to disruption by mutations. In an attempt to extenuate this problem, chloramphenicol concentrations in bacterial media were lowered to 15 µg/ml in agar plates and 8 µg/ml in LB. At these concentrations, growth of untransformed MegaX cells was still inhibited, while it became easier to obtain BACs from viruses that exhibited a mutator phenotype. Bacterial clones generated this way were inoculated for DNA preparation and subjected to high-throughput sequencing on an Illumina MiSeq platform. Sequencing of individual virus genomes obtained from passaged viruses revealed great differences in the mutation rates of different Pol mutants and WT virus (see figure 19 for graphic depiction and table 11 for actual numbers).

A

Pol Mutant	Passage	Mutations, individual clones	Mutations, average
WT	4	0	
	4	0	
	4	0	
	4	0	0
	7	0	
	7	2	
	7	1	
	7	3	1,5
	20	10	
	20	10	
	20	14	
	20	8	10,5
L755F	4	0	
	4	1	
	4	0	
	4	0	0,25
	7	3	
	7	1	
	7	2	
	7	0	
	7	2	1,6
	20	5	
	20	9	
	20	8	7,34
Y547S	4	2	
	4	0	
	4	3	
	4	2	1,75
	7	2	
	7	9	
	7	4	
	7	2	
	7	6	4,6
	20	33	
	20	33	
	20	37	
	20	31	
	20	24	31,6

B

Pol Mutant	Passage	Mutations, individual clones	Mutations, average
Y567F	0	31	
	0	45	
	0	39	
	0	44	39,75
	1	97	
	1	86	
	1	67	
	1	73	
	1	74	79,4
	3	90	
	3	83	
	3	100	
	3	80	88,25
	4	69	
	4	85	77
E360A	0	66	
	0	32	49
	1	107	
	1	105	106
D358A/E360A	0	42	
	0	39	40,5
	1	136	
	1	87	111,5

Table 11 Number of mutations in viral clones obtained from passaged viruses. **(A)** Absolute number of mutations in viruses with considerable exonuclease activity (Proofreading Pol mutants and WT). **(B)** Absolute number of mutations in viruses with significant loss of exonuclease activity (hypermutators).

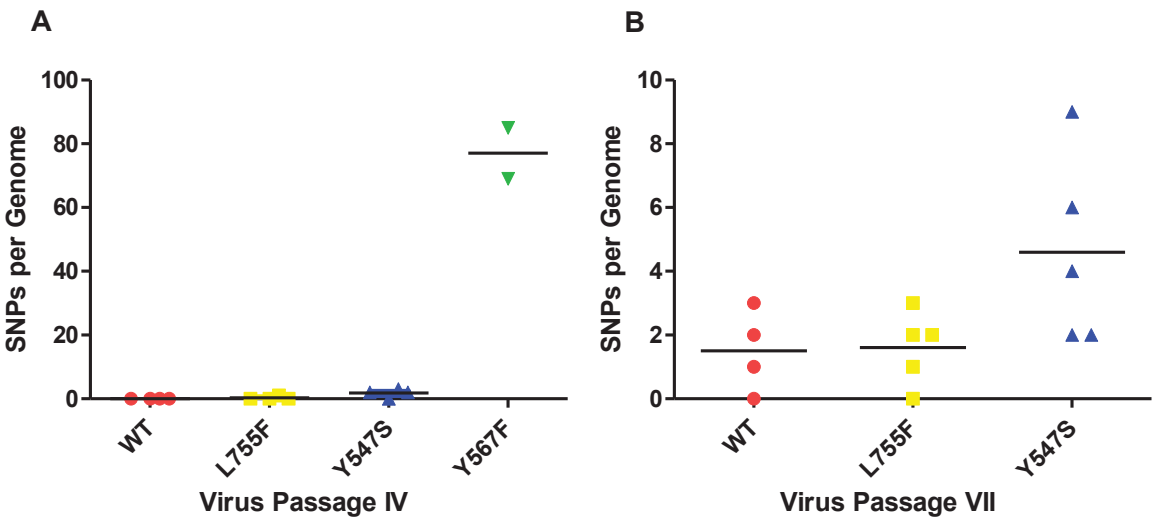

Figure 19 Number of mutations per individual viral genome in WT and different Pol mutants. Data obtained from whole genome sequencing of BAC clones from viral genomes after passaging in cell culture. **(A)** Clones from virus passage IV; WT, L755F and Y547S, n = 4; Y567F, n = 2. **(B)** Clones from cell culture passage VI; WT, n = 4; L755F and Y547S, n = 5.

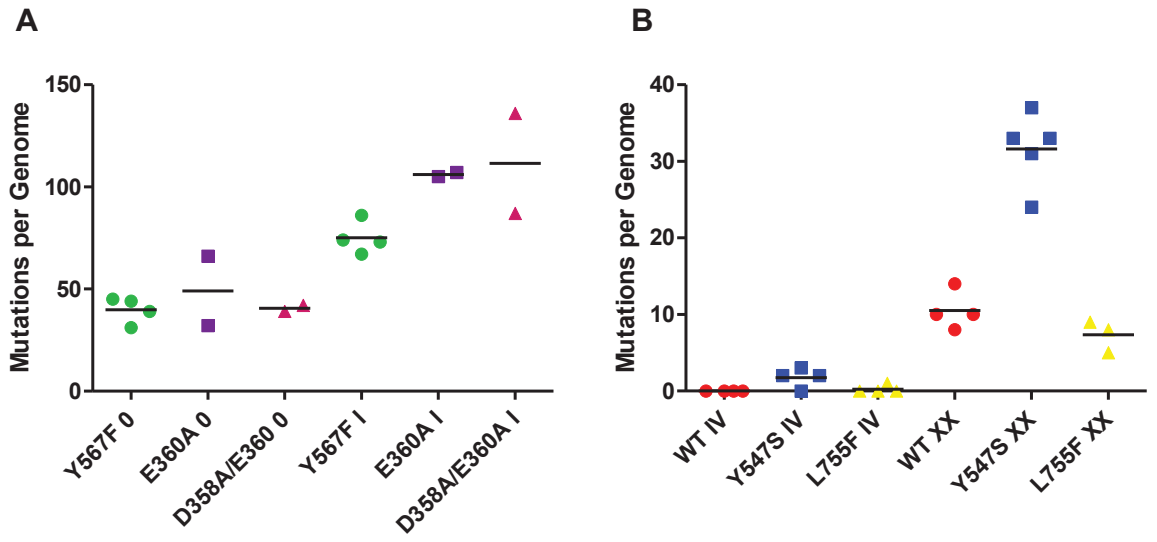

Figure 20 Accumulation of point mutations in viruses passaged serially in cell culture. Data was obtained from whole genome sequencing of BAC clones from viral genomes after cell culture passaging. **(A)** Number of mutations identified in genomes of viruses with hypermutator phenotype at passage 0 and I. Y567F, n = 4; E360A and D358A/E360A, n = 2 in both passages. **(B)** Number of mutations identified in genomes of WT and viruses with proofreading capability at passage IV and XX. Passage IV, WT, Y547S and L755F, n= 4. Passage XX, WT, n= 4; Y547S, n= 5; L755F, n= 3.

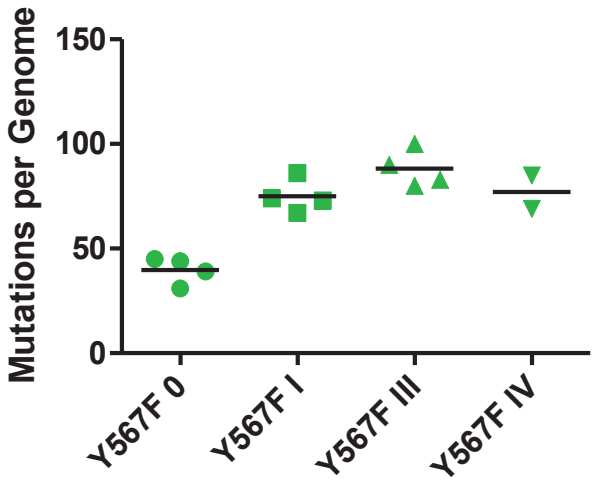

Figure 21 Number of mutations identified in an evolving viral population over time. Depicted is the number of point mutations in individual viral genomes obtained from Pol mutant Y567F at passage 0, I, III and IV in cell culture. No BAC clones were available from DNA isolated in passage II. Passages 0, I, III and IV, n = 4; passage IV, n = 2.

Results obtained from sequencing of individual viral genomes extracted from viral populations in cell culture and propagated as bacterial clones show a clear picture of the mutational properties of Pol mutant viruses. While mutations are absent or nearly absent in WT and L755F clones at passage IV and rare at passage VII, Y547S shows a slight increase in mutations at these passage numbers. Only much later, in passage XX, it becomes obvious that Y547S is indeed exhibiting significantly decreased replication fidelity compared to WT and L755F which, even at passage XX, have accumulated no more than 6-12 SNPs per individual genome.

This picture changes drastically for the Pol mutants Y567F, E360A and D358A/E360A. An immediate accumulation of mutations is obvious in all of the severely exonuclease deficient viruses. All of these viruses accumulate an avarage of 40-50 mutations within 7 days post transfection. Upon passaging, this accumulation of mutations continues and reaches an average of 80 mutations in Exo III mutant Y567F and more than 100 mutations per genome in the Exo I mutants. This is the last time at which DNA obtained from extrachromosomal extracts of Exo I mutant infected CECs was able transform E. coli to yield clonal viral genomes. For Exo III mutant Y567F, this was still achievable up to passage IV. In the case of Y567F, accumulation of mutations does not continue at the same pace, but rather stalls below 100 mutations per genome. Nevertheless, although mutations do not continue to accumulate, plaques disappear from cell culture plates and extracted viral DNA does not yeild any BAC clones in *E. coli* beyond passage IV.

High fidelity mutant L755F

Based on the sequencing of clonal viral genomes, an enhanced DNA replication fidelity of Pol mutant L755F cannot be doubtlessly confirmed. While a tendancy towards lower accumulation of muations might be concluded from sequencing of clones from passage XX, this is not significant and not sufficient to draw extensive conclusions on the overall replication fidelity of Pol mutant L755F.

2. DNA extraction from viral nucleocapsids

The second approach is based on a protocol published by Volkening and Spatz[107] and relies on the stability of the viral nucleocapsid. Cellular DNA is enzymatically digested while viral DNA stays protected inside the capsid and is subsequently released through enzymatic proteolysis. This approach should work for viruses that propagate well in cell culture. Cells are harvested late in infection, when plaque formation is extensive and the CEC monolayer is heavily disturbed. Mutants that do not propagate well in cell culture are not well suited for this type of DNA extraction. In NGS sequencing, extraction of viral DNA was considered successful when more than 20% of the reads obtained were of viral origin. Samples with viral reads below this threshold were of poor quality and had to be excluded from the final analysis.

