A new RHDV-2 vaccine based on recombinant baculovirus - Generation and characterization of induced immunity in rabbits

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A new RHDV-2 vaccine based on recombinant baculovirus - Generation and characterization of induced immunity in rabbits

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Abbreviations:

%	per cent
®	registered trade mark
°C	degree celsius
µ	micro
µg	microgramm
µl	microliter
a	anti
ab	antibody
AcMNPV	autographa californica multiple nucleopolyhedrovirus
ad	fill up to
ALT	alanine transaminase
AP	alkaline phosphatase
aqua dest.	aqua destillata (distilled water)
AST	aspartate aminotransferase
ATP	adenosine tri-phosphate
bac	baculovirus
BacBac	recombinant baculovirus for infection of insect cells
BacMam	recombinant baculovirus for transduction of mammalian cells
BHV-1	bovine herpes virus-1
bp	base pair
BSA	bovine serum albumin
BV	budded virus
C	cytosine
CAG(GS)	hybrid CMV enhancer/chicken ß-actin promoter
CCLV	Collection of Cell Lines in Veterinary Medicine
CD	cluster of differentiation
CIP	calf intestinal phosphatase
CMV	cytomegalovirus
conv	conventional
CSFV	classical swine fever virus
ct	threshold cycle
CU	codon usage
d	day(s)
D	dilution factor
DIC	disseminated intravascular coagulation
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
Dr	doctor
dsDNA	double stranded DNA
DTT	1,4-dithiothreitol
E.coli	escherichia coli
EBHS(V)	european bronw hare syndrome (virus)

EDTA	ethylenediaminetetraacetic acid
EG	European Community
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmatic reticulum
et al.	et alia (Latin: and others)
EU	European Union
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
Fig	figure
FITC	fluorescein isothiocyanate
FLI	Friedrich-Loeffler- Institute
G	gauge
g	gram
g	gravity
G	guanine
G1-G6	genogroups
GFP	green fluorescent protein
h	hour(s)
H.E.	hematoxylin and eosin
HA	hemagglutination
HBGA	histo-blood group antigen
HCMV	human cytomegalovirus
HU	hemagglutination unit
HZ	Hertz
i.m.	intramuscularly
IC	internal control
ICTV	International Committee on Taxonomy of Viruses
ie	immediate early
IFN	interferon
IgG	immunoglobuline G
IgM	immunoglobuline M
IL	interleukin
IP	isotonic phosphate
IPTG	isopropyl β-D-1-thiogalactopyranoside
kb	kilobase
kDa	kiloDalton
l	liter
LB	lysogeny broth
LDH	lactate dehydrogenase
LTR	long terminal repeat
M	molar
MEM	minimal essential medium
MHC	major histocompatibility complex
min	minute(s)

ml	milliliter
mm	millimeter
mM	millimolar
MOI	multiplicity of infection
n	number of positive wells
n.d.	not determined
NEAS	non-essential amino acids
NEB	new England biolabs
nm	nanometer
no	number
non-vacc	non-vaccinated
nt	nucleotide(s)
NTC	no template control
ODV	occlusion-derived virus
OIE	World Organisation for Animal Health
OPD	o-phenylenediamine dihydrochloride
ORF	open reading frame
p	number of parallel values
p	protruding
P10	baculoviral P10 promoter
PBS	phosphate buffered saline
PC	positive control
PCR	polymerase chain reaction
PE	phycoerythrin
PEG	polyethylene glycol
PEI	Paul-Ehrlich-Institute
PH	polyhedrin
pH	potential of hydrogen
pi	post infection
pmol	picomol
POD	peroxidase
qRT-PCR	quantitative real-time reverse transcription PCR
RCV	rabbit Calicivirus
RdRp	RNA-dependent RNA polymerase
rec	recombinant
RHD(V)	rabbit hemorrhagic disease (virus)
RHDV-1	rabbit hemorrhagic disease virus, classical variant
RHDV-2	rabbit hemorrhagic disease virus, variant emerged in 2010
RK	rabbit kidney
RNA	ribonucleic acid
rpm	rounds per minute
RSV	rous sarcoma virus
RT	reverse transcriptase
s	shell
s.c.	subcutaneous

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second(s)
SF	spodoptera frugiperda
SOA	super optimal broth without magnesium chloride and - sulfate
SOC	super optimal broth with glucose
ß-Gal	ß- galactosidase
StIKoVet	Ständige Impfkommission Veterinär
Suppl	supplementary data
T	triangulation number
TA	tris acetate buffer
Tab	table
TCID50	tissue culture infection dose 50
TE	tris EDTA buffer
TEMED	N,N,N′,N′-tetramethylethane-1,2-diamine
TEN tris	EDTA sodium chloride buffer
TM	trade mark
TNF	tumor necrosis factor
Tris	tris(hydroxymethyl)aminomethane
Tween20	polyoxyethylensorbitan monolaurate
U	unit
UV	ultraviolet
V	volt
v/v	volume/ volume
vacc	vaccine
VLP	virus like particle
vol	volume
VP	virus protein
VPg	virus-genome linked protein
W	watt
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
α	anti
α	alpha
β	beta
β-gal	β-galactosidase
γ	gamma
γ-GT	gamma-glutamyl transpeptidase
δ	delta

1. Introduction

Protection against severe clinical disease or mortality after an infection with highly virulent viruses is achieved by vaccination. Different approaches were used after the first successful vaccination trial by Dr. Jenner against smallpox virus (Rusnock, 2016), not only for human diseases, but also for pathogens of livestock.

In the last decades, the impact of several highly virulent pathogens on livestock could be reduced significantly by vaccination, like Rabies (Mähl et al., 2014), Classical swine fever (Postel et al., 2017), Foot-and-Mouth Disease (Paton et al., 2009) and others. However, when new viruses emerge in a naïve, unprotected host population, the risk for this population is very high, as seen with Rabbit hemorrhagic disease virus (RHDV) in European rabbits.

Detected for the first time in 1984 in China in rabbits imported from Germany the virus spread within 10 years rapidly and is now endemic in more than 40 countries worldwide where wild populations of European rabbits exist. Moreover, since its emergence several RHDV variants appeared (Abrantes et al., 2012). A genetically more different variant was found in 2010 in France, named RHDV-2 (Le Gall-Reculé et al., 2013).

RHDV is a highly contagious viral disease causing a severe hepatitis combined with high mortality in European rabbits. It was estimated that in Europe several 100 million rabbits in households and an unknown number of wild rabbits died. In Australia and New Zealand, where non-native populations of European rabbits exist, up to 95% of all rabbits died after initial introduction of RHDV as pest control agent (Abrantes et al., 2012).

To prevent the fatal outcome of this infectious disease several attempts were made to develop a successful vaccine. However, due to the fact that RHDV cannot be cultivated in cell culture, mainly inactivated RHDV vaccines prepared from livers of infected rabbits are available with the ethical problem that for vaccine production animals have to die from RHDV infection to protect others from the same infection.

In the present thesis the generation and optimization of a recombinant RHDV-2 vaccine, based on capsid protein VP60 expressed by recombinant baculoviruses, and the evaluation of its protective potential against RHDV-2 infection in rabbits is described and discussed.

2. Literature

2.2. Emergence, Prevalence and Importance of Rabbit Hemorrhagic Disease

Rabbit hemorrhagic disease (RHD) is a highly contagious viral infection of domesticated and wild European rabbits (*Oryctolagus cuniculus*). The first outbreak occurred in spring 1984 when a previously unknown disease killed Angora rabbits imported from Germany in the Jiangsu province of the People's Republic of China. Within 9 months several million rabbits died by this rabbit viral hemorrhagic disease (Liu et al., 1984; Xu and Chen, 1989; Xu, 1991). First, a picornavirus or a parvovirus were suggested to be the causative pathogen (Pu et al., 1985; Gregg and House, 1989; Xu and Chen, 1989; Xu, 1991). In the late 1980's/ early 1990's the aetiological agent was characterized as a Calicivirus (Granzow et al., 1989; Ohlinger et al., 1989; Ohlinger et al., 1990; Parra and Prieto, 1990; Meyers et al., 1991; Moussa et al., 1992) and the disease was named RHD caused by RHDV (Granzow et al., 1996).

The origin of RHDV is not fully understood. The pathogenic forms of this Calicivirus may have evolved from avirulent strains circulating asymptomatically in European rabbits (Capucci et al., 1996; Moss et al., 2002; Forrester et al., 2006, 2008; Strive et al., 2010). Moss et al. (2002) were able to prove that Caliciviruses were circulating in rabbits in Great Britain and most likely also in the rest of Europe at least 30 years before the first outbreak of RHDV in China emerged. Moreover, a common ancestor of Rabbit Calicivirus-like viruses (RCV) and RHDV circulating over 200 years ago was predicted, which mutated to the virulent RHDV strains that emerged in 1984 (Kerr et al., 2009). Another hypothesis postulates spillover infections of Caliciviruses found in small mammals close to wild rabbit populations (Merchán, et al., 2011; Abrantes et al., 2012; Le Gall-Reculé et al., 2013).

RHDV does not seem to have evolved from European brown hare syndrome virus (EBHSV) (Nowotny et al., 1997), another Calicivirus which occurred for the first time in 1980 in Denmark and Sweden (Gavier-Widén and Mörner, 1991) and causes a disease in European brown hares similar to RHD in rabbits. This is indicated not only by the limited amino acid sequence homology of about 76% between classical RHDV and EBHSV-capsid protein VP60 (Wirblich et al., 1994) but also by the fact that there is no cross-protection against RHDV in animals surviving an EBHSV infection (Lavazza et al., 1996).

Soon after the epidemics in China and in Korea in 1984 (Liu et al., 1984; Park et al., 1987; Xu, 1991) the first outbreak of RHD in Europe was reported in Italy in 1986 (Cancelotti and Renzi, 1991). Within the next ten years RHDV became endemic in most European countries. Especially for the wild rabbit population on the Iberian Peninsula, where European rabbits originated, RHDV caused a severe reduction of the population (Argüello et al., 1988; Villafuerte et al., 1995; Delibes-Mateos et al., 2007, 2008; Abrantes et al., 2012).

Already in 1988 RHDV was found in domestic rabbits in North Africa (Morisse et al., 1991). Also in 1988, it was introduced into Mexico, from where it was eradicated in 1992 most probably due to the absence of a susceptible wild rabbit population (Gregg et al., 1991). RHDV was first diagnosed in North America in 2000 followed by a limited number of outbreaks. Also in geographically distant regions, such as Cuba, Uruguay and Reunion Island, RHDV caused losses in domestic rabbits (Le Gall-Reculé et al., 2003; Farnós et al., 2007). The rapid dissemination of RHDV within one decade since the first detection, mainly due to the import of rabbits from already affected countries, resulted in the recent situation that RHDV is nowadays endemic in most parts of Europe, Asia, and parts of Africa (Cooke, 2002; Moss et al., 2002; Abrantes et al., 2012).

In contrast to the unwanted introduction of RHDV in all other countries, in Australia, where the European rabbit is an important 'pest species' and a major threat to the endemic wildlife (Gibb and Williams, 1994; Fenner, 2010), the Czech RHDV-1 strain V351 was introduced as a biocontrol agent on Wardang Island in Spencer Gulf, South Australia in 1991 (Cooke, 2002). In 1995, despite strict quarantine measures, RHDV escaped from the island (Cooke and Fenner, 2002) and spread all over southern Australia within two years (Mutze et al., 1998). In these areas, RHDV caused an up to 95% reduction of the rabbit populations (Abrantes et al., 2012).

In New Zealand, were it was initially decided not to follow the Australian example, RHDV was illegally introduced (as indicated by genetic analysis showing the similarity to the Czech V351 strain) with a comparable impact on the population of non-native European rabbits (Thompson and Clark, 1997; O'Keefe et. al., 1998).

Another problem with RHDV was the genetic variability after emergence in 1984. Since then several variants have been isolated with virulence ranging from avirulent, inducing no mortality but at least partial protection, to highly virulent with up to 100% mortality after infection (Capucci et al., 1996, 1998; Le Gall-Reculé et al., 1998, 2003). In 2010, a RHDV was isolated in France with only 82,4% nucleotide identity of the capsid protein VP60 gene to all known RHDV strains and grouped into the new cluster RHDV-2. The previous strains were afterwards grouped into the RHDV-1 cluster (Le Gall-Reculé et al., 2013; see 2.3.4.).