Virus	MDV copies per µl (Micrococcal nuclease)	MDV copies per µl (Hirt extraction)
L755F	$1.02x10^5$	$1.26x10^6$
WT	$4.52x10^4$	$9.00x10^5$
Y547S	$2.84x10^4$	$2.45x10^6$
Y567F	$1.59x10^3$	$7.59x10^4$
D461A	3.20	89.6
D571A	0.59	$1.75x10^2$
D358A	0.9	$3.65x10^3$
E360A	10	$6.08x10^3$
double	5	$4.89x10^3$

Table 12 Viral DNA copies originating from extractions of an MDV infected 100 mm CEC cell culture dish in passage II. Extraction of DNA from nucleocapsids (micrococcal nuclease) and extraction of extrachromosomal DNA were performed in parallel by splitting infected cells 1:1. DNA was eluted in 50 µl EB. Presented are means from two independent extractions.

Due to constraints in DNA extraction as demonstrated in table 12, sequencing of encapsidated DNA remained challenging, was not possible for some of the mutants and got impossible for others when viral titers dropped upon passaging. The success of DNA extraction remained highly variable even for viruses that grew well in cell culture. Sequencing results were variable and not conclusive when compared to the sequencing of clonal viral DNA. An example of sequencing results is shown in figure 22. While there is a trend towards higher accumulations of mutations in Pol mutants, the mutations detected in WT seems to be

a dramatic overestimation when compared to the sequencing of single clones. It appears that this method of DNA extraction and sequencing is not useful when trying to find rare SNPs in highly diverse viral populations.

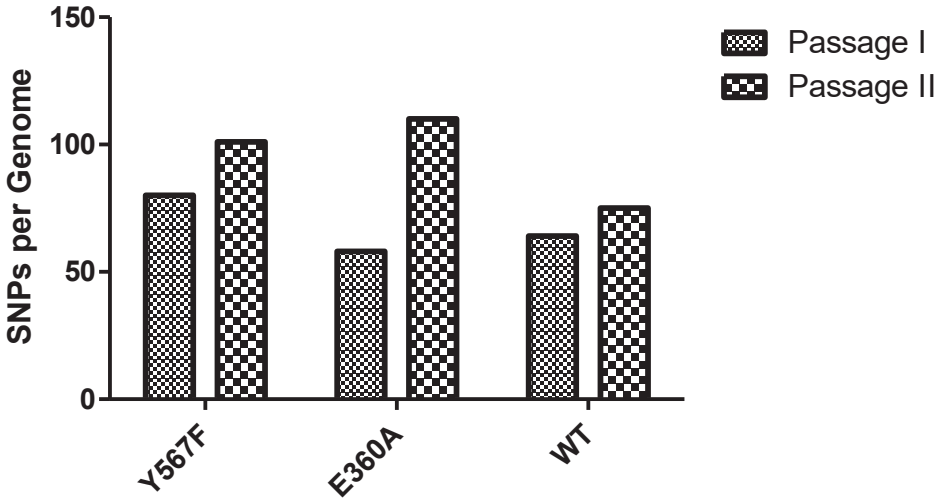

Figure 22 Mutations abundant in frequencies of >10% in NGS reads generated from DNA extracted from viral nucleocapsids in cell culture passage I and II.

6.6 Enrichment of viral DNA from infected cell cultures

To test the capacity of our newly developed tiling array to enrich viral DNA in the background of cellular DNA from chicken, we performed several tiling arrays on cell culture total DNA extracts (sarcosyl method, see methods section). We were able to enrich viral DNA against chicken DNA and achieved 100-1000 fold increase of viral reads in NGS sequencing when compared to non enriched samples. While this method reliably detected mutations with high abundance in the population (e.g. engineered Pol mutations), detection of rare SNPs remained impossible due to sequencing errors and the necessity to set a detection threshold of >10% abundance for mutations.

Sample	Total reads	On target reads	% on target	Fold coverage MDV genome	Fold enrichment
WT IV	792409	2019	0.25	2.7	n/a
WT IV enriched	**731457**	**693452**	**94.80**	**927**	**380**
Y547S IV	1499572	9874	0.66	13.2	n/a
Y547S IV enriched	**1144752**	**1121118**	**97.94**	**1499**	**148**

Table 13 Comparison of sequencing results with and without target enrichment. Target enrichment is performed using a custom designed tiling array targeting the entire sequence of MDV strain RB-1B.

6.7 Animal experiments

Poor growth in cell culture is a limiting factor for viruses to be tested in vivo. From the Pol mutants generated in this study only three were suitable for titrations and virus stock production. However, *in vitro* data supported the use of the three Pol mutants Y547S, Y567F, L755F for *in vivo* studies providing evidence for the existence of aberrant phenotypes. From the selected mutants, Y567F was inhibited in cell culture growth to an extent that enabled stock production but limited titers to relatively low values. Experiments with HVS-1 and HSV-2 have yielded higher titers for both viruses when cell cultures were treated with glucocorticoids, at least in certain cell types[159,160]. To enhance viral growth, infected cells were treated with the synthetic glucocorticoid dexamethasone as stated in methods section. Titers obtained from dexamethasone treated cell cultures were 3-6 times higher than untreated controls. Pol mutant Y567F and WT virus as a control were grown in the presence of dexamethasone to obtain the inoculum for an animal experiment. MDV is a strictly cell-associated virus, and therefore infection of animals can occur only with infected live cells. To avert potential side effects of dexamethasone, which has anti-inflammatory and immunosuppressive effects, on infected animals, the drug was removed from media of cultured cells 24 hours before preparation of the virus stock. While preparation of virus stock efficiently removes soluble dexamethasone, a carryover of intracellular dexamethasone is possible. To control for any unintentional secondary mutations introduced during the mutagenesis procedures, revertant viruses were generated, restoring the respective Pol mutations to WT sequence.

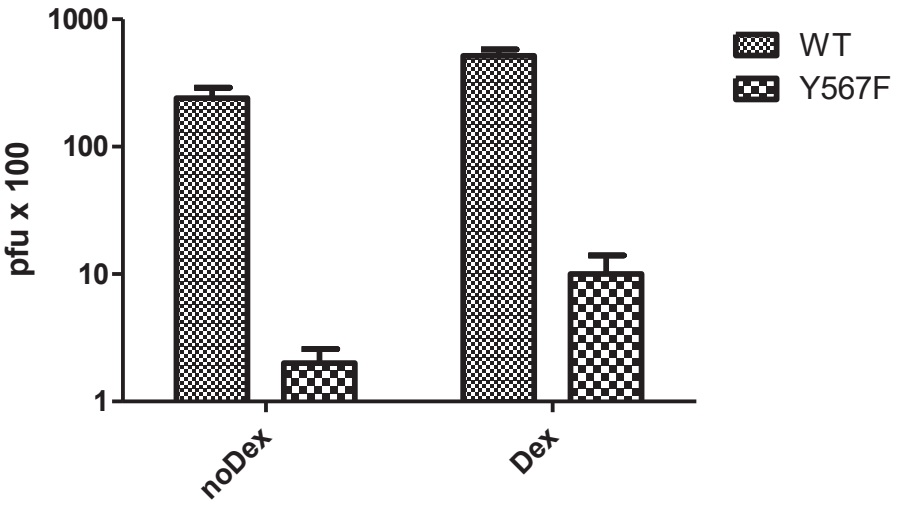

Figure 23 Titers obtained from WT and Pol Y567F mutant viruses grown in CEC in the presence of dexamethasone.

For the *in vivo* experiments, each group of 25 one-day old Valo SPF chickens was infected with one of the Pol mutant viruses. As controls 13 animals were infected with WT virus, 12 chickens were infected with WT virus that was grown in the presence of dexamethasone (WT Dex), and four groups of 13 chickens were infected with each of the revertant viruses. Chickens were infected intraabdominally with 4,000 pfu of the respective virus. The animals were housed in four experimental rooms. Chickens infected with mutant viruses were kept separately and chickens infected with WT and revertant viruses were housed together in one room. Ten uninfected chickens were housed in each room with the infected birds to monitor horizontal transmission of MDV. The experiment lasted 90 days. The animals were observed twice daily and terminated at the first clinical sign of MD. Blood was collected from 8 randomly selected infected chickens of each group at days 4, 7, 10, 14, 21 and 28 post infection, and from 8 uninfected chickens 21, 28, 35 and 42 days post infection.

MD incidence

The incidence of MD during the 90 day course of experiment is depicted in figure 24. While the differences in MD incidence observed in animals infected with Y547S, L755F and WT are not statistically significant; a dramatic decrease in MD incidence is observed for animals infected with Pol mutant Y567F. At this point it must be remembered, that the viral inoculum for Y567F was prepared using dexamethasone in cell culture as stated above. To control for any effect of the dexamethasone treatment, WT virus was grown in cell culture in the presence of dexamethasone exactly as Y567F and used as a control in the *in vivo* experiment.
Animals infected with dexamethasone-treated WT virus showed a pronounced decrease in MD incidence compared to the non-dexamethasone treated WT control. This result is surprising, because it is expected that if any intracellular dexamethasone was injected into the chicken with the viral inoculum, the immunosuppressive effects of the drug should promote the development of disease rather than prevent it. The difference between both the dexamethasone treated and non-treated WT is however not statistically significant ($p = 0.11$). The exact numbers for MD incidence during the 90 day observation period are: WT (81.8%), WT Dex (45.5%), Y547S (75%), L755F (57.9%), Y567F (4.5%)

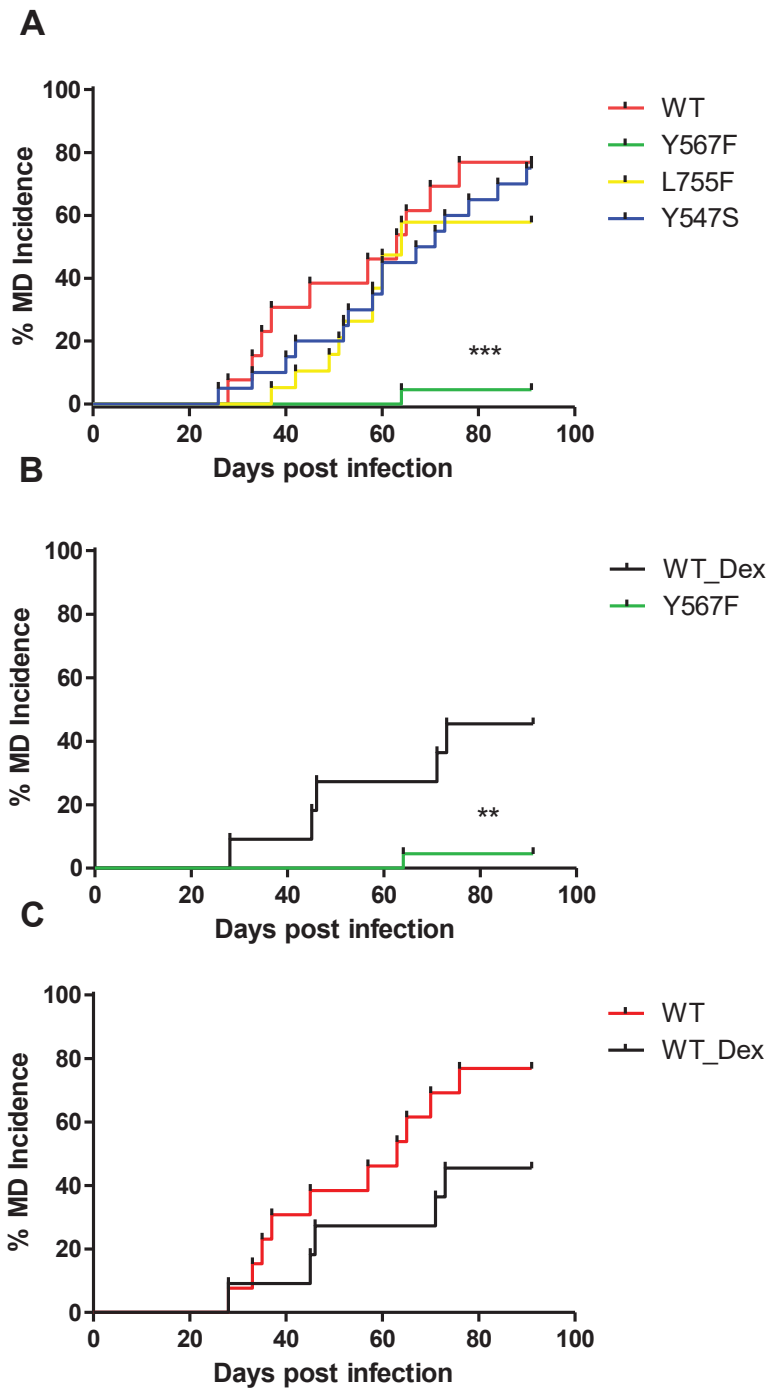

Figure 24 MD incidence diagnosed during a 90 days trial. **(A)** Comparison of chickens infected with Pol mutants Y567F, Y547S, L755F or WT. **(B)** Comparison of chickens infected with virus that was treated with dexamethasone in cell culture as described. **(C)** Comparison of animals infected with WT virus with or without cell culture dexamethasone treatment. Note that the comparison in graph C does not reveal a significant difference between the two groups (p=0.11).

In vivo replication kinetics

Blood was taken from eight birds on day 4, 7, 14, 21, 28 post infection for DNA extraction and qPCR analysis of MDV genome copy numbers. It must be noted that two of the eight birds designated for bleeding in the WT Dex group died before day 21 and 28 respectively while all other subjects survived for at least 29 days. DNA copy numbers of MDV over time are comparable for WT, Y547S and L755F. In contrast, Y567F seems to replicate undisturbed or even slightly better than the above mentioned viruses, until day 14 when replication drastically declines. This finding is in agreement with the observed suicidal phenotype in vitro. Despite higher survival rates in the birds that had received a dexamethasone treated WT inoculum, the virus was able to reach very large population sizes in some of the infected animals. Two animals in which the virus exhibited extreme lytic replication died prior to day 28.

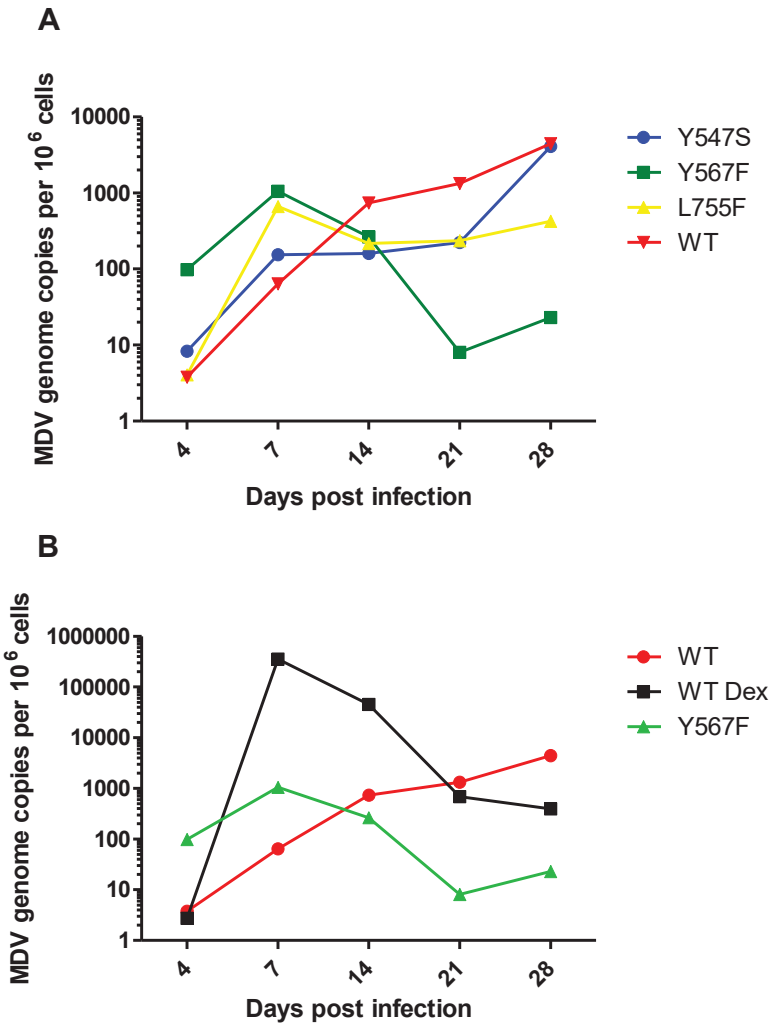

Figure 25 MDV replication kinetic in vivo depicted as viral genome copy numbers per 10^6 chicken cells on days 4, 7, 14, 21 and 28 post infection. MDV genome copy numbers for each timepoint are shown as mean of eight individual birds, except for WT Dex were two birds died before day 28 post infection (WT Dex: day 21, n = 7; day 28, n = 6). **(A)** Pol mutant viruses and **(B)** WT dexamethasone-treated viruses compared to non-treated WT.

Revertants

To control for any effect of secondary mutations potentially introduced during mutagenesis, revertant viruses were generated in which the introduced mutations had been removed and WT genotype restored using en passant mutagenesis. All revertant viruses were tested in vivo and no significant difference between WT and revertants was observed (figure 26). This validates the results obtained for the mutants.

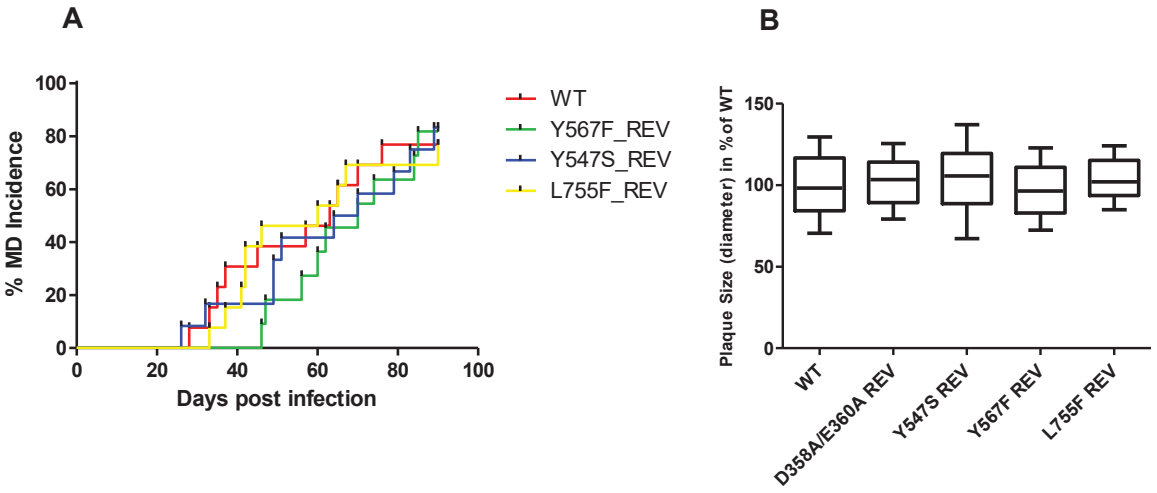

Figure 26 Characteristics of revertant viruses **(A)** MD incidence in chickens infected with WT virus or one of the three revertant viruses. **(B)** Plaque size of revertant viruses compared to WT using Kruskal-Wallis nonparametric test with Dunn's multiple comparison. No statistically significant differences were found.

MD incidence at final necropsies

In agreement with the regulatory approval, the animal experiment was terminated at day 91 post infection. All surviving birds were euthanized at this point and underwent post mortem inspection. Gross lesions of MD (visceral lymphomas, inflammation of peripheral nerves evidenced by marked swelling, ocular lymphomatosis) were recorded to identify subjects that were affected by MD but did not show clinical signs during the 90 day observation period. Overall, the data obtained at day 91 post infection is in good agreement with the data recorded during the 90 day observation period of the experiment. MD incidence in animals infected with Pol mutant Y567F was drastically reduced compared to WT and both other Pol mutants. Again, MD incidence in animals infected with WT virus that underwent dexamethasone treatment in cell culture was reduced when compared to the untreated WT. Comparing the groups infected with WT Dex and Pol mutant Y567F, MD incidence in Pol mutant Y567F is still perceptibly lower. All groups of animals infected with any of the revertant viruses showed MD incidences comparable to WT.

The relative numbers for MD incidence including animals that were diagnosed with MDV gross lesions during the final necropsy at day 91 are as follows.