The morbidity and mortality induced by RHDV is with up to 100% extremely high in unvaccinated rabbits. Therefore, the disease has a dramatic direct effect on wild rabbit populations with up to 95% decline when first introduced. Since the majority of RHDV infected rabbits die in their burrows underground, RHD is extremely hard to locate in the wild. RHD prevalence also varies depending on the season, breeding cycles and geographical location with some areas with high morbidity and mortality among its rabbit populations followed by calmer periods (Cooke, 2002; Mutze et al., 1998). Two intrinsic factors - maternal antibodies transmitted to the young as well as a not yet fully understood resistance of young rabbits - may be responsible for reoccurrence of RHDV outbreaks as some rabbits may develop immunity against RHDV strains, while others may endure persistent infections. However, the immunity is not maintained through the next generation, leaving open the possibility of further outbreaks in the population (Cooke et al., 2000; Marques et al., 2012).

Indirectly, RHDV affects ecosystems in Europe, where wild rabbits are an important food source for certain endangered predators, such as Iberian lynx (*Lynx pardinus)* (Delibes-Mateos et al., 2007, 2008; Anonymous, 2016). Moreover, used to control excessive numbers of wild, non-native European rabbits (*Oryctolagus cuniculus*) in Australia and New Zealand, it may also influence the endemic fauna positively by the subsequent reduction of predator populations which formerly hunted rabbits (Anonymous, 2016; Pedler et al., 2016).

Finally, RHDV causes important economic losses in the rabbit meat and fur industry. Here, in the last two decades several 100 million rabbits died after RHDV infection (Abrantes et al., 2012). These dramatic economical losses highlight the need for the development of vaccines against RHDV.

2.3. The Rabbit Hemorrhagic Disease Virus (RHDV)

2.3.1. Classification

First trials to identify the RHD causing viral pathogen were hampered because RHDV cannot be cultivated in cell culture. In the beginning the virus was suspected to be a picornavirus (An et al., 1988), a parvovirus (Gregg and House, 1989) or a parvo-like virus (Xu, 1991). In the early 1990s it was finally identified as a member of the *Caliciviridae* family (Ohlinger et al. 1990; Parra and Prieto, 1990; Rodák et al., 1990; Meyers et al., 1991; Abrantes et al., 2012).

Four genera in the *Caliciviridae* family are recognized by the International Committee on Taxonomy of Viruses (ICTV) at the moment: Lagovirus, Vesivirus, Norovirus and Sapovirus. Three more genera are not recognized by the ICTV yet, but are nominated as part of this family. These are: Nabovirus or Becovirus (Oliver et al., 2006) Recovirus (Farkas et al., 2008) and Valovirus (L’Homme et al., 2009). Caliciviruses cause different diseases like gastroenteritis (Norovirus, Sapovirus), hemorrhagic diseases (Lagovirus) and reproductive failures, vesicular lesions and respiratory infections (Vesivirus). Several animal species and humans serve as hosts (Abrantes et al., 2012) (Fig. 1).

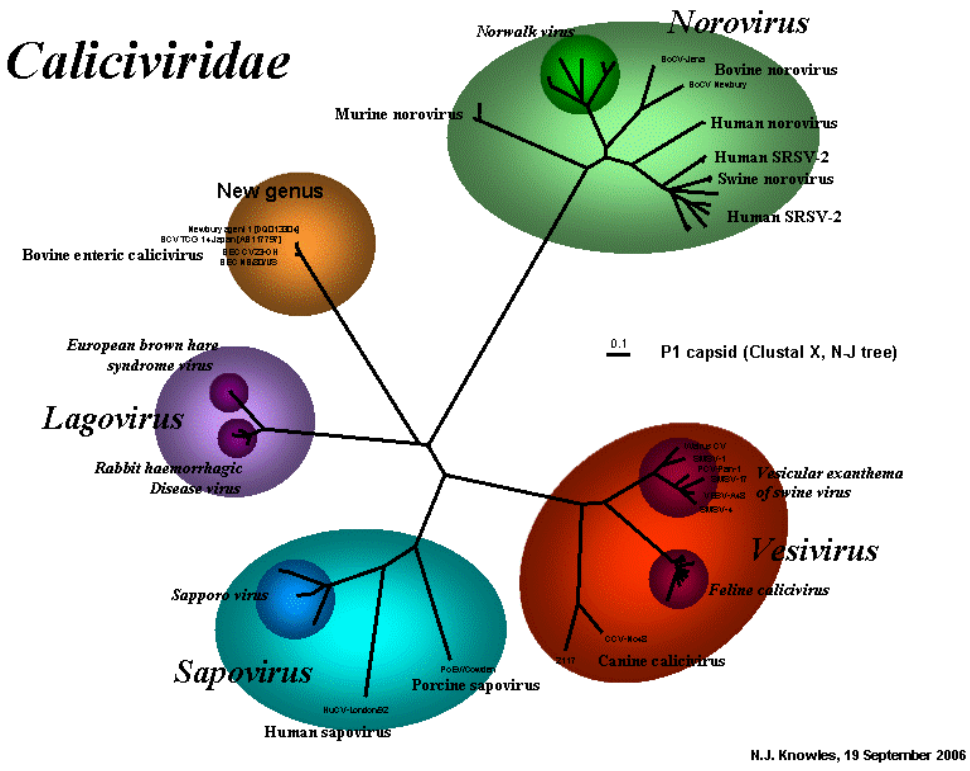

Fig. 1. Overview over Calicivirus genera (with permission of N.J. Knowles, Pirbright Institute, UK)

Currently, two virus species are assigned to the genus *Lagovirus*: RHDV and European brown hare syndrome virus (EBHSV). EBHSV was first detected in Sweden in the early 1980s (Gavier-Widén and Mörner, 1993). This virus is closely related to RHDV but represents a distinct species and only hares (*Lepus europaeus* and *Lepus timidus*) are susceptible to infection. Clinical symptoms, (histo-)pathological alterations, mortality rates, virion morphology and antigenicity are similar to RHDV, but there is no cross-species infection and cross-species protection (Capucci et al., 1991; Marcato et al., 1991; Chasey et al., 1992; Fuchs and Weissenböck, 1992; Wirblich et al., 1994; Lavazza et al., 1996; Abrantes et al., 2012).

2.3.2. Genome organization and replication

Caliciviruses are non-enveloped single stranded RNA viruses with a genome of positive polarity (Granzow et al., 1989). The genome consists of a genomic and subgenomic RNA (Meyers et al., 1991; Abrantes et al., 2012). In contrast to other Calicivirus genera, the

7437 nt genomic RNA of Lagoviruses encompasses 2 slightly overlapping ORFs, instead of 3 ORFs as in other Calicivirus genera. ORF1 encodes a polyprotein, which consists of non-structural proteins (p16, p23, p29, a helicase, RNA-dependent RNA polymerase, VPg and a protease) and the major structural capsid protein VP60 (Fig. 2).

After translation, the polyprotein precursor is cleaved by the viral trypsin-like cysteine protease. The helicase and RNA-dependent RNA polymerase are important for viral replication, whereas the role of p16, p23 and p29 is not known yet. ORF2 encodes VP10, a minor structural protein. The subgenomic RNA is 2,2kb in size and translated into structural proteins VP10 and VP60 (Wirblich et al., 1996; Abrantes et al., 2012), the latter used for virus assembly.

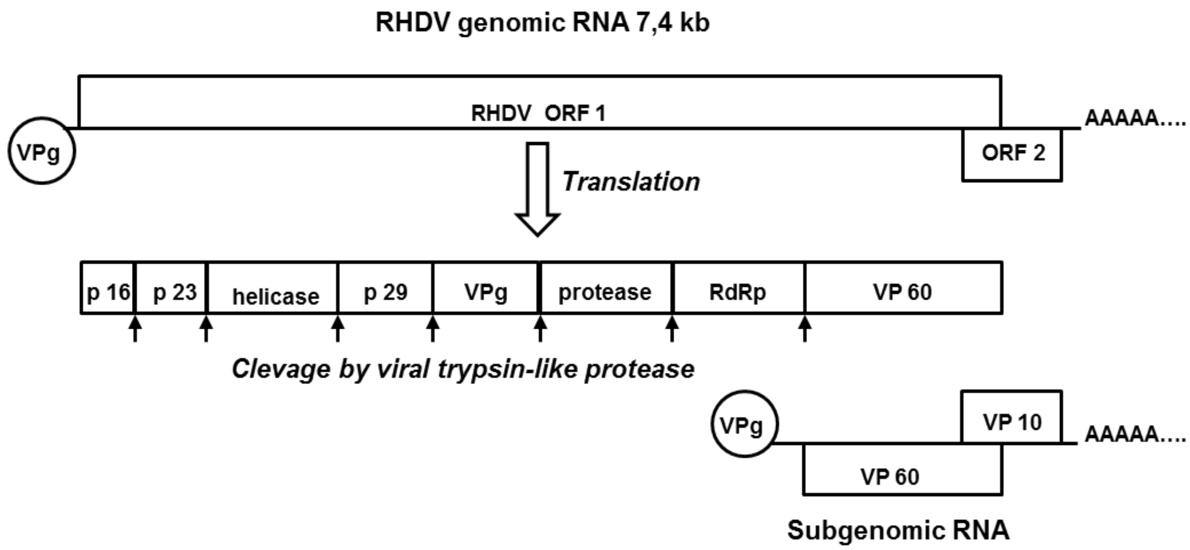

Fig. 2. Genomic organization of RHDV (Abrantes et al., 2012; modified)
The RHDV genome consists of two slightly overlapping ORFs, ORF1 and ORF2. ORF1 encodes a polyprotein which is cleaved by the viral trypsin-like protease (arrows) into non-structural proteins (p16, p23, helicase, p29, VPg, protease and RdRp) and the major structural protein VP60. ORF2 encodes the minor structural protein VP10. Subgenomic RNA encoding both VP60 and VP10 can be found in the viral particle, too. Both RNA species are poly-adenylated at their 3'end with the covalently attached viral protein VPg at the 5'end.

In contrast to other Caliciviruses, VP10 is not necessary for infectivity of RHDV but seems to induce apoptosis in host cells for virus release, downregulates VP60 expression and decreases the level of genome replication (Liu et al., 2008; Chen et al., 2009). Both, genomic and subgenomic RNA have a polyadenylated 3' terminus. At the 5' terminus a virus-genome linked protein (VPg) is attached (Wirblich et al., 1996; Abrantes et al., 2012) which may play a role in translation (Goodfellow et al., 2005; Wang et al., 2013a).

The first step of viral entry in Calicivirus infections involves recognition of histo-blood group antigens (HBGAs) by the P-domain L1 loop of VP60 (see 2.3.3.) (Ruvoën-Clouet et al., 2000;

Chen et al., 2011; Wang et al., 2013b). After attachment and internalization into the cell, the genomic RNA becomes uncoated and is translated into a polyprotein precursor, which is then processed and cleaved by the viral trypsin-like cysteine protease into the non-structural proteins and VP60 (Fig. 3). The protease, helicase, RNA-dependent RNA polymerase and VPg form a replication complex which synthesizes either antigenomic RNA from genomic RNA or subgenomic RNA from antigenomic RNA.

Antigenomic RNA is also used as template for genomic RNA which can be translated again into a polyprotein precursor or becomes packaged in new virus particles. The release mode is not fully known yet, but apoptosis seems to be involved (Rohayem et al., 2010; Abrantes et al., 2012).