Controls: WT (90.9%), WT Dex (54.5%)
Pol mutants: Y547S (90%), L755F (73.68%), Y567F (22.7%)
Revertants: Y547S_Rev (84.6%), L755F_Rev (84.6%), Y567F_Rev (100%)

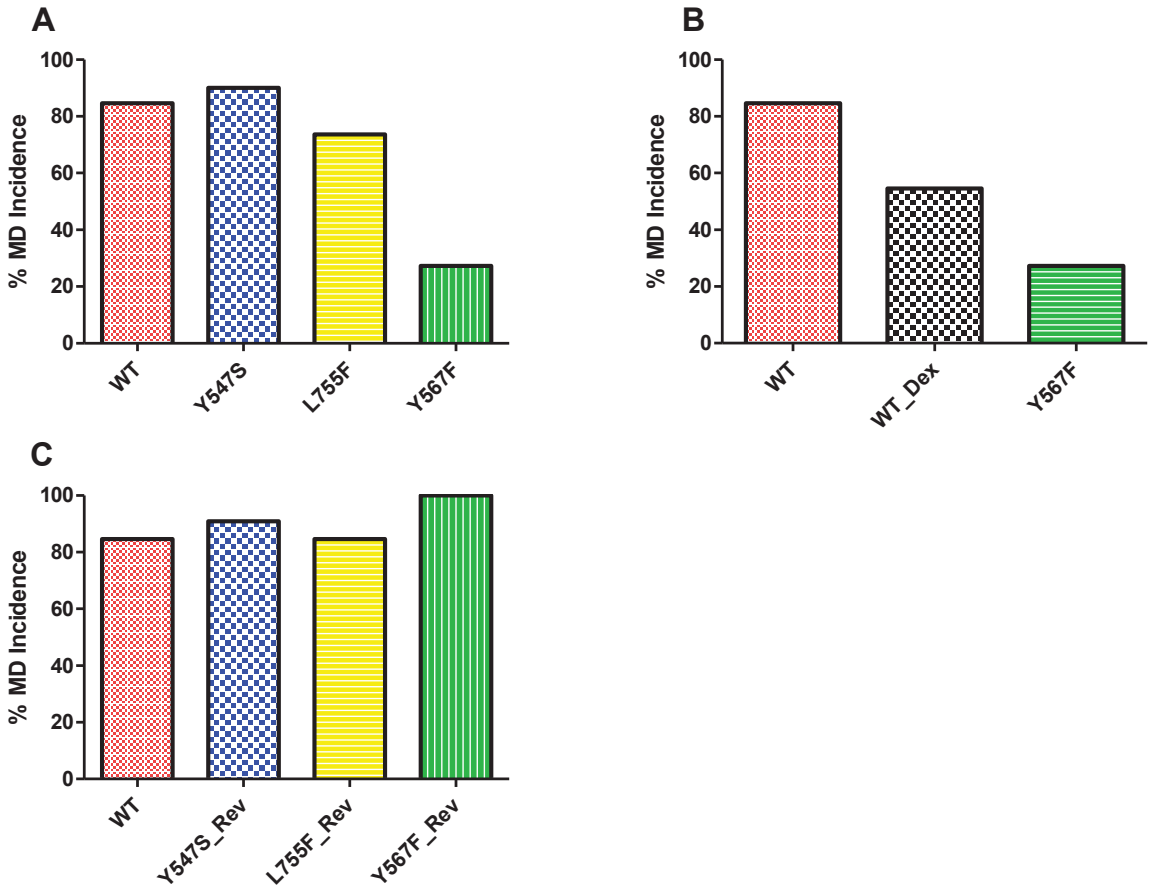

Figure 27 Total MD incidence observed during 91 days post infection with MDV. Numbers include all chickens that were diagnosed with MD during the 90 days observation period as well as all animals that presented gross lesions of MD during post mortem examination at day 91 post infection. **(A)** MD incidence in WT and Pol mutant viruses **(B)** MD incidence in WT, WT treated with dexamethasone in cell culture prior to infection of animals and Pol mutant Y567F. **(C)** MD incidence in WT and revertants of all Pol mutants used for infection of animals.

In vivo transmission – Survival of contact animals

In each of the four experimental rooms (A-D see table 9) uninfected chickens ("contact birds") were housed to control for natural virus transmission. During the 90 day trial, none of these birds showed clinical signs of MD and only one contact animal (housed with the Y547S infected birds) showed gross pathological signs of MD in the final necropsy after termination of all surviving animals.

6.8 Enrichment of viral DNA from tissue extracts and genetic diversity *in vivo*

Total DNA was extracted from different animal tissues and enriched for viral sequences as described in the materials section. The tiling array described in the introduction showed excellent characteristics in its ability to specifically enrich viral sequences from DNA extracts that contained less than 0.5 % target sequences.

Animal ID / Organ	Total reads	On target reads	% on target	Fold coverage MDV genome	Fold enrichment
1820 spleen	5645277	22025	0.39	36	n/a
1820 spleen enriched	**3371618**	**1581526**	**47.18**	**2635**	**120**
1883 spleen	1562740	4498	0.29	8	n/a
1883 spleen enriched	**2608071**	**1501351**	**58.64**	**2502**	**202**
1866 skin/feather	1485962	4986	0.28	8	n/a
1866 skin/feather enriched	**1975802**	**1427534**	**72.25**	**2290**	**258**

Table 14 Enrichment of viral DNA from samples of animal origin. Compared are sequencing results of libraries prepared with or without target enrichment. More than 100-fold enrichment was achieved in all cases.

Genetic diversity of viral DNA obtained from animal tissues

While genetic diversity was comparable among WT, Y547S and L755F viruses, Exo III mutant Y567F showed a significant increase in sequence diversity. Since only one animal infected with Y567F showed clinical signs of MD, the sample size is naturally limited. The amount as well as the distribution of mutations was analyzed in different tissues. This revealed very limited sequence divergence in different tissues, regarding overall amount as well as in the distribution of mutations.

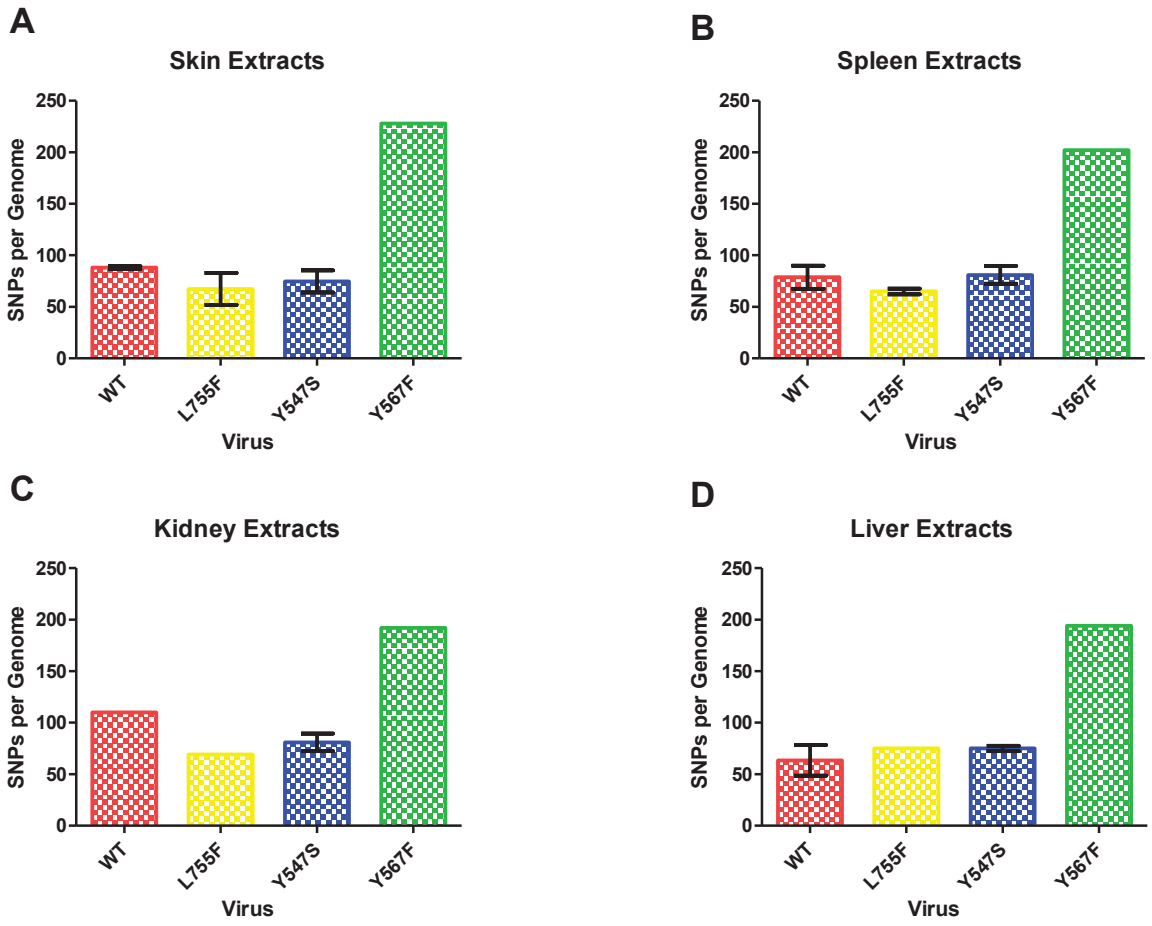

Figure 28 Number of point mutations per genome introduced during in vivo replication, compared to the original BAC sequence. Shown are SNPs that were apperent in at least 10% of NGS reads. All DNA extractions were performed on animals that were dignosed with MD during the 90 days observation period. **(A)** DNA extracted from skin, WT n = 2, Y547S n = 3, L755F n = 5 and Y567F n = 1 **(B)** DNA extracted from spleen WT n = 4, Y547S n = 3, L755F n = 4 and Y567F n = 1 **(C)** DNA extracted from kidney WT n = 1, Y547S n = 3, L755F n = 2 and Y567F n = 1 **(D)** DNA extracted from liver WT n = 2, Y547S n = 3, L755F n = 1 and Y567F n = 1.

7. Discussion

7. 1 Viability of Pol mutant viruses

Despite several attempts none of the HSV-1 Pol Exo I and Exo II mutants have yielded viable viral progeny[161,162]. Thus, to the best of our knowledge, our study is the first to describe reconstitution of viable herpesvirus mutants with amino acid substitutions in Exo domains I and II of the viral DNA polymerase and offers a unique chance to determine the impact of these mutations on DNA replication fidelity and viral viability. The fact, that we were able to generate viable herpesviruses with mutations in the Pol Exo I domain, also argues against the hypothesis which proposes that DNA polymerases of herpesviruses fundamentally differ from other proofreading DNA polymerases in being less distinct in polymerase and exonuclease functions, a theory that is discussed in literature[162].

While the relatively quick loss of viability of Exo II mutant D461A likely resulted from an impairment of its polymerase activity (see table 2), the extinction of Exo I mutants D358A/E360A and E360A can be attributed to a continuous accumulation of mutations in viral genomes over time. Judging based on our data, it seems that the maximum number of mutations that hypermutator viruses can still tolerate is approximately 100 mutations per genome. If the MDV genome is mutated above this threshold, the loss of genetic information leads to extinction of viral populations. While Exo I mutants seem to reach this barrier within the first three passages, the Exo III mutant Y567F appears to be genetically slightly more stable. It is obvious that this virus is approaching the “error threshold” with considerable pace within one passage post transfection but since then it hovers at the edge of extinction. Based on the full genome sequencing of virus clones, this could be due to secondary mutations in the Pol gene that potentially repair exonuclease activity and compensate for the Y567F mutation. Although a reversion of the Y567F mutation is easily achieved by a single base substitution (TTC->TAC), sequencing data suggest that a complex array of mutations within the Pol gene might also be responsible for the reversion of the proofreading deficiency. It is likely that a combination of reversion and suppression contributes to enhanced replication fidelity on a population level in later passages. Such rescue that would avert extinction of a declining population was not observed in Exo I mutants. There are three possible explanations. First, the mutation rate in these viruses might be too excessive to allow timely selection of viable mutants with repaired exonuclease function. Second, no suppressor mutations are possible that would compensate the loss of amino acids that are needed for the coordination of an essential Mg^{2+} ion in the catalytic core of the exonuclease. Third, the reversion of the implemented mutations is less feasible than in the case of Y567F because it would require two or four base substitutions. Knowing that population size plays an important role in overcoming evolutionary obstacles, a dramatic increase of viral population size post transfection might enable recovery of viable Exo I repaired or reverted viruses. Such an experiment should be attempted in future studies and could help to find possible ways of repairing mutations in the Exo I domain, which in turn would deepen our understanding of exonuclease function.