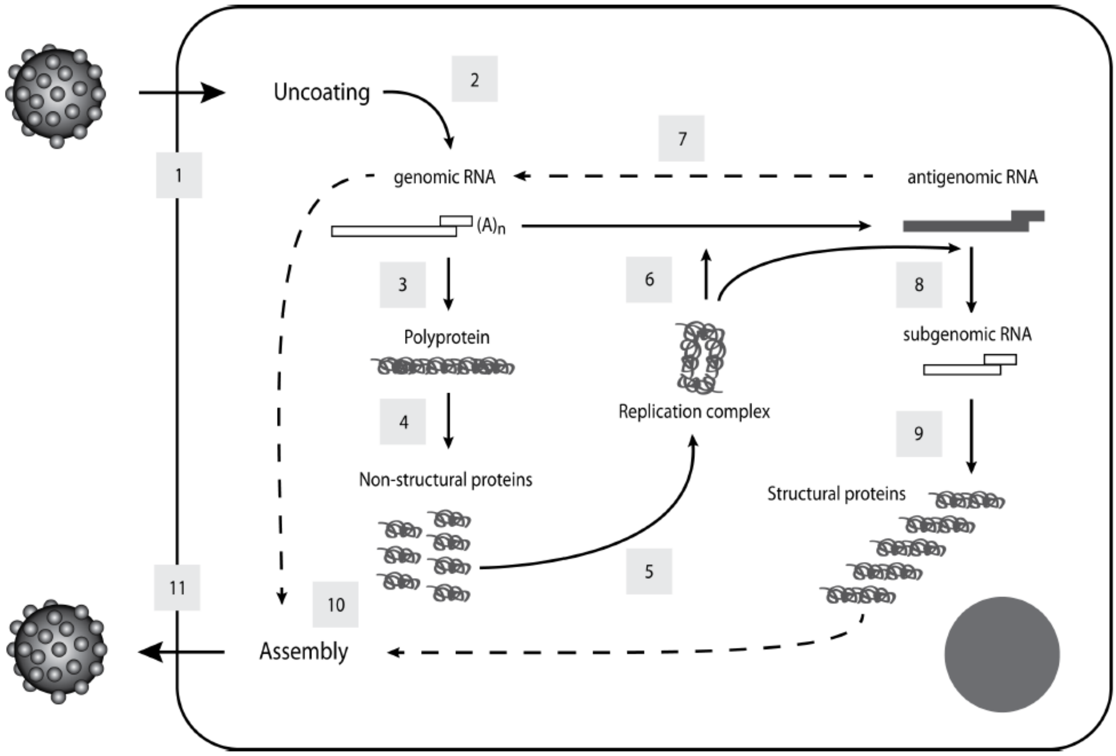

Fig. 3. The replication cycle of Caliciviruses (from Abrantes et al., 2012; modified)
1: Attachment and internalization; 2: Uncoating of viral genome; 3: translation of genomic RNA into polyprotein precursor; 4: cleaving into non-structural proteins and VP60 by viral protease; 5: formation of replication complex by non-structural proteins; 6: synthesis of antigenomic RNA; 7: antigenomic RNA as template for genomic RNA; 8: antigenomic RNA as template for subgenomic RNA; 9: translation of subgenomic RNA into structural proteins VP60 and VP10; 10: assembly of structural proteins and packaging of genomic RNA in assembled viral protein core; 11: release of mature virions

2.3.3. Viral particles, antigenicity and stability

RHDV mature virions are spherical, non-enveloped, icosahedral particles of 32-40nm in diameter, whose capsid consists of 90 dimers of capsid protein VP60. These dimers form 32 cup shaped depressions on the surface which are arranged in a T = 3 icosahedral symmetry (hence the family name *Caliciviridae* as calix means cup in Latin) (Granzow et al., 1989; Valícek et al., 1990; Thouvenin et al., 1997; Luque et al., 2012). Each VP60 monomer consists of a shell (S) domain and a protruding (P) domain. The S-domain is buried and includes the N-terminus. The P-domain is protruding on the surface and encompasses the C-terminus. Both are connected by a hinge domain. The P-domain (Fig. 4) is subdivided into two subdomains: P1 (stem of arch) and P2 (top of arch) (Prasad et al., 1994; Capucci et al., 1995; Bárcena et al., 2004; Hu et al., 2010; Abrantes et al., 2012).

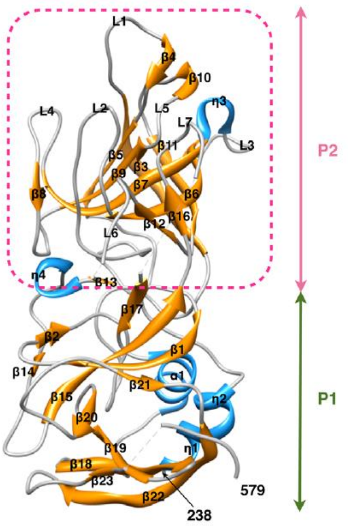

Fig. 4. Crystal structure of RHDV-VP60 P-domain (from Wang et al., 2013b; modified)
Ribbon representation of the crystal structure of the RHDV-VP60 P-domain. P1 (green) and P2 (pink) sub-domains are indicated and colored according to their secondary structure elements.

The P2-subdomain is located at the most exposed region of the capsid. The Loop L1 contributes to host interaction and contains one of the main neutralizing epitopes (Wang et al., 2013b). Therefore and due to selection pressure resulting from recognition by host antibodies, this region displays the greatest genetic and antigenic variation (Capucci et al., 1995; Martínez-Torrecuadrada et al., 1998; Bárcena et al., 2004; Abrantes et al., 2012) and tends to evolve faster to escape from the selective pressure (Esteves et al., 2008; Kinnear and Linde, 2010).

The virus itself is very resistant and remains infectious in the environment for a long time. When exposed to normal environmental conditions it can last up to 10 days in dried states. While in animal carcasses it can even last for 3 months (Henning et al., 2005). Durability is dependent on weather conditions. According to OIE, RHDV is infectious in carcasses for up to 20 days at 22°C and in dried states on clothes for at least 3 months at room temperature under experimental conditions. It also survives in organ suspensions > 7 months at 4°C or at least 2 days at 60°C in organ suspensions and dried states. Unprotected virus is resistant to temperatures of 50°C for 1 hour and also to freeze-thaw cycles. RHDV is stable at pH 4,5-10,5, but can also survive pH of 3,0. It can be inactivated for example by pH >12,0, formalin (1-2%), sodium hydroxide (1%), 0,5% sodium hypochlorite or substituted phenolics (Smíd et al., 1991; OIE, Technical Disease Card, 2009; Anonymous, 2016).

2.3.4. Appearance of RHDV variants

The existence of three main RHDV groups is indicated by genetic and antigenic comparison and epidemiological data (OIE, Terrestrial Manual, 2016):

a) "classical RHDV" (RHDV-1): Virus of genogroups G1–G5, first reported in 1984 in China (Liu et al., 1984) and since then spread to other areas in Asia, Africa, Americas, Europe and Oceania. Nowadays these viruses are endemic where European rabbits live naturally or are domesticated.
b) RHDVa/G6: Identified in Europe in 1996 (Capucci et al., 1998; Schirrmeier et al., 1999) and currently detected also in Oceania, Asia and Americas. Nucleotide identity of VP60 between classical RHDV and RHDVa was found to be about 93% (Capucci et al., 1998).
c) RHDV-2: Emerged in France in 2010 in wild and farmed vaccinated rabbits (Dalton et al., 2012; Le Gall-Reculé et al., 2011a, 2013), then rapidly spread in Europe, the Mediterranean basin (Malta and Tunisia), and also in Australia in 2015. The nucleotide identity of VP60 between RHDV-1/RHDVa and RHDV-2 was determined to be 82,4% and between EBHSV and RHDV-2 70,4%, confirming that it is indeed a new RHDV variant.

The G1-G6 RHDV genogroups of the serotype RHDV-1 do not cluster by regional but by temporal appearance or year of emergence. Originally groups G1-G3 were identified. Later G1 and G2 disappeared in many regions. G3 turned into G4 and new clusters G5 and G6 emerged with subtype G6 being a distinct antigenic variant (RHDVa) (Le Gall-Reculé et al., 1998, 2003).

RHDV-2 originated of unknown origin and seems not to derive from classical RHDV (Le Gall-Reculé et al., 2013). It is classified as a second RHDV serotype (Fig. 5).

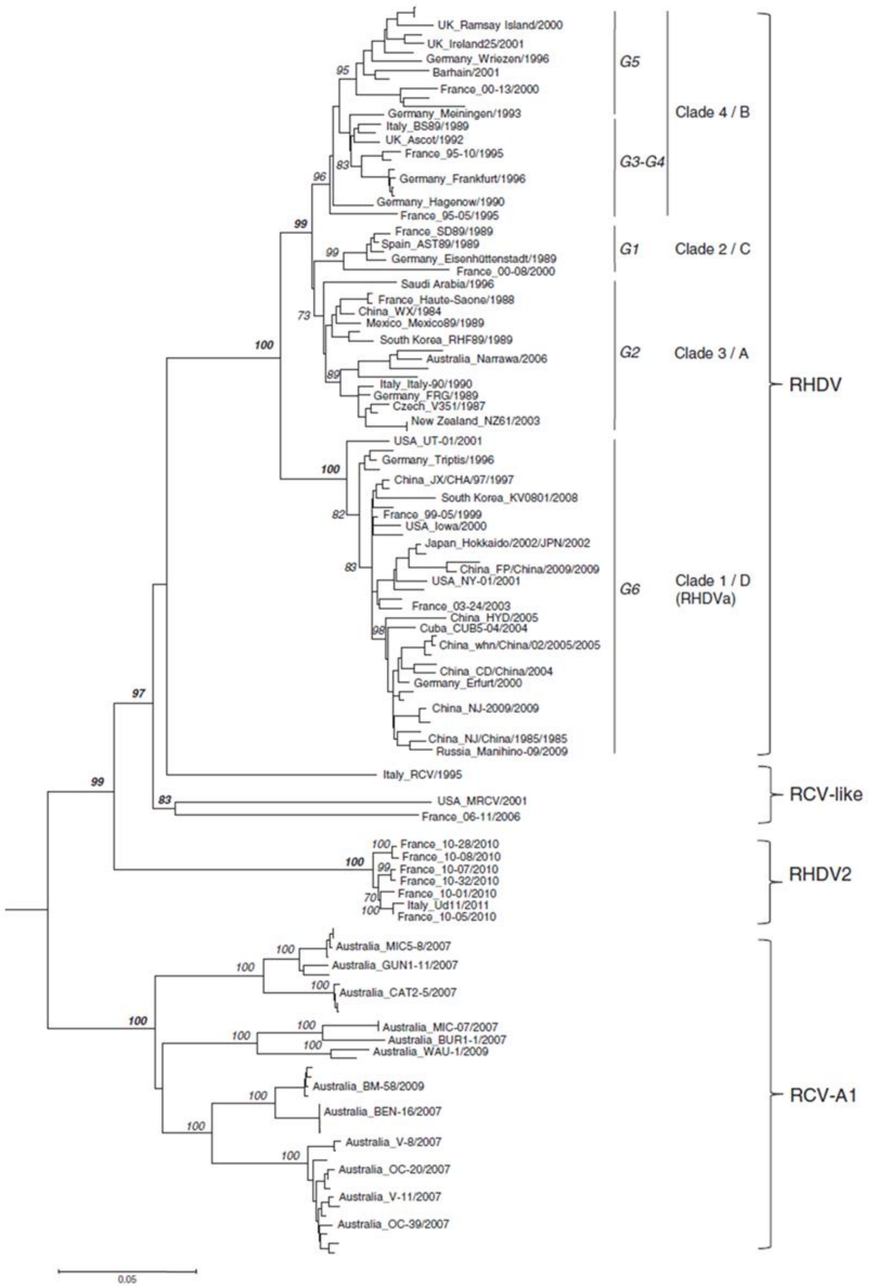

Fig. 5. Phylogenetic tree derived for RHDV-VP60 gene nucleotide sequences of 127 rabbit lagoviruses including 7 RHDV-2 (from Le Gall-Reculé et al., 2013; modified)
The tree was obtained using the Neighbor-Joining method and was drawn to a scale of nucleotide substitutions per site. The percentages greater than 70% of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are given in italics before each major branch node. The European brown hare syndrome virus (EBHSV) strain GD (Z69620) was used as an out-group to root the tree. The names of some representative strains from different countries are shown. For RHDV, the genetic groups G1 to G6 according to Le Gall-Reculé et al., 2003 and clade 1 to 4, or A to D, according to Kerr et al., 2009 or to Kinnear et al., 2010, respectively, are annotated.

After RHDV-2 discovery in north western France in summer of 2010 and its detection in samples collected in April 2010 from a rabbitry in western France, further cases appeared in southern France in February 2011 and in north eastern Italy in summer 2011. The virus was not only found in rabbitries but in wild populations as well (Le Gall-Reculé et al., 2013).