The only Exo mutant that reliably supports viral replication and does not have a strong tendency towards reversion or suppression of the introduced mutation, is the Exo III mutant Y547S. The exonuclease function is in this virus only slightly impaired, leaving a substantial proofreading capacity. This leads to a relatively slow accumulation of mutations, and the viral

population is far away from the “error threshold” even after twenty passages in cell culture. Whilst the mutation rate of this virus is still significantly above the WT, the relative genetic stability of this mutant makes this virus an ideal choice for future long-term evolutionary experiments. The reason for the growth deficit of Pol mutant D571A could not be determined. Despite a slight reduction in polymerase activity, the homologue of this mutant was among the two Exo III mutants that were reconstituted in HSV-1[155].

The exact reason for the differences in phenotype between Pol mutant MDV and HSV-1 viruses could not be addressed in this study. The fact, that a herpes virus with mutations in Exo I and II is capable of replication, is paradigm shifting and should be of interest in the future.

A detailed analysis of the different changes in the Pol gene that potentially influence replication fidelity and lead to a reversion of the implemented proofreading deficiency could be a very interesting area of study in the future and deepen our understanding of (herpes-) DNA polymerase function.

7.2 Expression, purification and in vitro activity measurement of MDV DNA polymerase

The expression and purification of sufficient amounts of MDV DNA polymerase has proved to be a challenging endeavor. Literature indicates that HSV-1 produces only limited amounts of DNA polymerase during infection[145,146,163]. Interestingly, the processivity factor encoded by UL42 seems to be relatively abundant in infected cells[163]. The absence of detectable WB bands for His- and FLAG tagged DNA polymerase expressed from its native locus indicates very low levels of viral DNA polymerase in MDV infected cells. Difficulties in expressing detectable amounts of MDV DNA Pol during in vitro T’N’T suggest either instability of mRNA or inefficient transcription or translation. Attempts to express MDV DNA Pol in prokaryotic systems, either cell-free or in E. coli, resulted in protein bands of about 140 kDa, readily detectable on a Coomassie stained gel. However, all of the proteins expressed in prokaryotic systems lacked enzyme activity when tested *in vitro*. Transient transfection of eukaryotic expression plasmids, harboring a C-terminally FLAG tagged UL30 insert under the control of the IE CMV promoter did, in several cell lines and CEC, not reliably result in detectable bands on a WB. When trying to express DNA Pol under the control of the IE CMV promoter in the MiniF region of the RB-1B BAC, no detectable amount of infectious virus was produced following transfection of the BAC into CECs. It appears, that overexpression of its own DNA Pol prevents efficient replication of MDV in cell culture. It can only be speculated what the reason for this could be. The fact that the virus tightly controls the natural expression of its Pol gene suggests that replication requires only a limited amount of DNA Pol, while a comparatively large amount of the accessory subunit encoded by UL42 seems to be required. It is possible, that disturbing the natural balance between the gene products of UL30 and UL42 prevents efficient DNA replication leaving a replication incompetent construct. It surprising to see, that an N-terminally labeled construct could not be expressed under the control of the IE CMV promoter in the context of HVT infection. It is possible that the very similar DNA Pol of MDV (amino acid similarity is 82% between the DNA Pols of HVT and MDV) interferes with the DNA replication of HVT, possibly by binding to its accessory

subunit. That an interference with the processivity factor encoded by UL42 of HVT could be the reason for our inability of overexpressing MDV DNA Pol in an HVT construct is supported by the fact that it was possible to detect and purify a C-terminally FLAG tagged version of MDV DNA Pol. It is possible that the C-terminal tag prevents efficient binding of HVT UL42. Admittedly, all of these explanations are very speculative; the exact reason for the difficulties to express functional MDV DNA Pol in several different backgrounds remains elusive.

The C-terminally FLAG tagged protein that was produced in HVT infected cells and purified via the FLAG tag was functional *in vitro*. Although herpesviruses typically require an accessory subunit for efficient DNA replication in vivo, the catalytic subunit encoded by UL30 is *in vitro* a highly processive DNA polymerase, even in the absence of its processivity factor[151]. Consequently, comparative analysis of enzymatic properties could be conducted on purified pUL30 in absence of pUL42. The results obtained from our *in vitro* measurements largely confirm the results of Kühn and Knopf[112]. While the tendency of our measurements with regard to polymerase and exonuclease activity is in accordance with published results for HSV-1, exact values differ to some extent (see table 10). This can likely be explained by natural biologic divergence of two related but evolutionary distinctly separated viruses. While this explanation is satisfactory for mutants Y547S and Y567F, it is surprising, that exonuclease activity was not reduced to zero in mutants E360A and D358A/E360A. These mutations should disable coordination of a Mg^{2+} ion essential for exonuclease activity[113] and thus reduce excorporation of nucleotides to zero. Marginal residual exonuclease activity in E360A and D358A/E360A may not be reflecting an actual activity of the respective enzyme but could be a contamination of exonuclease activity from purified extracts. Negative control for the radioactive assay was heat inactivated purified WT MDV DNA polymerase in which all enzymatic activity should have been depleted. It is possible that the native protein extracts contained a very slight exonuclease activity unrelated to the enzymatic properties of MDV DNA Pol that causes the observed effect. However, the values obtained for exonuclease activity of Exo I mutants E360A and D358A/E360A are still only slightly above background. Overall, the raw data (radioactive counts per minute, cpm) indicates lower enzymatic activity in our purified proteins compared to the extracts measured by Kühn and Knopf[112]. This is possibly due to the fact that protein purification was less stringent in the case of Kühn and Knopf, which could have led to a carryover of pUL42 or other viral or cellular factors enhancing enzyme activity. In the case of our measurements, a partial loss of enzymatic activity due to the relatively long and intensive purification procedure cannot be excluded.

7.3 Mutation frequencies in Pol mutant viruses

There are several, partially contradicting, reports on the influence of proofreading/exonuclease activity on replication fidelity in HSV-1[155,164,165]. Hwang and colleagues report an up to 800-fold increase in mutation rates in Exo III mutant Y577H/D581A[155], while the same authors can only find a 4- to 5-fold increase in mutations using another assay[166]. This uncertainty about the true contribution of exonuclease activity to overall DNA replication fidelity is, at least partially, due to the lack of accurate determination of mutation frequency. With the aid of NGS approaches, we were able to accurately determine mutations in whole viral genomes following viral replication in cell culture. The ability to generate bacterial clones of passaged virus gives us the opportunity to accurately

determine the accumulation of mutations over time in progeny of individual virus clones. In this study, this method was by far superior to any other way of determining mutation rates. Our data suggest that the total loss of exonuclease activity results in severely increased mutation rates while mutants with only impaired exonuclease exhibit lower mutation rates which are still significantly higher than WT.

Pol Mutant	Passage 0	Passage I	Passage XX	Mutations per passage	Mutation frequency relative to WT
E360A	49	106	n.d.	51	102
D358A/E360A	40.5	111.5	n.d.	48	96
Y567F	39.8	79.4	n.d.	39.8	79.5
Y547S	n.d.	n.d.	31.6	1.6	3.2
WT	n.d.	n.d.	10.5	0.5	1
L755F	n.d.	n.d.	7.3	0.4	0.8

Table 15 Comparison of mutation frequencies in MDV with WT or mutated DNA polymerase. Mutation frequencies from passage 0 to I were not determined for Y547S, WT and L755F because these viruses had accumulated less than 2 mutations per genome at passage IV. In contrast, mutation frequencies for E360A, D358A/E360A and Y567F could not be determined in passages later than I, because viral populations collapsed or suppressed/reverted the deficiencies of the exonuclease activity. Mutations per passage were calculated as the average number of mutations per genome per passage. It was determined based on all available full genome sequences; see table 11 for detailed information.

Mutational analysis of enriched samples

Employing a custom made tiling array, our ability to sequence MDV from chicken samples goes beyond even the most elaborate extraction procedures that have been reported previously[143]. This has resulted in *de novo* sequencing of several field strains of MDV and enabled an evolutionary analysis regarding the evolution of virulence in MDV[167].

The sensitivity for the detection of mutations that are rare in viral populations was severely limited by the overall fidelity of the sequencing process. To avoid the inclusion of errors that were introduced by PCR or sequencing, only variants that accounted for more than 10% of the high quality reads at a respective position were accepted in the final analysis. Consequently, this most likely results in the loss of the majority of the variants in the analysis. However, without the application of a detection threshold, results of any genetic variant analysis became impossible due to the relatively high number of mutations introduced during library preparation and sequencing. Even under these unfavorable circumstances, the strong mutator phenotype of Pol mutant Y567F could be confirmed. In contrast, the slightly increased mutation rate in Pol mutant Y547S was not confirmed by the mutational analysis of enriched samples from animal origin.

7.4 Phenotype of Pol mutant viruses

The phenotype of Pol mutant viruses falls into three groups:

Group I – Mutant viruses have severe growth defects immediately after transfection. Into this group falls the Exo II mutant D461A as well as Exo III mutant D571A. While the reduction of the Pol activity is likely the reason for the poor growth of the mutant virus D461A, the HSV-1 homologue of D571A is one of the few HSV-1 Exo mutants that support viral replication[155]. The reason for the growth deficit of the MDV Pol mutant D571A could not be determined.

Group II – Mutant viruses show an unimpaired phenotype during the first days post transfection but lose replication fitness within a few passages in cell culture. To this group belong the Exo I mutants E360A, the double mutant D358A/E360A, as well as the Exo III mutant Y567F. Mutational analysis of single viral genomes suggest that the accumulation of mutations inflicted by the mutant DNA polymerase is causing a rapid loss of replication fitness in cell culture. It is fair to assume that the majority of mutations introduced by the loss of DNA replication fidelity is detrimental[168] for viral fitness. Thus, it cannot surprise that viruses that exceed an evolutionary “error threshold” exhibit a suicidal phenotype.

Group III – Virus mutants do not differ in their replication properties in cell culture from the WT parent during 20 passages in cell culture and cause disease in chickens with kinetics similar to the WT. These are the finger domain mutant L755F and the Exo III mutant Y547S. While the replication fidelity of the HSV-1 homologue of L755F was higher than of the WT[119], this could not be conclusively demonstrated for its MDV homologue in this study. While a slight decrease in mutations in passage XX was observed, this result is insufficient to allow the unambiguous determination of the true effect of the finger domain mutation L755F on replication fidelity. If there is any effect at all, it clearly is only minor. In contrast, Exo III mutant Y547S did show a significant increase in mutations when compared to WT in passage XX. This 3-fold increase in mutation rate does not seem to affect the fitness of viral populations in any way. While this is indicative of an increase in mutation rate that remains stable below the error threshold, it must be noted that the observation period in our experiment is, in evolutionary dimensions, almost negligible. It would be intriguing to study the evolution of viral populations that carry this mutation in a much larger time frame.

7.5 Remarkable diversity in a stable population of partially repaired or reverted Y567F

All experiments except one are suggesting that highly reduced proofreading ability associated with the accumulation of point mutations over time are deleterious or lethal to viral populations. However, one experiment offers a truly remarkable perspective on population dynamics under circumstances of reduced fidelity DNA replication. While populations of Pol mutants with hypermutator phenotype typically eroded with time and ceased to grow within three to five passages post transfection, one population escaped eradication and formed a diverse population, cumulatively regaining WT fitness in cell culture. It seems obvious that population size is a major factor contributing to the rescue of a viable virus population from a suicidal hypermutator phenotype. A critical mass of infectious viruses, and thereby genetic diversity, has to be available to allow not only reversion or repair of the inserted Pol mutation

but also the selection of phenotypes that are replication competent despite carrying a considerable amount of mutations inflicted by the proofreading deficient DNA Pol. It is a very interesting observation that genetic and phenotypic diversity seems to be maintained in this viral population during cell culture passaging. It is not absolutely clear if the diversity that is still observed in passage XVI is a result of the genetic variation initially established by the original Pol mutant Y567F in early passages or if increased genetic variation still arises within the population in later passages from continued hypermutation due to a remaining part of the population that still carries the inserted Pol mutation and has not fully restored WT proofreading capacity by compensatory mutations. This question could be answered by measuring enzymatic properties of Pol enzymes *in vitro* that still carry the original Y567F mutation along with secondary point mutations in the Pol gene. Noteworthy is the propagation of a large amount of viral sequences that does not seem to be able to give rise to infectious virus upon transfection. Whole genome sequencing of two apparently replication-incompetent genomes has revealed intact genome architecture with point mutations distributed over the entire genome. So far, no principle difference, apart from the different point mutations, between replication competent and replication incompetent viral genomes could be determined and neither frame shifts nor stop mutations could be identified as the reason for replication incompetence. If part of the viral population still exhibit mutator properties, these replication-incompetent genomes could just be the rejects of error prone replication. If these replication-incompetent genomes would indeed be maintained within the replication-competent population this would strongly suggest that they play a role in achieving higher mean population fitness by contributing certain factors to the replication of other genotypes. At this point, we are indeed observing the **rise of a diverse quasispecies from a proofreading deficient virus clone** replicating in cell culture. The fact that the mean population fitness of the diversified viral sequence cloud is higher than the fitness of some individual clones obtained from this cloud strongly supports the argument. With the limited data we have obtained from cell culture, it is not possible to conclusively answer this interesting question. Not only will more sequencing work be necessary to explore the genetic diversity of the population deeper, it could also greatly enhance our understanding of evolutionary processes. This would be particularly true if we could study behavior of the intact population (high diversity), viruses derived from single BAC clones (no diversity) and combinations of clones with different phenotypes (low diversity) under selection pressure – in an immunocompetent chicken. It would be intriguing to not only determine the outcome of an animal experiment with regard to survival of chicken, but also to see in which way diversity is influenced by a strong selective pressure.

Since evolution of higher virulence in MDV is a major impetus to our work, a brief discourse about genetic diversity of field samples is not misplaced here. The sequence diversity observed between different field strains is not extremely high[167], at least compared to viruses that evolve as quasispecies. Although limited interstrain variability in MDV argues against increased genetic variation as a driving force of evolution towards higher virulence, it is quite possible that sequence diversity in infected hosts is so far underestimated due to poor sequencing capabilities. Even with our newly developed target enrichment, detection of minor variants is limited to sequences that are present in more than 10 % of sequencing reads. Many field isolates do not grow in cell culture which limits our ability to study their mutation rate and diversity. and most of the published MDV sequences are obtained from cell culture adapted viruses, which certainly is a bottleneck to sequence diversity. However,

the relatively high nucleotide identity between sequenced field strains still argues for an at least not huge diversity of circulating viral strains.

7.6 The effect of dexamethasone

Dexamethasone was used in cell culture to enhance the growth of Pol mutant Y567F and has helped to increase titers of this virus for the infection of animals. Although every attempt was made to remove pharmacologically active dexamethasone from the inocula, a group of birds was infected with WT virus treated exactly as the Pol mutant Y567F to control for any effect of the dexamethasone treatment. Surprisingly, animals infected with WT virus that had been grown in the presence of dexamethasone in cell culture showed a marked, albeit not statistically significant, reduction in MDV incidence during the 90 days trial.

Although MD incidence in animals infected with Pol mutant Y567F is still significantly lower than in the WT Dex group and the WT Dex group is statistically not different from the WT group, there is an obvious trend towards an effect of dexamethasone on MD infection of birds. Potential reasons for this observation are discussed in the following section.

Dexamethasone is a synthetic fluorinated glucocorticoid that has an about 29-times higher anti-inflammatory and gluconeogentic potential than hydrocortisone[169]. Glucocorticoids are steroid hormones, the natural cortisole is produced as one of the major stress response hormones in the adrenal cortex. There is an extensive amount of literature on glucocorticoid actions and cellular responses to glucocorticoid exposure and there is a remarkable amount of conflicting information within this literature. It is, however, indisputable that glucocorticoids have major anti-inflammatory and immunosuppressive effects, and are extensively used in medicine[170]. The effects on the immune system are very diverse and range from stimulation of inflammatory gene expression at low doses[171] and upregulation of several pathways involved in innate immunity to the suppression of the adaptive immunoresponses[172]. A biphasic model of glucocorticoid action has been proposed, according to which low doses of glucocorticoids exhibit utterly different effects than high doses[173]. In therapeutic concentrations, the suppression of pro-inflammatory cytokine production, antigen presentation by dendritic cells, T-cell responses and proliferation as well as the suppression of humoral immunity is observed[174]. Mechanistically, those effects seem to be mediated by three independent mechanism of glucocorticoid action. First and foremost, there is a genomic action of glucocorticoids. Due to their lipophilicity, glucocorticoids diffuse freely through the plasma membrane and bind the cytosolic glucocorticoid receptor which is expressed in nearly all nucleated cells. Upon binding of a glucocorticoid, the liganded receptor translocates to the nucleus where it can alter gene expression in three different ways:

1. Binding to glucocorticoid responsive elements (GREs)
2. Protein-protein interactions with other transcription factors
3. Composite binding of DNA or protein substrates

Importantly, all three mechanisms can have positive and negative effects on gene expression[174]. Beyond the genomic actions, there are non genomic effects of glucocorticoids. These are mediated through either membrane bound glucocorticoid receptors[175] or

nonspecific interactions with the cellular membrane[176] and likely responsible for the immediate glucocorticoid response that is observed under certain conditions[177].

The classical medical view is that viral infections represent a relative contraindication for glucocorticoid treatment[178]. Immunosuppressive effects are thought to promote viral replication, leading to prolonged viremia and inferior clinical outcomes to the point of fatal outcomes of otherwise controllable viral diseases such as chickenpox and measles[179,180]. For herpesviruses, cell culture experiments generally evidence moderate gain in titers after glucocorticoid treatment[181]. Still there are a number of case reports that indicate benefits in glucocorticoid treatment of viral diseases that have an immune mediated component such as HSV encephalitis, where therapy, however, always includes an antiviral component such as acyclovir[182].

The reasons for the observed effect of dexamethasone treated virus inoculum on survival of chickens remain speculative. There is the possibility that dexamethasone treatment leads to rapid attenuation in cell culture and that the observed effect is thereby inherent to the virus. This however, is not supported by sequencing data, there are no specific or globally increased mutations observed in dexamethasone treated virus which would suggest attenuation of the virus. More likely seems a pharmacologic effect elicited by traces of dexamethasone injected with the virus. Although dexamethasone is known for a prolonged activity when compared to natural cortisol and its exact pharmacokinetic in birds is unknown, pharmacological effects in mammals do not last longer than 72 h, with the exception of crystalline intramuscular depot injections[183]. Cortisol suppression can last for several days after a single administration of water soluble dexamethasone[184]. Given the fact that dexamethasone was withdrawn from cell cultures 24 h prior to stock production and several washes were performed afterwards, it can be excluded that a significant amount of free dexamethasone was applied with the virus. Consistent with the biphasic model of glucocorticoid action, it would be possible that a low amount of dexamethasone initially increased innate immune response and phagocyte function, imposing a stronger pressure on the virus that may lead to enhanced virus clearance and improved clinical outcomes. This hypothesis, however, seems unlikely in the light of qPCR results indicating that virus replication was initially not impaired in the dexamethasone treated groups and viral titers began to drop only later in infection.

Another possibility is that the observed effect of dexamethasone treatment was provoked by a possible high dexamethasone content of infected cells. The cells used for infection have likely retained some dexamethasone that was potentially still pharmacological active at the time of infection. It seems most likely that this caused the differences that were observed later. This would mean that the effect was conducted by dexamethasone action very early in infection. It is possible that phagocytes that incorporated injected cells received a significant amount of dexamethasone, causing changes in the chain of immunologic events following phagocytosis of virus infected cells. However, qPCR data do not support the early inhibition of viral replication as possible explanation for the observed effect in the group in question. One possibility is that the initial alteration of immunological events caused a deregulated viral replication early in infection which later on provoked a more vigorous immune response leading to improved clinical outcomes.

Although glucocorticoids are known for their decisive effects on T-cells[174] and are used therapeutically to treat lymphoproliferative disorders[185] a direct therapeutic effect of dexamethasone on lymphomagenesis is unlikely. Transformation of T-cells is not thought to occur prior to day 7 post infection[36], a time by which all pharmacological actions of dexamethasone should have worn off. An effect of dexamethasone on the development of T-cells in infected chicken prior to transformation seems more likely. A change in the T-cell population could potentially lead to altered infection kinetics which could account for the improved clinical outcome observed in birds that received dexamethasone treated virus inoculum.

Furthermore, it cannot be excluded that besides its immunomodulatory properties, dexamethasone can exhibit effects on viral replication and gene regulation. Herpesvirus genomes are known to contain GREs, these however have been held responsible for improved viral replication and reactivation from latency[186,187]. To my knowledge, there is no report on any direct action of glucocorticoids on a viral level that would be detrimental to viral growth. There is no GRE in the genome of MDV that shares the consensus sequence GGAACAnnnTGTTCT published[188] for the GRE. Notably, there are two negative glucocorticoid responsive elements (nGRE) sharing the published consensus sequence CTCC$(n)_{0\text{-}2}$GGAGA within the genome of MDV. As the name suggests, nGREs are known for transrepression of gene activation[189]. Most interestingly, nGREs within the genome of MDV are located within the meq and U_S8 genes. The role of meq as major oncogene has been mentioned in the introduction. U_S8 is encoding glycoprotein E (gE) with putative roles in cell to cell spread, virion transport in neurons and anti-host defenses[14]. This preliminary analysis of the MDV genome with respect to glucocorticoid responsive elements opens another very interesting perspective on how loss of virulence in dexamethasone treated viruses could be explained.