Liver samples of rabbits and also cape hares in Sardinia collected between April and October 2011 (Puggioni et al., 2013) were also tested positive for RHDV-2. In Spain RHDV-2 was confirmed after testing of liver samples collected in September 2011 (Dalton et al., 2012). In November 2012 RHDV-2 was found in livers of both of the European rabbit subspecies in Portugal (Abrantes et al., 2013). In 2014 RHDV-2 cases in Scotland and Wales and in 2014 England were confirmed (Baily et al., 2014; Westcott et al., 2014). Westcott and Choudhury (2015) even traced back the occurrence of RHDV-2 in Great Britain to 2010. In late 2014 RHDV-2 was detected on the Azores islands and therefore for the first time outside of continental Europe (Duarte et al., 2015a, b). In 2015 first cases were described in Australia with a strain closely related to another one that is currently present in Portugal and the Azores islands (Hall et al., 2015). To date, RHDV-2 continues to spread and seems to replace the classical strains of RHDV in some regions, e.g. in the Iberian Peninsula (Dalton et al., 2014), Portugal (Lopes et al., 2014a) and France (Le Gall-Reculé et al., 2013).
First cases in Germany were proved in samples from a rabbitry in North Rhine Westphalia in RHDV-1 vaccinated rabbits in 2013 (Fig. 6).

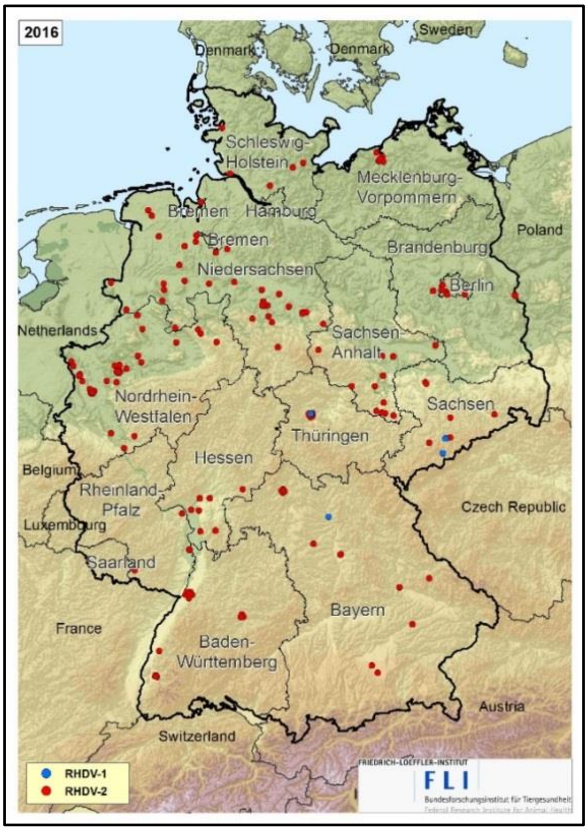

Fig. 6. Overview of classical RHDV (RHDV-1) and RHDV-2 cases in Germany in 2016
Only rabbits examined at FLI- Insel Riems are depicted. (Overview by N. Neumann, kindly provided by Dr. P. König, FLI- Insel Riems)

In March 2014, it was detected in Rhineland Palatinate and in August outbreaks occurred already in middle and eastern parts of Germany (FLI- Insel Riems). By the end of 2016, RHDV-2 had spread across Germany and most of the liver samples of deceased rabbits that were examined in 2016 at FLI- Insel Riems were tested positive for RHDV-2 (Fig. 6).
In contrast to full cross-protection between RHDV-1 and RHDVa (Capucci et al., 1998; Schirrmeier et al., 1999), only a partial cross-protection between RHDV-1/RHDVa and RHDV-2 (Le Gall-Reculé et al., 2013) was found in infection trials.
In addition to the pathogenic variants of classical RHDV, RHDVa and RHDV-2, there are several strains of non-pathogenic rabbit Caliciviruses circulating in the wild rabbit population. An Italian non-pathogenic strain isolated in 1996 was called Rabbit Calicivirus (RCV) and was the first evidence of non-pathogenic Caliciviruses in rabbits (Capucci et al., 1996). For non-pathogenic Caliciviruses the new term rabbit Calicivirus-like viruses (RCV-like) was introduced by Kerr et al. (2009). Together with isolates like Ashington (Moss et al., 2002) or 06-11 (Le Gall-Reculé et al., 2011b), the Italian RCV belongs to a new group of rabbit Caliciviruses which is distinct from the RHDV-1/RHDVa group (Strive et al., 2009). Another important non-pathogenic strain is the Australian strain RCV-A1 (Strive et al., 2010), which is genetically distinct also from other RCVs and forms a separate group (Strive et al., 2009) (Fig. 5). Non-lethality has been confirmed for the Italian RCV and RCV-A1, whereas it is only assumed for other non-pathogenic Caliciviruses (Le Gall-Reculé et al., 2011b). These non-pathogenic RHDV strains are transmitted by the fecal-oral route and have a different organ tropism. They do not replicate in the liver but in the intestine of rabbits. However, the Italian RCV was also found in liver and spleen in a few rabbits in small amounts (Capucci et al., 1996) and the RCV-A1 virus was detected in the liver of one and the spleen of two animals after infection (Strive et al., 2009). Rabbits infected with RCV do not display any RHD like symptoms. Importantly, RCV is able to induce antibody titers in rabbits which can lead to complete cross-protection against classical RHDV infection. However, these RCV cannot infect hares and there is no cross-protection between RCV and EBHSV (Capucci et al., 1996). RCV-A1 induces only a partial cross-protection against classical RHDV (Strive et al., 2009). The amino acid identity of VP60 of the Italian RCV to the classical RHDV is 91,5%. The average nucleotide identity between RCV-A1 and classical RHDV is 78%, and between RCV-A1 and EBHSV 71% at the genomic level.
Some non-pathogenic strains, however, do not induce any protection against classical RHDV, for example the strain 06-11 (Le Gall-Reculé et al., 2011b), although the nucleotide identity of VP60 between RCV strain 06-11 and classical RHDV is 83% (Le Gall-Reculé et al.,

2011b). High antibody levels for non-pathogenic, non-protective strains were detected in rabbit sera but those animals did not survive a classical RHDV infection (Marchandeau et al., 2005; Abrantes et al., 2012).

The pathogenic forms seem to have evolved from non-pathogenic Caliciviruses (Capucci et al., 1996; Moss et al., 2002; Forrester et al., 2006; Strive et al., 2010). Moss et al. (2002) demonstrated that rabbit Caliciviruses were circulating in Great Britain and most likely also in the rest of Europe at least 30 years before the first outbreak of RHDV in China. There seems to be a common ancestor of RCV-like viruses and RHDV over 200 years ago and it is suggested that virulent RHDV emerged in the early 20th century, as the most plausible explanation for the sudden occurrence of pathogenic RHDV (Kerr et al., 2009). Another hypothesis for the occurrence of pathogenic rabbit Caliciviruses is a species jump as RHDV was found in small mammals (*Mus spretus*, *Apodemus sylvaticus*) close to wild rabbit populations (Merchán et al., 2011; Abrantes et al., 2012; Le Gall-Reculé et al., 2013). There is still a lot unknown regarding the importance of non-pathogenic strains for the variation of RHDV (Marchandeau et al., 2005). A current example of ongoing mutual influence of different RHDV strains regarding protection against RHDV can be observed in Australia. The non-pathogenic Australian strain RCV-A1 induces partial cross-protection against the pathogenic Czech RHDV-1 strain V351 that was released in Australia in 1996 in order to eradicate rabbit populations therefore interfering with success of this project. However, the Korean RHDVa strain named K5 is in turn able to break the protection against RHDV-1 build by RCV-A1 which makes it a useful tool for further decimation of rabbits (www.pestsmart.org.au).

2.4. Rabbit Hemorrhagic Disease (RHD)

2.4.1. Susceptibility and transmission

Wild and domestic European rabbits (*Oryctolagus cuniculus*) from the age of 9 weeks are fully susceptible to classical RHDV and develop severe clinical signs within 20–48h after infection (Xu and Chen, 1989). Other lagomorphs like European brown hares (*Lepus europaeus*), cottontails (*Sylvilagus floridanus*) (Lavazza et al., 2015), black-tailed jackrabbits (*Lepus californicus*) and volcano rabbits (*Romerolagus diazzi*) seem not to be susceptible to classical RHDV (Merchán et al., 2011). However, in dead wild Iberian hares (*Lepus granatensis*) collected during an outbreak in the 1990s classical RHDV-RNA was recently detected (Lopes et al., 2014b). In some rodents like wood mice (*Apodemus sylvaticus*) and Algerian mice (*Mus spretus*), collected in the vicinity of warrens that contained RHDV infected wild rabbits, viral RNA was detected in internal organs (Merchán et al., 2011). No evidence of RHDV replication was found in any other mammals tested so far, including rabbit predators, although some of those animals did seroconvert (Leighton et al., 1995; Parkes et al., 2004; Merchán et al., 2011; Anonymous, 2016).

The new virus variant RHDV-2 seems to have a broader host range as this virus infects not only European rabbits but also Cape hares (*Lepus capensis var. mediterraneus*) and Italian hares (*Lepus corsicanus*) (Le Gall-Reculé et al., 2013; Puggioni et al., 2013; Camarda et al., 2014). While initially no evidence was found of infected European brown hares (Puggioni et al., 2013) more and more cases were detected recently (Velarde et al., 2016; Hall et al., 2017; FLI- Insel Riems), suggesting another species jump. A possible explanation for overcoming species barriers could be the genetic variation of the capsid protein VP60 which alters the binding to histo-blood group antigens that are discussed to be important entry ways for the virus (Le Gall-Reculé et al., 2013; Puggioni et al., 2013). HBGAs are found in the upper respiratory tract and intestines of rabbits, and RHDV is able to bind to these receptors. Different types of HBGAs were found in rabbits and different virus strains show variable affinity to the different HBGAs, suggesting that there is a constant adaptation of the host as well as the virus. By changing those attachment factors, e.g. through mutations, individual animals or even complete species can become more or less susceptible to the virus (Nyström et al., 2011; Le Pendu et al., 2014; Velarde et al., 2016).

The virus is transmitted mainly orally, but also by the nasal, conjunctival or parenteral route by direct contact with live or dead animals, or indirectly by contaminated equipment, food, water and clothes as well as insects (Xu and Chen, 1989; Ohlinger et al., 1993; Asgari et al., 1998). Infectious virus can persist in flies for up to 9 days and already a few virus particles can infect rabbits via the conjunctival route. Virus can be deposited via fly spots (oral or anal excretions of flies) on vegetation where it is then consumed by rabbits (Asgari et al., 1998). RHDV is supposed to be transmitted with most secretions and excretions, e.g. urine, feces and respiratory secretions from infected animals and can be shed by surviving animals for at least one month after their recovery. Viral RNA has been detected in rabbits for at least 15 weeks after infection (Gall et al., 2007; Anonymous, 2016). RHDV remains infectious in carcasses for long periods of time and even rabbit fur can contain infectious virus (Xu and Chen, 1989; Xu 1991; McColl et al., 2002; Henning et al., 2005).

2.4.2. Clinical course and pathology

The incubation period of RHD usually ranges between 20-48h with rabbits dying in most cases within 12-36h after onset of fever, which can rise over 40°C. Four different clinical courses are distinguished: peracute, acute, subacute and chronic (Xu and Chen, 1989; Abrantes et al., 2012). In the peracute form, animals die suddenly without any clinical signs. Sometimes foamy hemorrhagic nasal discharge and vaginal hemorrhages are seen. High fever, anorexia, apathy, congestion of the palpebral conjunctiva and death within 48-72h post infection are characteristics of the acute form. Also neurological symptoms like opisthotonus, excitement, paralysis and ataxia were observed. In the moribund stage tracheitis, dyspnea and cyanosis as well as foamy and bloody nasal discharge, lacrimation, ocular hemorrhages and epistaxis can be seen. In subacute forms of the disease rabbits display similar, but milder clinical symptoms and most animals survive. Characteristically, rabbits surviving the RHDV infections develop high RHDV specific antibody titers which confer a long-lasting protection from re-infection (Patton, 1989). A low percentage of RHDV infected rabbits develop a chronic form of the disease with severe and generalized jaundice, anorexia and lethargy (Capucci et al., 1991). Of these chronically infected animals some die within 2 weeks post infection (Lavazza and Capucci, 2008), but those that survive develop high RHDV specific antibody titers (Capucci et al., 1991; Abrantes et al., 2012). For RHDV-2 similar symptoms, but more prolonged courses of the disease are described (Le Gall-Reculé et. al., 2013). Mortality rates range from 5-60% (Velarde et al., 2016) in contrast to mortality rates between 70-100% of RHDV-1.