Given the likely low amount and single time administration of dexamethasone in this trial, the cause of reduced MD incidence in the WT Dex group remains elusive and most certainly requires further experimental evaluation.

7.7 The phenotype of Pol mutant L755F

Pol mutant L755F was constructed based on a report of a homologues mutation in HSV-1 that has led to an acyclovir (ACV) resistant phenotype and increased replication fidelity[57]. While ACV resistance was confirmed for the MDV homologue, an increase in DNA replication fidelity could not be conclusively demonstrated.

If at all, the enhancement of replication fidelity of the Pol finger domain mutant L755F is below our detection threshold. The number of mutations in L755F after 20 passages in cell culture is slightly lower compared to WT at the same time point. This, however, is at most, a tentative trend and should not be over-interpreted. The question of potentially enhanced replication fidelity in L755F could be addressed by further passaging of L755F and WT to compare the mutation rate at a later time point. If this was to be done, it should be considered that the MiniF region harboring antibiotic resistance and bacterial origin of replication is not indefinitely stable. We typically observe a marked loss of bacterial transformation efficiency in extrachromosomal DNA obtained from BAC derived viruses in

cell culture beyond passage XX. The reason for this can be a partial or complete loss of the MiniF or mutations that affect replication in *E. coli* or the antibiotic resistance.

Phenotypic, Pol mutant L755F was indistinguishable from WT in cell culture as well as in animals. Due to the, if at all, very limited effect of this finger domain mutation on DNA replication fidelity, no clear conclusion can be drawn with regard to the question whether or not increased DNA replication fidelity has an influence on the viral phenotype.

8. Conclusions

Regarding the three main goals of this project, the following can be concluded

1) Establishing a system to enable high-throughput sequencing of the strictly cell associated MDV to asses genome variability

With the development of a custom made tiling array, our ability to sequence MDV from chicken samples goes beyond the most elaborate extraction procedures that have been reported to date[143]. For the first time, we can obtain a complete MDV genome sequence from various sources, ranging from cultured virus to dust samples from chicken farms. This has resulted in *de novo* sequencing of several MDV field strains which in turn facilitated an analysis on the evolution of virulence in Eurasia and North America[167]. While this technique will certainly remain useful for future sequencing endeavors, its ability to detect genetic variation in viral populations is limited by the overall fidelity of the sample preparation and sequencing process. Rare genotypes with an abundance of less than 10% of the total population could not be reliably detected.

2) Determine virus genome variability as a function of viral DNA polymerase fidelity using viruses with altered replication fidelity in cultured cells

This study is the first to report the successful generation of viable herpesviruses with mutations in different parts of the 3'->5' exonuclease included in the viral DNA polymerase. It was conclusively shown that the replication capacity of most Exo mutants in cell culture correlates with their proofreading ability. Viruses that lost most of this ability introduced around 100 times more mutations per cell culture passage than WT virus. Viruses with a mutator phenotype are suicidal. They quickly lose fitness in cell culture, leading to extinction of viral populations typically within less than 6 passages. Despite this, it seems to be possible for viruses that exhibit mutator phenotypes to escape the error catastrophe by a combination of restoration of exonuclease activity and reversion of the implemented mutation. The diverse population rising from this event has regained WT fitness in cell culture and will be the object of further studies. Indeed, it seems possible that a proofreading deficient herpesvirus, in an environment with a large virus population and low selection pressure, can give rise to a genetically hyperdiverse population that could function as a quasispecies. This being said, it is clear that much more work will be necessary to deeper understand this phenomenon. Furthermore, a virus with slow mutator phenotype, exhibiting a mutation rate only 3 times higher than WT, was identified. Up to passage XX this virus is indistinguishable from WT and can be considered an excellent candidate for long-term evolutionary experiments.

3) Examine pathogenicity of polymerase mutants in the natural host and determine sequence space in different compartments of the host

Due to the limited growth of proofreading deficient viruses in cell culture, only a small selection of Pol mutants could be tested in vivo. Clearly, a mutation rate exceeding the natural mutation rate by approximately 80 times (Y567F) proved to be a disadvantage for viral fitness during infection of a susceptible host. By contrast, a 3 fold increase in mutation rate left the Pol Y547S mutant virus with pathogenicity comparable to WT. Sequencing of MDV samples isolated from different organs showed a marked increase in sequence

diversity in the chicken that developed MD following infection with hypermutator Pol mutant Y567F. This, however, did not reflect the extent of increase in mutation rate observed in single viral genomes obtained from virus in cell culture. The reason for this is a lack of sensitivity in sequencing of large populations. To exclude sequencing errors and artifacts introduced during sample preparation from the final analysis, only SNPs with an abundance of at least 10% of NGS reads where accepted. This, of course, causes a drastic loss of sensitivity in the detection of rare SNPs. The comparison of sequences obtained from different compartments of one and the same host did not reveal major differences in the amount or distribution of SNPs.

Animal experiments clearly demonstrated that a hypermutator phenotype is a disadvantage during infection of a susceptible host. While the hypermutator Pol mutant Y567F caused disease in only one chicken, mutants Y547S and L755F are statistically indifferent from WT regarding their ability to cause disease in susceptible hosts.

9. Outlook

The results obtained in preparation of this thesis will certainly be of further scientific interest. Right now, three projects that build on knowledge and practical expertise generated throughout this project are ongoing or planed for the near future.

1. Employing the developed tiling array for targeted enrichment of MDV sequences from a variety of field samples, including, but not limited to dust, which was collected over a period of time from poultry farms in the USA. To achieve the best possible outcome, an auspicious collaboration with scientists from the Pennsylvania State University is already established.
   While this project is already ongoing, it will not remain the only way to exploit the possibilities of the now established target enrichment. Within the next months, the author will be screening the chicken collection of the Museum für Naturkunde Berlin, which carries one of the world's biggest zoological collections, for the occurrence of MDV DNA in chicken that died in the beginning of the 20th century. Targeted enrichment and sequencing of MDV genomes from this period would enable unprecedented insights in the evolution of MDV.

2. The highly diverse population of MDV that arose from the reversion/repair of Pol mutant Y567F will be further studied within the next year. These studies will include further characterization of clonal viruses obtained from this population as well as animal experiments which will allow us to test if high genetic diversity represents an advantage for viral populations and a quasispecies-like behavior can be confirmed.

3. Among the viruses that were generated and characterized in this study, Pol mutant Y547S should not be overlooked. While this virus retains a WT like growth in cell culture as well as comparable kinetics during infection of the host, it does have a "leaky" proofreading that leads to an accumulation of mutations that is considerably

faster than in WT. This virus will be an interesting subject for further evolutionary studies as it could enable us to study the evolution of MDV on a fast track. Its increased genetic variation may enable faster adaptation to different environments and selection pressures. From this virus, we could learn, if the amount of errors made during genome replication correlates in any way with the speed and feasibility of adaption. Only one example of an interesting experiment would be to evaluate if we see a “turbo” attenuation of Y547S in cell culture passaging and vice versa if the passage of Y547S in chickens would result in a loss of the ability to obtain cultivable isolates for cell culture.

Of great advantage for future studies on evolution and genetic diversity in viral populations is our ability to generate individual clones from passaged viral populations. Together with the targeted enrichment of MDV sequences established during this study, it will enable us to follow adaptation processes and development of genetic diversity in progeny of individual virus clones as well as changes in diverse virus populations.

Aside from the prospects mentioned above, the sequencing data generated throughout this study will be a valuable source for the design of new studies targeting mutations in several MDV genes. Not only were we able to observe an array of so far undescribed mutations in different domains of MDV Pol that potentially restore replication fidelity in Exo deficient Pol mutants, we also observed patterns of characteristic changes in several MDV genes upon infection of animals. Among them are the major oncogene *meq*, the glycoprotein B encoded by UL 27 and the ubiquitin specific protease encoded by UL 36 to only name a few. Finally, the first description of a viable herpesvirus with mutations in the Exo I motif of the viral DNA polymerase could also influence studies involving other herpesviruses.

10. Summary

Gallid herpesvirus 2, also known as Marek's disease virus, is the causative agent of Marek's disease in chickens that can cause up to 100 % mortality in unvaccinated hosts. Vaccination against MDV is one of the most successful vaccination campaigns in the history of veterinary medicine, reducing disease incidence by more than 99%. Despite this success, MDV is still prevalent in chicken flocks worldwide and has shown a remarkable increase in virulence over the past decades. A major reason for the persistence of MDV could be the fact that vaccination against MD is not inducing sterilizing immunity but is permissive for (reduced) viral replication and shedding. It is argued that the imperfection of vaccination drives viral evolution towards higher virulence by selecting for viral phenotypes that maintain lytic replication and thereby the ability to be shed and transmitted in the presence of vaccine-induced immune response. The phenotypes selected in this way could ultimately benefit from vaccination, as vaccinated chickens which survive the infection shed the most replication competent viruses for a prolonged time, and thus contribute to the spread and evolution of particularly virulent virus strains. As a result, the development of MDV vaccines is caught in a vicious circle – vaccination drives selection of rapidly replicating escape mutants, which requires development of new vaccines based on viral strains that can replicate in the vaccinated host. This scenario has indeed been observed with vaccines of the first and second generation. In the light of these possibilities, the increase in virulence observed during the last decades is undoubtedly alarming.

In the context of selection for higher virulence, genetic variation of MDV in vaccinated hosts could provide a selective advantage similar to what is known for some RNA viruses, which have evolved error-prone genome replication and form highly diverse quasispecies. As large DNA virus, MDV is believed to be genetically relatively stable, employing a proofreading DNA polymerase for genome replication. There is, however, evidence for remarkable genetic variation among several large DNA viruses, including herpes viruses such as HCMV and HSV-1.

The objectives of this study were 1) to develop a NGS sequencing strategy for this highly cell associated virus 2) to determine, if genetic variation in MDV is a function of the fidelity of its DNA polymerase and 3) to examine replicative fitness and pathogenicity of proofreading-deficient viruses *in vivo*.