Main (histo-)pathological alterations are seen in the liver, lungs, spleen, kidneys and serosal surfaces. In the liver an acute necrotizing hepatitis is seen due to apoptosis of liver cells induced by the virus (Alonso et al., 1998). It usually appears swollen, yellow/grey to-red, fragile and reticulated. Disseminated hepatic necrosis is seen with fatty degeneration. Petechial hemorrhages are also seen in the mucosa of gall bladder. Kidneys often are enlarged, congested with hyperemia or petechial hemorrhages, (glomerulo-)nephritis can be found in some cases. Additionally, hyaline thrombi and hyaline degeneration are seen in glomerular capillaries. Splenomegaly due to congestion as well as depletion of lymphocytes due to necrosis as characterized by karryorhexis and karyolysis of the lymphocytes and reticuloendothelial cells is found. In the lungs hemorrhages, hyperemia and alveolar edema are found with presence of macrophages and neutrophils in the airway and alveoli, while in the trachea bloody foam and hyperemia of the mucous membrane are seen. Hemorrhages and congestions can also be observed in other organs, like brain, thymus and heart (Xu and Chen, 1989; Marcato et al., 1991; Park et al., 1995; Abrantes et al., 2012).

2.4.3. Pathogenesis

After entry, the virus presumably attaches to HBGA receptors in the upper respiratory and digestive tract (Nyström et al., 2011). The main target cells are hepatocytes. In animals older than 9 weeks, virus antigen was found in the liver already from 12h pi to 24h pi, mainly in periportal areas. During a massive rise of antigen amounts over the next 24h, symptoms of apoptosis begin (Prieto et al., 2000).

Already in an early infection stage, viral antigen is found in neutrophils surrounding infected hepatocytes. Antigen is also detected in Kupffer cells, circulating monocytes, lymphocytes and macrophages in the red and white pulp of the spleen, lung macrophages, glomerular mesangial cells of the kidneys and lymphocytes in the thymus and lymph nodes (Ramiro-Ibáñez et al., 1999; Prieto et al., 2000; Kimura et al., 2001). However, it remains unclear whether replication takes place in these cells (Prieto et al., 2000) but the possibility was discussed in another study (Kimura et al., 2001). It is suggested, that macrophages and circulating monocytes play an important role in virus dissemination in the body (Ramiro-Ibáñez et al., 1999; Kimura et al., 2001).

In the end, animals die from acute liver failure and disseminated intravascular coagulation (DIC) which leads to total organ failure (Ueda et al., 1992; Park et al., 1995). Liver failure results as virus induces apoptosis in hepatocytes (Vallejo et al., 2014; Trzeciak-Ryczek et al., 2015). After apoptosis there is little to no regeneration of liver tissue which leads to loss of function and an increase of liver enzymes can be seen in the blood (AST, ALT, bilirubin, γ-GT, AP, LDH) (Ferreira et al., 2006; Trzeciak-Ryczek et al., 2015). Bilirubin rises already 18h pi, AST and ALT from 24-36h. AST values of > 6000 IU/l result in death in the next 6h. Hypoglycemia, probably due to damage of mitochondria during apoptosis, is also an important finding which is assumed to be responsible for seizures before death (Ferreira et al., 2006). In the terminal phase of the disease a decrease of thrombocytes, leukocytes, fibrinogen, antithrombin, coagulation factors V, VII, X and an increase of fibrin is observed. Additionally a prolonged activated partial thromboplastin time and prothrombin time can be measured (Plassiart et al., 1992; Ueda et al., 1992). Severe leukopenia is explained by cytotoxic effects of the virus to white blood cells, the migration of cells to the liver and reduced production of white blood cells due to a cytopathic effect of the virus to the bone marrow (Ferreira et al., 2006). DIC means a wide spread activation of the coagulation system in the body. The internal and external coagulation pathway is activated which leads to an increased coagulation rate in the whole body. This results in formation of blood clots in small blood vessels and therefore organ failure and consequently in the consumption of thrombocytes and coagulation factors which in turn leads to heavy bleeding. DIC can be caused by many reasons, for example through trauma, bacterial or viral infections, intoxication etc. In RHDV-infected animals, DIC was already observed between 24h and 30h after infection. Its pathogenesis remains unclear (Trzeciak-Ryczek et al., 2015) and there have been many suppositions made about it. It is concluded that DIC appears together with liver necrosis, because rabbits with mild hepatitis do not develop DIC, whereas rabbits with heavy acute necrotizing hepatitis do (Plassiart et al., 1992). DIC seems to be caused by liver dysfunction, which leads to activation of the external coagulation pathway by tissue thromboplastin (external pathway) or activation of coagulation factors in serum (internal pathway) due to endothelium damage, to reduced formation of coagulation factors in the liver, a reduced clearing of coagulation factors because of liver and spleen damage and a reduction of coagulation inhibitors leading to increased coagulation (Plassiart et al., 1992; Ueda et al., 1992; Park et al., 1997). It is unknown, whether the endothelium is damaged by the virus itself, by antigen-antibody complexes or because of aggregation of infected monocytes at the endothelium (Park et al., 1997; Ramiro-Ibáñez et al., 1999).

2.4.4. Age dependent resistance

Rabbits younger than 9 weeks display a not yet fully understood resistance against a RHDV infection which seems to be independent of maternal antibodies, but involves the innate immune system. They do not exhibit any clinical symptoms (Mikami et al., 1999; Marques et al., 2012, 2014). After infection at an early age, rabbits achieve a long-term resistance like surviving adult rabbits (Ferreira et al., 2005; Marques et al., 2012). In 2 week old rabbits, aggregates of macrophages, lymphocytes and heterophils in the liver increase from 24h pi. Near these aggregates necrotic hepatocytes are detected. Similar findings are seen in 4 week old rabbits but with more severity suggesting that 4 week old rabbits become already more susceptible than younger animals. RHDV-antigen is only found in hepatocytes and macrophages in the liver in these young rabbits, and in contrast to adult rabbits only a few are infected (Mikami et al., 1999). Also, the number of thrombocytes and coagulation factors do not change and liver enzymes ALT and AST increase only slightly (Ferreira et al., 2004). From 24h pi large numbers of heterophils are found in the liver of 4 week old rabbits whereas from 48h pi mostly B- and T-cell lymphocytes as well as liver macrophages are detected with most hepatocytes being intact. At that time of the infection in fully susceptible animals, large amounts of heterophils and damaged hepatocytes are usually found (Ferreira et al., 2005; Marques et al., 2012). While adult rabbits display leukopenia with severe decrease not only of heterophils but also of lymphocytes in the final stage of the disease, young rabbits show only a transient decrease of heterophils (Ferreira et al., 2004, 2006). The resistance of young animals seems to be based on innate immune mechanisms in early immune response with activation of pro- and anti-inflammatory cytokines and IFNα (Ferreira et al., 2005; Marques et al., 2012). When immuno-suppressed, young rabbits infected with RHDV show the same clinical symptoms and pathological alterations as adult rabbits as well as an increase of cytokines and heterophils in the liver (Marques et al., 2014). With increasing age rabbits become more susceptible to a RHDV-1/RHDVa infection. The reasons for the increasing susceptibility are still unknown. It could be connected to a change in molecular structures on the surface of hepatocytes or changes in HBGA patterns which are also made responsible for differences in susceptibility of different species as was mentioned earlier (Ferreira et al., 2005; Nyström et al., 2011; Abrantes et al., 2012). The new variant RHDV-2 infects and kills young rabbits from the age of 4 weeks, sometimes even younger (Dalton et al., 2012). The basis for this early susceptibility to this virus variant is also still unknown.

2.5. Control of Rabbit Hemorrhagic Disease

2.5.1. Treatment

No treatment is available to cure infected rabbits once clinical symptoms appear. A metaphylactic passive immunization is useful only for animals with subclinical or no clinical signs to gain protection for a short time (Abrantes et al., 2012).

2.5.2. Protection by sanitation and hygiene management

For control of RHDV a proper hygiene management and vaccination are the most important tools. To limit distribution and prevent disease, especially in the rabbit industry, biosecurity measures such as sanitation, disinfection and quarantine are highly recommended. These measures are even more important in countries with circulating RHDV in wild rabbits where eradication cannot be achieved, while RHDV-free countries could place restrictions on importation of rabbits and rabbit products. A strict hygiene management can help to prevent spreading of the virus among the animals. Before integrating new animals in consisting groups, quarantine is recommended. Correct hygiene management of RHDV outbreaks is dependent on the epidemiological situation of the region in which they occur. In order to determine the right management measure, viral evolution in the field should be monitored to detect new genetic and antigenic variants early (Argüello- Villares, 1991; Abrantes et al., 2012; Le Gall-Reculé et al., 2013; Anonymous, 2016).

2.5.3. Protection by vaccination

Vaccines are supposed to protect organisms against diseases by stimulation of a specific anti-pathogen immune response (Aoshi et al., 2011). There are two principle forms of vaccination: passive and active. For passive immunization pathogen-specific, neutralizing antibodies (immunoglobulin preparations from animals of the same species) are applied to provide a "lent" immunity. This form is mainly used as metaphylactic treatment when a naïve host is infected by pathogens causing severe diseases like rabies (Both et al., 2012).

Active immunization is achieved by vaccines composed of either attenuated live or inactivated pathogens. Conventional live attenuated vaccines contain former virulent agents that are attenuated in vitro either by a mutagenic agent or by different culture conditions or they contain non-pathogenic field strains. Live vaccines induce a long-lasting immune response by mimicking a natural infection. The problem with attenuated vaccines is the possibility of reversion to virulence by passaging in the host.

Conventional killed vaccines contain inactivated pathogens or only immunogenic parts of them. The induced immune response is usually short-lived. To maintain a protective immune status, multiple doses and booster immunizations are frequently necessary. However, the advantage of these vaccines is that the antigen cannot replicate or reverse to virulence. Additionally, they can be stored easily in a freeze-dried state and refrigeration like for live vaccines is not necessary (Babiuk, 2002).

2.5.3.1. Conventional vaccines against RHDV

Vaccines against the classical variants RHDV/RHDVa are usually made of liver material of infected rabbits followed by chemical inactivation of the virus (Argüello-Villares, 1991; Smíd et al., 1991). An exception is the recombinant vaccine "Nobivac Myxo-RHD" (Intervet International BV, Netherlands) which contains a myxoma virus vector that expresses RHDV-1-VP60. Examples of liver-derived vaccines against RHDV licensed in Germany are shown in table 1.

In September 2016, the first liver-derived vaccine against the new variant RHDV-2 was introduced into the European market (Eravac, Laboratorios Hipra S.A., E) followed by a second in March 2017 (Filavac VHD K C+V, FILAVIE, F Roussay) which covers RHDV-1

and RHDV-2. Further liver-derived RHDV-2 vaccines are available with only national authorization in Spain (Novarvilap, Ovejero Laboratorio; Cunipravac RHD variant, Hipra, veterinary faculty Utrecht) (StIKoVet, FLI, state 28.06.2016 + 08.05.2017).
The RHDV-1 vaccine “CUNIVAK RHD” provides an early long-lasting protection against RHDV-1. Moreover, a partial cross-protection against RHDV-2 was seen in rabbits after prime-boost vaccination 7 days after a second vaccination. This cross-protection lasts for 3 months as well as for 6 months as 89,5% and 83,3% of prime-boost vaccinated rabbits survived a challenge with RHDV-2 (Dr. H. Schirrmeier, FLI- Insel Riems, personal communication; Dr. M. Müller, IDT, personal communication).

Tab. 1. Overview of liver-derived vaccines against RHDV licensed in Germany (PEI, state 12.07.2017)

vaccine	containing virus strains	manufacturer	date of accession	accession number
Lapimed RHD	classical RHDV strain AG88, inactivated	Merial GmbH	08.04.1995	499a/91
Dercunimix	myxoma virus strain SG3, attenuated classical RHDV strain AG88, inactivated	Merial GmbH	20.12.2001	PEI.V.01945.01.1
RIKA-VACC RHD	classical RHDV strain Eisenhüttenstadt, inactivated	Ecuphar AG	04.09.2003	200a/91
CUNIVAK RHD	classical RHDV strain Eisenhüttenstadt, inactivated	IDT Biologika GmbH	11.05.2004	206a/92
RIKA-VACC Duo	myxoma virus strain CAMP V-219, attenuated classical RHDV strain CAMP V-351, inactivated	Ecuphar NV	12.06.2008	PEI.V.03071.01.1
CUNIVAK COMBO	myxoma virus strain CAMP V-219, attenuated classical RHDV strain CAMP V-351, inactivated	IDT Biologika GmbH	05.08.2009	PEI.V.07962.01.1
Eravac	RHDV-2 strain V-1037, inactivated	Laboratorios Hipra S.A., E	26.09.2016	EU/2/16/199
Filavac VHD K C+V	RHDV-1 strain IM.507 SC.2011, inactivated RHDV-2 strain LP.SV.2012, inactivated	FILAVIE, F Roussay	13.03.2017	PEI.V.11900.01.1

2.5.3.2. Recombinant vaccines

There is a growing interest in the use of molecular methods to obtain novel safe and efficient vaccines. The goal is to avoid the risks associated with live vaccines but to maintain the efficient induction of an immune response by a biologically active agent that can replicate in the host. Specific genes can be deleted, which results in reduced risks of reversion. This concept is used in so called marker vaccines that also allow differentiation between organisms infected with wild type or vaccine virus. Another possibility is to use modified viruses as vectors for other pathogens, therefore allowing immunization against more than one pathogen (Babiuk, 2002). For RHDV this approach was used for the recombinant vaccine “Nobivac Myxo-RHD” (Intervet International BV, Netherlands, source PEI, state 12.07.2017) in which a myxoma virus vector expresses RHDV-1-VP60 and induces protection against both myxomatosis and classical RHDV.

Another type of genetically engineered vaccines are sub-unit vaccines. They contain single proteins or peptides which are derived from infectious virus material or produced in recombinant expression vector systems (Babiuk, 2002). Single proteins have the disadvantage of being less immunogenic than vaccines containing the whole virus particle, therefore being more expensive in manufacturing because higher amounts of antigenic protein is needed than in conventional vaccines (Noad and Roy, 2003). Special kinds of sub-unit vaccines are Virus like particle (VLP) vaccines (Noad and Roy, 2003). VLPs are virus particles that lack viral genome. They are not infectious but because of their similarity to infectious particles by structure and antigenicity, they have the ability to induce a strong immune response. Structural proteins can assemble spontaneously to VLPs with their immunogenic potential being higher than that of non-assembled proteins. That is also the reason why less antigen is needed than in classical sub-unit vaccines. VLPs can induce not only a humoral but also a cellular immune response (Grgagic and Anderson, 2006; Chen and Lai, 2013). Processing of VLPs by dendritic cells can lead to activation of the innate and adaptive immune system (Grgagic and Anderson, 2006; Chen and Lai, 2013). VLPs taken up by antigen presenting cells can be presented by MHC class II molecules after processing. This leads to activation of dendritic cells, abundant cytokine release and stimulation of $CD4^+$ T-cells. VLPs are also presented by MHC class I molecules by antigen presenting cells, after their processing in the cytosol, leading to activation of cytotoxic $CD8^+$ T-cells (Kushnir et al., 2012; Chen and Lai, 2013). Due to their size, VLPs can spread easily to lymph nodes where even more T-cells can interact with them. Some VLP types are shown to induce maturation of dendritic cells which in turn produce cytokines and activate $CD8^+$ T-cells (Chen and Lai, 2013). VLPs induce also B-cell responses with generation of memory B-cells leading to high antibody titers and long-lasting immune responses (Chen and Lai, 2013). Because of this great immunogenic potential, VLPs are explored for use in many different fields for diagnostic, prophylactic or therapeutic use like vaccines, gene therapy or immunotherapy (Kushnir et al., 2012).

VLPs for vaccine development are often generated by using different expression systems like baculoviruses, yeast, Escherichia coli or Vaccinia virus (Noad and Roy, 2003). Presently, different vaccines based on VLPs are commercially used. In veterinary medicine, for example, two VLP based vaccines against porcine circovirus type 2 are on the market: Ingelvac CircoFLEX®, Boehringer Ingelheim and Porcilis PCV, Intervet International B.V., Netherlands/MSD (Crisci et al., 2012; van Oers et al., 2015; PEI, state of 15.02.2017).

For the development of RHDV-1 vaccines based on the recombinant capsid protein VP60, different heterologous expression systems and recombinant viruses (Bertagnoli et al., 1996a, b; Fischer et al., 1997; Bárcena et al., 2000; Fernández et al., 2011; Rohde et al., 2011) were established. As expression systems served Escherichia coli (Boga et al., 1994; Guo et al, 2016), cultured insect cells (Laurent et al., 1994; Marín et al., 1995; Nagesha et al., 1995; Plana-Duran et al., 1996; Gromadzka et al., 2006; López-Vidal et al., 2015), yeast (Farnós et al., 2005), plants (Castañón et al., 1999; Mischkofsky et al., 2009) and insect larvae (Pérez-Filgueira et al., 2007). The immunogenic potential of recombinant VP60 by induction of a protective humoral immune response was proven in different studies. However, low production costs, high yields and the potential of scaling up need to be taken into consideration when aiming for commercial use (Abrantes et al., 2012).

2.6. Recombinant baculoviruses

2.6.1. Baculovirus

Baculoviruses are DNA viruses of the family *Baculoviridae* with about 700 known members. Their natural hosts are insects mainly of the order Lepidoptera to which butterflies and moths belong. They cannot infect and replicate in mammalian cells but can be internalized by vertebrate cells.

Baculoviruses are divided into four genera: α-, β-, γ- and δ-baculovirus. They are rod shaped, enveloped viruses, of about 30-60 x 250-300nm in size, and contain a circular double stranded DNA with a genome of 80-180kb (Airenne et al., 2013).

There are two virus forms, BV (budded virus) and ODV (occlusion-derived virus). ODV is surrounded by a so-called occlusion body which is composed of polyhedrin and is the viral form which can persist in the environment. After ingestion by insects, the polyhedrin occlusion body dissolves and the virus then infects intestinal cells by direct fusion with the cell membranes of the midgut. The DNA genome is replicated and transcribed in the nucleus. After translation and assembly of nucleocapsids, the BV form leaves the cell by budding at the plasma membrane. This budded virus is infectious and can infect more cells in the same host. Very late in the infection progress, the nucleocapsids bind to the membrane of the nucleus and are embedded in the polyhedrin matrix (ODV form). These virus forms are released again into the environment after cell death and can endure for years before infecting a new host (Hu, 2005; Airenne et al., 2013; Clem and Passarelli, 2013). ODV forms of β-baculoviruses only contain one virion per occlusion body whereas α-, γ- and δ-baculoviruses contain several virions in their occlusion bodies reflected by the former name Polyhedroviruses (Airenne et al., 2013).

The most widely used baculovirus is the *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) which is 25 x 260nm in size and has a genome of 134kb. It belongs to the genus α-baculovirus and its genome has been sequenced. Since they only infect insect cells, baculoviruses can be handled at low bio safety levels (Airenne et al., 2013). Genome expression in AcNMPV is under temporal control (Rohel and Faulkner, 1984) with (immediate) early, (delayed) early, late and very late promotors for different phases of gene expression. In the very late phase, proteins polyhedrin and P10 are expressed under two strong promotors, the polyhedrin promotor and the P10 promotor, respectively. Both proteins are non-essential, thus these two promotors are widely used in baculovirus expression systems for directing expression of foreign proteins (van Oers et al., 2015).

2.6.2. Baculovirus expression system

Because of their large DNA genome which can be modified easily, and convenient laboratory handling characteristics, protein expression systems based on baculoviruses as vectors were developed in the 1980's. The first protein that was produced by recombinant baculoviruses was human IFN-β, expressed under control of the polyhedrin promotor (Smith et al., 1983). Since then the baculovirus expression system has been developed further and has become an important tool for protein expression. In 1993, the nowadays widely used "bacmid system" was developed (Luckow et al., 1993). It uses a bacterial artificial chromosome ("bac") that carries the entire AcMNPV genome sequence with which recombinant baculoviral genomes are generated in Escherichia coli faster to develop recombinant baculoviruses or expression vectors more effectively (van Oers et al., 2015). A well-known commercially used baculovirus expression system that uses this technique is the Bac-to-Bac® System by Life Technologies (Fig. 7).

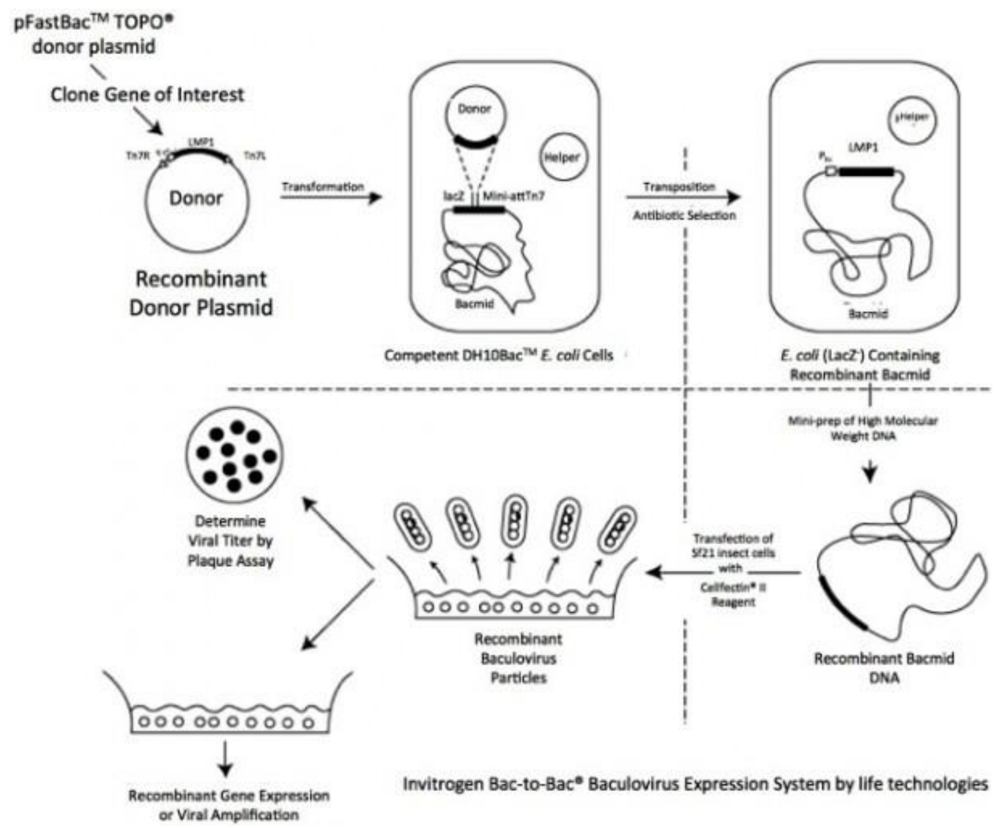

Fig. 7. Overview of generation of recombinant baculoviruses with Baculovirus Expression System "Invitrogen Bac-to-Bac®" by Life Technologies (with permission of Life Technologies/ Thermo Fisher Scientific)

Upper row from left to right: cloning of gene of interest from donor plasmid into a recombinant donor plasmid; transformation of purified plasmid DNA into DH10Bac™ E.coli cells containing Bacmid DNA; Transposition of gene of interest into Bacmid DNA of E.coli and antibiotic selection of E.coli containing recombinant Bacmid DNA

Lower row from right to left: Isolation of recombinant Bacmid DNA; transfection of insect cells with recombinant Bacmid DNA; generation of recombinant baculoviruses; determination of viral titer, recombinant gene expression or viral amplification

Insect cell lines used for infection by recombinant baculoviruses are often SF9 and SF21 cells, derived from ovarian tissue of *Spodoptera frugiperda,* or BTI-TN-5B1-4 cells (High V; Invitrogen), derived from ovarian tissue of *Trichoplusia ni* (Hu, 2005).

Protein expression by baculoviruses in insect cells has many advantages. Proteins can be produced in large amounts and, since baculoviruses can only infect some cells of Lepidoptera species, they provide no risk to mammalians (Noad and Roy, 2003).

However, not only insect cells are suitable for protein expression by recombinant baculoviruses, but also mammalian cells. Hofmann et al. (1995) transduced successfully mammalian cells (human hepatocytes) with modified baculoviruses that expressed proteins under a cytomegalovirus (CMV) immediate early promotor, while Boyce and Bucher (1996) did the same in different types of mammalian cells under a Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter-β galactosidase (β-gal) gene cassette. Shoji et al. (1997) developed the strong CAG(GS) enhancer/promotor element, which consists of the CMV immediate early enhancer promotor, the chicken β-actin promoter and a rabbit β-globin polyadenylation signal, for transduction. Transduction of mammalian cells with recombinant baculoviruses is called “BacMam” system.