Following the development of a tiling array for highly specific capture of viral sequences from infected chicken cell extracts, we were able to sequence whole viral genomes from a variety of samples ranging from infected chicken embryonic cells to dust collected from chicken farms. Next, we constructed MDV mutants with point mutations, in the exonuclease and finger domain of Pol (UL30), that could enhance or reduce replication fidelity. The observed level of residual exonuclease activity correlated with the capacity of mutated viruses to replicate in cell culture. Viruses that encoded a DNA Pol which lacked the majority of its inherent exonuclease activity proved to be suicidal in cell culture, losing their replication fitness within a few passages after reconstitution from BAC DNA. Sequencing of clonal genomes obtained from virus propagated in chicken cells revealed that Pol mutants indeed exhibited higher mutation rates than wild type virus. Following *in vitro* characterization, three Pol mutants – a hypermutator (Y567F, mutation rate approximately 80-fold higher than WT), a weak mutator

(Y547S, mutation rate approximately 3-fold higher than WT) and a putative hypomutator (L755F, mutation rate possibly slightly lower than WT) were examined *in vivo*.
The survival of chickens indicates that a hypermutator phenotype (Y567F) is detrimental for viral pathogenicity while no significant difference between Y547S, L755F and WT was observed.
Sequencing of MDV DNA enriched from different chicken tissues showed that this difference in virulence correlates with a higher mutation rate in the Y567F virus. Increasing the mutation rate through reducing MDV Pol fidelity seems to be deleterious for the replicative capacity and fitness of MDV *in vitro* as well as *in vivo* through generation of an excessive number of mutations. Nevertheless, the potential of escaping this “error catastrophe” by partial repair of exonuclease function and establishment of a highly diverse viral population with WT like fitness was observed in cell culture for one of the hypermutator mutants (Y567F). The formation of functional and hyperdiverse populations by Pol mutant herpesviruses should be further investigated with special respect to potential quasispecies population dynamics.

11. Zusammenfassung

Titel der Arbeit: Über den Einfluss der Replikationsgenauigkeit der viralen DNA Polymerase auf genetische Variabilität und Pathogenität des Virus der Marekschen Krankheit.

Das Gallide Herpesvirus typ 2, auch bekannt als Virus der Marekschen Krankheit (Marek's Disease Virus, MDV) ist Erreger der Marekschen Krankheit des Huhns, einer Krankheit, die bei nicht geimpften Tiere bis zu 100% Mortalität verursachen kann. Die Impfkampagne gegen Mareksche Krankheit zählt zu den erfolgreichsten in der Geschichte der Veterinärmedizin. Trotz dieses großen Erfolges zirkuliert das Virus der Marekschen Krankheit weiterhin unter Hühnern weltweit und hat in den letzten Jahrzehnten eine bemerkenswert gesteigerte Virulenz erkennen lassen. Einer der Hauptgründe für dieses Phänomen könnte die Tatsache sein, dass eine Impfung gegen Mareksche Krankheit zwar den Ausbruch der Krankheit effektiv verhindert, jedoch keine sterilisierende Immunität erzeugt. Die Impfung ist permissiv für eine, wenn auch reduzierte, Replikation des Virus und lässt auch weiterhin eine Virusausscheidung und damit die Ansteckung weiterer Tiere zu. Derzeit wird diskutiert, ob diese imperfekte Impfung die Steigerung der Virulenz des Virus antreibt, indem sie Phänotypen selektiert die auch unter dem durch die Impfung gesteigerten Selektionsdruck noch in der Lage sind effizient lytisch zu replizieren und damit ausgeschieden werden können. Die so selektierten Phänotypen könnten am Ende von der Impfung profitieren, da geimpfte Hühner die Infektion überleben, länger infektiöses Virus ausscheiden und so zu einer Ausbreitung besonders virulenter Virusstämme beitragen. Dies könnte zu einem Teufelskreis führen, indem die Impfung zur Ausbreitung immer virulenterer Viren führt bis diese schließlich den Impfschutz durchbrechen. In der Tat konnte dieses Szenario bereits bei Impfstoffen der ersten und zweiten Genration beobachtet werden. Im Lichte dieser Möglichkeiten ist die beobachtete Virulenzsteigerung zweifelsohne besorgniserregend.
Im Zusammenhang mit der Selektion immer virulenterer Viren, könnte eine hohe genetische Variabilität des Virus im geimpften Wirt einen Selektionsvorteil bedeuten. Vergleichbares ist bei vielen RNA Viren zu beobachten welche eine fehleranfällige Replikation ihres Erbguts evolviert haben und sehr diverse Quasispezies bilden. Von einem großen DNA Virus, wie dem Virus der Marekschen Krankheit, ist grundsätzlich eine hohe genetische Stabilität zu erwarten. Die primäre Mutationsrate wird durch den Einsatz einer DNA Polymerase mit Korrekturlesefunktion limitiert. Dennoch gibt es Hinweise auf bemerkenswerte genetische Variabilität auch bei DNA Viren, darunter auch Herpesviren wie HCMV und HSV-1.
Die Ziele dieses Projektes waren 1) die Entwicklung einer Hochdurchsatzsequenzierungsstrategie für das hochgradig zellassoziierte MDV 2) die Klärung der Frage, ob die genetische Variabilität des MDV in direktem Zusammenhang mit der Genauigkeit der DNA Replikation steht und 3) herauszufinden welchen Einfluss das Fehlen der Korrekturlesefunktion der DNA Polymerase auf die Replikationsfitness und die Pathogenität des Virus hat.
Nach der Entwicklung eines hochspezifischen Anreicherungsverfahrens (tiling array) für virale DNA, sind wir in der Lage ganze virale Genome aus den verschiedensten Materialen, von infizierten Hühnerembryozellen bis hin zu auf Hühnerhöfen gesammeltem Staub, anzureichern und zu sequenzieren. Weiterhin haben wir Viren mit Punktmutationen in

der Exonuklease- und Fingerdomäne der viralen DNA-Polymerase erzeugt, welche die Genauigkeit der viralen DNA Replikation vermindern oder, in einem Fall, erhöhen soll. Das Ausmaß der Reduktion der Exonukleaseaktivität korrelierte dabei mit der Fähigkeit des Virus in Zellkultur zu replizieren. Viren, die den größten Teil der Korrekturlesefunktion ihrer DNA Polymerase verloren hatten, zeigten eine drastische Abnahme ihrer Replikationsfähigkeit innerhalb weniger Passagen nach Rekonstitution aus BAC DNA *in vitro*. Die Sequenzierung klonaler Virusgenome die aus in Zellkultur passagierten Viren gewonnen wurden, zeigte in der Tat, dass Pol mutante Viren eine höhere Mutationsrate als der WT aufweisen. Nach Charakterisierung der Pol mutanten Viren in vitro, wurden drei dieser Viren für einen Tierversuch ausgewählt. Darunter ein hypermutierender Phänotyp (Y567F, Mutationsrate übersteigt WT um das etwa 80-fache), ein mutierender Phänotyp (Y547S, Mutationsrate übersteigt WT um das etwa 3-fache) und ein Mutant der Fingerdomäne der DNA Pol (L755F) mit im Vergleich zum WT möglicherweise leicht erniedrigter Mutationsrate.
Im Infektionsversuch zeigt der hypermutierende Phänotyp (Y567F) eine erheblich verminderte Virulenz, während sich die anderen getesteten Mutanten im Hinblick auf ihre Virulenz nicht signifikant vom WT unterscheiden. Die Sequenzierung von aus verschiedenen Geweben angereicherter viraler Erbinformation zeigt, dass der beobachtete Unterschied in der Virulenz mit einer erhöhten Mutationsrate des Y567F Virus einhergeht. Demnach hat die Erhöhung der Mutationsrate durch eine Reduktion der Genauigkeit der DNA Replikation und die damit verbundene Anhäufung von Mutationen im Laufe der Zeit einen negativen Einfluss auf die dauerhafte Replikationsfähigkeit und Gesamtfitness des Virus sowohl *in vitro* als auch *in vivo*. Nichtsdestoweniger zeigte sich die Möglichkeit eines Entkommens aus dieser „Fehlerkatastrophe“ durch partielle Reparatur der Exonukleaseaktivität verbunden der Entstehung einer diversen Viruspopulation mit einer dem WT vergleichbarer Fitness in Zellkultur. Die Eigenschaften dieser funktionalen und hochgradig diversen Populationen sollten zukünftig insbesondere im Hinblick auf mögliche Quasispeziesdynamiken untersucht werden.

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13. List of publications

13.1 Peer reviewed journals

Kheimar A, **Trimpert J**, Groenke N, Kaufer BB. Overexpression of cellular telomerase RNA enhances virus-induced cancer formation. Oncogene 2018, published online Oct 19 doi:10.1038/s41388-018-0544-1

Eschke K, **Trimpert J**, Osterrieder N, Kunec D. Attenuation of a very virulent Marek's disease herpesvirus (MDV) by codon pair bias deoptimization. PLoS Pathog. 2018 Jan 29;14(1):e1006857. doi: 10.1371/journal.ppat.1006857.

Trimpert J, Groenke N, Jenckel M, He S, Kunec D, Szpara ML, Spatz SJ, Osterrieder N, McMahon DP. A phylogenomic analysis of Marek's disease virus (MDV) reveals independent paths to virulence in Eurasia and North America, 2017 Dec; 10(10): 1091–1101.

Ogasawara S, Daddona JL, **Trimpert J**, Stokol T. Effect of recombinant canine interleukin-6 and interleukin-8 on tissue factor procoagulant activity in canine peripheral blood mononuclear cells and purified canine monocytes. Vet Clin Pathol. 2012 Sep; 41(3):325-35.

Stokol T, Daddona JL, Mubayed LS, **Trimpert J**, Kang S. Evaluation of tissue factor expression in canine tumor cells. Am J Vet Res. 2011 Aug; 72(8):1097-106.

13.2 Conference contributions

2013 Poster presentation: 24th Annual Meeting of the Society for Virology (GfV), Alpbach, Austria *"Quasispecies or just variability of herpesvirus genomes: the role of the DNA polymerase fidelity on genetic variation of Marek's disease virus"*

2016 Poster presentation: 26th Annual Meeting of the Society for Virology (GfV), Münster, Germany
"Impact of altered fidelity of DNA polymerase on herpesvirus genomic variability and pathogenicity in vivo."

2016 Oral presentation: 11th International Symposium on Marek's Disease and Avian Herpesviruses, Tours, France
"Impact of altered fidelity of DNA polymerase on herpesvirus genomic variability and pathogenicity"

2016 Oral presentation: 11th MiniHerpes Workshop, Berlin, Germany
"The role of DNA polymerase fidelity on genetic variation and pathogenicity of Marek´s disease virus"

2017 Poster presentation: 2017 International Herpesvirus Workshop (IHW), Gent, Belgium
"The Role of DNA Polymerase Fidelity on Genetic Variation and Pathogenicity of Marek's Disease Virus."

2017 Oral presentation: 2017 International Conference for Graduate Students, Nanjing China
"Impact of altered fidelity of DNA polymerase on herpesvirus genomic variability and pathogenicity in vivo."

2018 Oral presentation: 12th International Symposium on Marek's Disease and Avian Herpesviruses, Yangzhou, China
"Unexpected consequences of artificially expanded herpesvirus genetic diversity on viral fitness and pathogenicity"

2018 Oral presentation: 13th MiniHerpes Workshop, Hamburg, Germany
"Unexpected consequences of artificially expanded herpesvirus genetic diversity on viral fitness and pathogenicity"

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15. Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig und aussschließlich unter Zuhilfenahme der genannten Quellen und Hilfen angefertigt habe.

Jakob Trimpert Berlin, den 26.11.2018