2.6.3. Recombinant baculovirus based vaccines

Protein expressed by recombinant baculoviruses are used as vaccines commercially, for example, Porcilis Pesti (Intervet International BV, Netherlands), against Classical swine fever containing E2- glycoprotein of CSFV (van Oers et al., 2015; PEI, state 15.02.2017). A recombinant baculovirus-derived vaccine for use in humans, Cervarix (GlaxoSmithKline Biologicals S.A.) is directed against human papilloma virus. Many more vaccine candidates containing protein expressed by baculovirus expression system are in clinical tests (Vicente et al., 2011; van Oers et al., 2015).

Beside other advantages mentioned earlier (2.6.2), live baculoviruses are supposed to have an immunogenic effect in the vaccinated organism by inducing interferon α (Gronowski et al., 1999). This could be an asset in induction of immune responses by recombinant baculovirus vaccines.

3. Aims of the thesis

Development of effective vaccines was a major breakthrough to protect rabbits from RHD in the early 1990’s (Argüello-Villares, 1991), which was however accompanied with the death of thousands of rabbits needed to produce the vaccines from infected rabbit livers. With the appearance of the new virus variant RHDV-2 among rabbit populations and due to the insufficient protection of available vaccines, it was necessary to develop new ones that protect rabbits against the fatal outcome of RHDV-2 infection.

Therefore, the aims of this thesis were

a) to develop a recombinant baculovirus-derived RHDV-2-VP60 vaccine to replace the ethically questionable conventional vaccine production from liver preparations of infected rabbits,
b) to optimize the recombinant baculovirus vaccine to combine high production yields, easy and effective purification with a good antigenicity based on the self-assembly of the structural protein VP60 of RHDV-2 into VLPs
c) to establish an effective immunization protocol to induce a protective long-lasting immunity with a minimal dose early after a single immunization,
d) to analyze the onset and duration of immunity against RHDV-2 and cross-protective capacity against classical RHDV (RHDV-1) of the induced immunity and
e) to characterize the humoral and cellular immune response against RHDV-2 in rabbits immunized with the newly established recombinant vaccine in comparison with a conventional RHDV-2 vaccine.

4. Material

4.1. Cell lines

Sf9	Insect cell line from ovary tissue of the moth *Spodoptera frugiperda*
High V	Insect cell line from ovary tissue of the moth *Trichoplusia ni*
RK13	Rabbit kidney cell line

All cell lines were obtained from Collection of Cell Lines in Veterinary Medicine (CCLV) FLI, Insel Riems.

4.2. Virus strains

Recombinant baculoviruses:

BacMam-ieGFP	recombinant baculovirus, expresses GFP under control of the HCMV major ie promotor, FLI
CO107 Baculo-p10GFP	recombinant baculovirus, expresses GFP under control of the baculoviral P10 promotor, FLI

The following recombinant baculoviruses were generated in this study (6.1):

BacBacVP60-2/BHV1	recombinant baculovirus, expressing VP60 of RHDV-2 with the codon usage of BHV-1 under control of the promotor P10 and GFP under control of the promotor HCMVie in insect cells
BacBacVP60-2/AcMNPV	recombinant baculovirus, expressing VP60 of RHDV-2 with the codon usage of AcMNPV under control of the promotor P10 and GFP under control of the promotor HCMVie in insect cells
BacMamVP60-2/BHV1	recombinant baculovirus, expressing VP60 of RHDV-2 with the codon usage of BHV-1 under control of the hybrid promotor CAG(GS) and GFP under control of the polyhedrin promotor in mammalian cells
BacMamVP60-2/AcMNPV	recombinant baculovirus, expressing VP60 of RHDV-2 with the codon usage of AcMNPV under control of the hybrid promotor CAG(GS) and GFP under control of the polyhedrin promotor in mammalian cells

RHDV challenge viruses

RHDV-2 strain “Werne”	wild type virus prepared from the liver of a RHDV-2 “Werne” infected rabbit (FLI)
RHDV-1 strain “Eisenhüttenstadt”	wild type virus prepared from the liver of a RHDV-1 “Eisenhüttenstadt” infected rabbit (FLI)

4.3. Media and solutions for cell cultivation

ZB5

5,32 g Hank’s Salts
4,76 g Earle’s Salts
1,25 g $NaHCO_3$
0,12 g Na-pyruvate
10 ml nonessential amino acids (NEAS)
100 ml fetal calf serum (FCS)
ad 1 l aqua dest.
100 U/ml penicillinG
100 µg/ml streptomycin
pH 7,2

ZB12

2,7 g lactalbumine-hydrolysate
3,75 g Leibovitz L15 (Gibco)
1,26 g $NaHCO_3$
15 mg phenol red
75 ml Hank’s salts

ZB15

46,12 g Grace´s Insect powder medium (Serva)
3,3 g lactalbumine-hydrolysate (Difco)
3,3 g yeast extract (NeoLab)
ad 900 ml aqua dest.
100 ml fetal calf serum (FCS)
100 U/ml penicillinG
100 µg/ml streptomycin
pH 6,5

trypsin solution

32,0 g NaCl
0,8 g KCl
10 g trypsin
5 g EDTA
0,8 g KH_2PO_4
4,6 g $NaH_2HPO_4 \times 2H_2O$
64 mg phenol red
pH 7,2 – 7,4
ad 1 l aqua dest.

High V medium

Insectomed SF express-medium
(Biochrome)

Media ZB5, ZB15 and ZB12 were received as complete preparations from CCLV FLI, Insel Riems.

4.4. Bacteria

C600 Escherichia coli Genotyp: F– supE44 thi-1 thr-1 leuB6 LacY1 tonA21 Lambda- hsdR-hsdM+ (FLI)

DH10Bac™ Escherichia coli Genotype: F– mcrA (mrr-hsdRMS-mcrBC) 80lacZ M15 lacX74 recA1 endA1 araD139 (ara, leu)7697 galU galK – rpsL nupG /pMON14272 / pMON7124 (Invitrogen)

All bacteria were incubated in LB-medium while shaking or on LB agar plates at 37°C.

4.5. Media and solutions for bacterial cultures

LB-Medium

10 g bacto- tryptone
5 g yeast extract
8 g NaCl
ad 1 l aqua dest.

LB^+-Medium

LB-Medium with
10 mM KCl
20 mM $MgSO_4$

SOC-Medium

10 ml SOA-medium
100 µl 1M $MgSO_4$
100 µl 1M $MgCl_2$
200 µl 1M Glucose
ad 500 ml aqua dest.

SOA-Medium

10 g peptone 140
2,5 g yeast extract
1 ml 5M NaCl
1,25 ml 1M KCl
ad 500 ml aqua dest.

selection medium

markers for selection were added in following concentrations:

ampicillin 100 µg/ml
gentamicin 7 µg/ml
kanamycin 50 µg/ml
tetracycline 10 µg/ml

LB-agar

LB-medium with 1,5 % agar and selection markers in the following concentrations:

ampicillin 100 µg/ml
gentamicin 7 µg/ml
kanamycin 50 µg/ml
tetracycline 10 µg/ml

IPTG 40 µg/ml
X-Gal 100 µg/ml in dimethylformamid

4.6. Plasmids

The following two plasmids were generated by GeneArt (Regensburg, Germany) using the provided sequences of RHDV-2-VP60 of strain 10-05 (GenBank accession no. FR819781; Suppl. 7) (this study):

14ABWG4P_RHDV-2_VP60_ BHV1_Cod_pMK-RQ	vector containing the synthetic ORF encoding RHDV-2-VP60 with the BHV-1 codon usage
14ABWG6P_RHDV-2_VP60_ ACNPHV_pMK-RQ	vector containing the synthetic ORF encoding RHDV-2-VP60 with the AcMNPV codon usage
pFBD-P10Uhis-ieGFP	cloning vector for gene integration of the P10 promoter controlled expression cassettes into the baculovirus genome, contains a GFP expression cassette controlled by the major ie promotor of HCMV (FLI)
pCAGGS-PHGFP	cloning vector for gene integration of hybrid CAG(GS) promotor controlled expression cassettes into the baculovirus genome, contains a GFP expression cassette controlled by the baculoviral polyhedrin promotor for GFP expression (FLI)

The following plasmids were generated using the above mentioned vectors (this study):

pFBD_RHDV-2_VP60_BHV_Cod	vector coding for viral VP60 protein of RHDV-2 with BHV-1 codon usage under control of the promotor P10, derived from cloning vector pFBD-P10Uhis-ieGFP
pFBD_RHDV-2_VP60_AcMNPV	vector coding for viral VP60 protein of RHDV-2 with AcMNPV codon usage under control of the promotor P10, derived from cloning vector pFBD-P10Uhis-ieGFP
pMBCAGGS-RHDV-2_VP60 _BHV1_Cod	vector coding for viral VP60 protein of RHDV-2 with BHV-1 codon usage under control of the hybrid promotor CAG(GS), derived from cloning vector pCAGGS-PHGFP
pMBCAGGS-RHDV-2_VP60 _AcMNPV	vector coding for viral VP60 protein of RHDV-2 with AcMNPV codon usage under control of the hybrid promotor CAG(GS), derived from cloning vector pCAGGS-PHGFP

4.7. Antibiotics

ampicillin	Serva
gentamicin	Sigma
kanamycin	Sigma
penicillin G	Biochrom
streptomycin	Biochrom
tetracycline	Sigma

4.8. Enzymes, nucleic acids, DNA/ protein size markers

alkaline phosphatase	Roche
calf intestinal phosphatase (20 U/µl)	Roche
DNA Polymerase I, Large (Klenow) Fragment	New England BioLabs

DNA-size marker "1kb-ladder"	Invitrogen
dNTP Mix 10 mM	Promega
internal control RNA (IC-RNA)	FLI
lysozyme	Sigma
prestaind protein ladder protein size marker Page Ruler™	Thermo Scientific
protein kinase K	Roche
restriction enzymes	Biolabs
RNase A	Sigma
T4-DNA-Ligase	Roche

4.9. Sera and purified antigen

fetal calf serum (FCS)	Invitrogen
horse serum	Biochrom
purified RHDV-1 antigen	FLI
purified RHDV-2 antigen	FLI
rabbit normal serum	FLI
rabbit serum RHDV-1 positive	FLI
rabbit serum RHDV-2 positive	FLI

4.10. Antibodies

goat α-rabbit IgG, peroxidase-conjugated	Dianova
αGFP IgG, polyclonal rabbit serum	FLI
αVP60_1 IgG, polyclonal rabbit-ab	FLI
DYLight 488 conjugated anti-mouse IgG1	Rockland
R-Phycoerythrin –conjugated anti-mouse IgM	Jackson Immuno Research
R-Phycoerythrin –conjugated anti-mouse IgG2a	Jackson Immuno Research

4.11. Chemicals and bioreagents

1,4-Dithiothreitol (DTT)	Roche
2-mercaptoethanol	MP Biomedicals
agar	Difco
agarose	Invitrogen
ATP	Sigma
bacto tryptone	Invitrogen

binary ethylenimin	Sigma
bovine serum albumin (BSA)	NEB
bromophenol blue	Serva
cesium chloride	Invitrogen
EDTA	Sigma
EGTA	Sigma
ethidium bromide	Serva
FuGENE® HD	Roche
IPTG	Roche
o-Phenylenediamine dihydrochloride (OPD, 4 mg/tbl)	Sigma
Pancoll animal, density 1,077 g/ml	Pan-Biotech
PEG	Sigma
ROX (1:200 in 10 mM Tris-HCl pH 8)	Invitrogen
SDS	Serva
sucrose	Serva
TEMED	Roth
tris	Invitrogen
trypsin (powder)	Difco
Tween 20	Sigma
X-Gal	Invitrogen
yeast-extract	Difco

4.12. Kits

Clarity™ Western ECL Substrate	Bio-Rad
Ingezim RHDV DAS R.17.RHD.K2	Ingenasa
Plasmid Midi Kit	QIAGEN
QIAamp Viral RNA Mini Kit	Qiagen
SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase	Invitrogen

4.13. Buffers and solutions

Buffers used in different methods:

10x PBS

80 g NaCl

2 g KCl

11,5 g Na_2HPO_4 x H_2O

2 g KH_2PO_4

ad 1 l aqua dest.

pH 7,4

PBS^+

140 mM NaCl

2,7 mM KCl

8 mM Na_2HPO_4

1,5 mM KH_2PO_4

0,9 mM $CaCl_2$ x $2H_2O$

0,5 mM $MgSO_4$

pH 7,4

Buffers used for DNA preparation and cloning:

10x TA

330 mM tris

660 mM potassium acetate

100 mM magnesium acetate

1 mg/ml BSA

5 mM DTT

pH 7,9 with acetic acid

50x TA (for DNA-agarose gels)

2 M tris

0,05 M Na-acetate

pH 7,8 with glacial acetic acid

DNA-marker

30 µl 1kb ladder (1000 µg/ml)

40 µl 10x TA

330 µl aqua dest.

100 µl sample buffer

heat for 10min at 56°C

sample buffer (for DNA-marker)

40 % sucrose

0,05 % bromophenol blue

0,1 % SDS

1 mM EDTA

TE buffer

10 mM tris pH 7,5

1 mM EDTA pH 7,5

Buffers used for purification of plasmid DNA:

Solution I

10 mM EDTA, pH 8,0

20 mM tris, pH 8,0

50 mM glucose

2 mg/ml lysozyme

Solution II

0,2 M NaOH

1 % SDS

Solution III

3 M Na-acetate pH 4,8

Buffers and solutions used for SDS-PAGE:

SDS 10 % separating gel

9,6 ml 30 % acrylamide / 0,8 % bisacrylamide

7,5 ml 4x Lower Tris

12,9 ml aqua dest.

60 µl 10 % ammonium peroxodisulfate

30 µl TEMED

SDS 4,5 % stacking gel

3 ml 30 % acrylamide/ 0,8 % bisacrylamide

5 ml 4x Lower Tris

12 ml aqua dest.

60 µl 10 % ammonium peroxodisulfate

60 µl TEMED

4x Lower Tris

1,5 M tris-HCl pH 8,8

0,4 % SDS

4x Upper Tris

0,5 M tris-HCl pH 6,8

0,4 % SDS

4x protein lysis buffer

40 % sucrose

12 % SDS

62,5 mM 4x Upper Tris

0,025 % bromophenol blue

ad 100 ml aqua dest.

10x running buffer

144 g/l glycine

30 g/l tris

10 g/l SDS

Buffers used for Western Blot:

transfer buffer

1,514 g tris

7,21 g glycine

0,5 g SDS

100 ml 30 % methanol

ad 500 ml aqua dest.

washing buffer I

1x PBS with 0,3 % Tween 20

washing buffer II

1x PBS with 0,1 % Tween 20

Buffers and solutions used for VLP purification:

40 % sucrose

40 g D^+ - sucrose

ad 100 ml 0,2 M tris-HCl

1 M tris-HCl

60,57 g tris

ad 500 ml aqua dest.

pH 6,8 with concentrated HCl

CsCl solution

4,2 g CsCl in 10 ml PBS end volume

Buffers used for antigen purification:

TEN buffer

20 mM tris

1 mM EDTA

150 mM NaCl

pH 7,6

Buffers and solutions used for antibody-ELISA:

Coating buffer Tris-NaCl

2,422 g tris

8,766 g NaCl

ad 1 l aqua dest.

pH 7,6

washing buffer

1x PBS with 0,05 % Tween 20

substrate buffer

solution A:

0,1 M citric acid ad 100 ml aqua dest.

solution B:

0,2 M Na_2HPO_4 x $2H_2O$ ad 100 ml aqua dest.

substrate solution

2,43 ml solution A

2,57 ml solution B

5 ml aqua dest.

1 tablet OPD is dissolved in 10 ml substrate solution, add 15 µl 30% H_2O_2 immediately before use.

Buffer used for hemagglutination test:

0,15 M Isotonic phosphate buffer (IP)

8,28 g NaCl

1,19 g Na_2HPO_4 x $2H_2O$

0,2 g KH_2PO_4

ad 1000 ml aqua dest.

Real time RT-PCR Mastermix:

Mastermix RT-PCR RHDV-2

2,4 µl RNAse free water

12,5 µl Rxn Mix (2x)

1,0 µl SS III RT/ Platinum Taq Mix

2,0 µl RHDV-2 Mix

2,0 µl IC-Mix

0,1 µl ROX

Mastermix RT-PCR RHDV-1

2,4 µl RNAse free water

12,5 µl Rxn Mix (2x)

1,0 µl SS III RT/ Platinum Taq Mix

2,0 µl RHDV-1 Mix

2,0 µl IC-Mix

0,1 µl ROX

Buffer used for flow cytometric analysis (FACS):

FACS buffer

1x PBS with 0,01% 1mM EDTA

4.14. Primers and probes

All primers and probes (Tab. 2) were obtained from Eurofins Genomics (Germany) and used in concentrations of 100pmol/µl.

Tab. 2. Primers and probes used for real time RT-PCR

Primer/Probe Mix	Sequence 5' - 3'	reference
RHDV-specific qRT-PCR		
RHDV-1 Mix		Gall et al., 2007
vp60-7_forward primer	ACYTGACTGAACTYATTGACG	
vp60-8_reverse primer	TCAGACATAAGAAAAGCCATTGG	
vp60-9_FAM probe	FAM-CCAARAGCACRCTCGTGTTCAACCT-TAMRA	
RHDV-2 Mix		unpublished
FRA-korr-forward primer	ACTTGTCAGACCTTGTTGACA	
FRA-reverse primer	TCAGACATAAGAAAAGCCATTAG	
FRA _v2-FAM probe	FAM-CCACAAGCACGCTTGTGTACAACTTG-BHQ1	
IC-specific qRT-PCR		
IC-Mix		Hoffmann et al., 2006
EGFP12-F primer	TCGAGGGCGACACCCTG	
EGFP10-R primer	CTTGTACAGCTCGTCCATGC	
EFGP-Hex probe	HEX-AGCACCCAGTCCGCCCTGAGCA-BHQ1	

4.15. Monoclonal antibodies

Tab. 3. Monoclonal antibodies specific for leukocyte differentiation markers used for FACS analysis

clone	antigen	expressing leukocytes	isotype	reference
RTH2A	not defined	T-cells	G1	Davis et al., 2008
RTH26A	isoform of CD5	T-cells	G2a	Kotani et al., 1993
RTH1A	CD4	T_{helper} cells, monocytes	G1	Jacobson et al., 1993
ISC27A	CD8	$T_{cytotoxic}$ cell	G2a	Davis et al., 2008
ISC29E	CD8	$T_{cytotoxic}$ cell	G1	Davis et al., 2008

4.16. Equipment and devices

agarose gel apparatus	FLI
BioPhotometer	Eppendorf
electrophoresis power supply	Pharmacia Biotech
ELISA microplate reader Spectra	Tecan
ELISA microplate washer HydroFlex	Tecan
Eppendorf Thermomixer 5436	Eppendorf

fluorescence microscope Eclipse Ti-S with digital camera	Nikon
gyratory shaker Duomax 1030	Heidolph
incubator for bacterial cultures	Heraeus
incubator MAX Q 8000	Thermo Scientific
light microscope	Leitz
Microm HM 340E	Microm International
Mini Protean Tetra System	Bio-Rad
multichannel pipettes, pipettes	Eppendorf, Gilson
polarizing light microscope Zeiss Axio Scope.A1	Zeiss
qPCR system MX3005P	Stratagene
Tissue Lyser II	Qiagen
Tissue processor Leica ASP 300S	Leica Biosystems
Trans-Blot®-SD Semi-Dry Transfer Cell	Bio-Rad
ultrasound waterbath	Branson
UV-Transilluminator	Herolab
VersaDoc™ Imaging System	Bio-Rad
vortex mixer	Bachofer
water jacketed CO_2 incubator for cell culture	Forma Scientific

<u>Centrifuges</u>

centrifuge 5415R	Eppendorf
centrifuge 5430R	Eppendorf
centrifuge 5810R	Eppendorf
centrifuge Rotina 420R	Hettich
J2-HS Centrifuge	Beckman
Minifuge 4400 GL	Heraeus Christ
Optima™ LE-80K Ultracentrifuge	Beckman
Optima™ Max-XP Ultracentrifuge	Beckman
Wifug centrifuge	Lab Centrifuges

4.17. Consumables

96 well U-bottom microplates	Greiner
BD Microtainer® Blood Collection Tubes	Becton, Dickinson
cell culture plates + flasks	Greiner, Costar®

cellulose chromatography paper 3MM	Whatman®
centrifuge tubes	Beckman
EDTA pretreated tubes, 1,6mg EDTA/ml blood	Sarstedt
FACS tubes	Becton Dickinson
filter paper	Schleicher Schuell
medium binding 96well ELISA plates Microlon® 200 96W Microplate	Greiner
N-ACHROPLAN objectives	Zeiss
needles Sterican® 21G and 24G	Braun
nitrocellulose membrane 0,2 µm	Whatman® Protran®
PCR plates 96well with Flat Cap Strips	Kisker Biotech
reaction tubes	Eppendorf
self-adhesive PCR aluminium foil seal	SLG Süd-Laborbedarf Gauting
stainless steel beads, 5mm	Qiagen
syringes	Braun
tubes 2.0 ml, sterile, DNA-, DNase-, RNase and Pyrogen free	Biozym
tubes, black cap, 12ml	Greiner

4.18. Software

Chemiluminescence: QuantityOne	Bio-Rad
ELISA microplate washer software: Hydrocontrol 4.1	Tecan
ELISA reading software: E.A.S.Y win	Herolab GmbH
qRT-PCR: MxPro	Stratagene
FACS: CellQuestPro	Becton Dickinson

4.19. Animals

Rabbits, hybrids “Zimmermannkaninchen” (“ZI-KA”) from a commercial rabbit farm

5. Methods

5.1. Generation of recombinant baculoviruses

5.1.1. Purification of plasmids coding for RHDV-2-VP60 ORFs

Two plasmids containing the open reading frames for VP60 of RHDV-2 were synthesized by GeneArt (Regensburg, Germany) based on the codon usages of autographa californica multiple nucleopolyhedrovirus (CU AcMNPV) and bovine herpesvirus 1 (CU BHV-1), respectively. The sequences of the respective ORFs were deduced from the RHDV-2-VP60 amino acid sequence (Le Gall-Reculé et al., 2013, GenBank accession number FR819781 RHDV; see supplementary data) and designed using the codon usage tables available at http://www.kazusa.or.jp/codon/.

In a first step, the plasmids were purified from 200µl E.coli suspended in 50ml LB medium + kanamycin cultivated overnight using the Qiagen Plasmid Midi Kit. Briefly, bacterial cells were pelleted by centrifugation with a Heraeus Christ centrifuge at 4°C with 3000rpm for 30min. The pellet was resuspended in 4ml buffer P1 and P2 each and incubated at room temperature for 5min before adding 4ml buffer P3 and incubation on ice for 15min. Centrifugation was performed at 4°C with 15000rpm for 30min using a JA17 rotor of a J2-HS centrifuge. A Qiagen tip was equilibrated with 4ml QBT buffer. The supernatant was added to the Qiagen tip and the tip was washed with 10ml QC buffer twice afterwards. DNA was eluated with 5ml QF buffer and then aliquoted into 1ml samples. DNA was precipitated at room temperature by adding of 0,7ml isopropanol followed by centrifugation at 4°C with 14000rpm for 15min with an Eppendorf centrifuge. Pellets were washed with 1ml 70% ethanol and centrifuged at 4°C with 14000rpm for 5min. After drying of the pellets at 56°C, DNA was resuspended in 125µl TE buffer.

5.1.2. Preparation of transfer vectors

pFBD-P10Uhis-ieGFP (kindly provided by C. Klopfleisch) and pCAGGS-PHGFP were used as transfer vectors. Both transfer vectors contain a GFP expression cassette which facilitates isolation and titer determination of the respective baculovirus recombinants (Keil et al., 2009). Transfer vector pFBD-P10Uhis-ieGFP was cleaved with SmaI while transfer vector pCAGGS-PHGFP was cleaved with EcoRI. 5µg DNA was cleaved with 2U of the respective restriction enzyme per µg DNA in a final volume of 100µl containing 10µl 10x reaction buffers NEB2 (EcoR1) or Cut Smart (SmaI), respectively. The reaction mixture with EcoR1 was incubated for 1,5 hours at 37°C, while the one with SmaI was incubated at 25°C for the same time. Cleavage was controlled by agarose gel electrophoresis.

5.1.3. Cleavage of plasmids by restriction enzymes

In the provided plasmids the synthetic open reading frames for RHDV-2-VP60 were flanked by EcoRI cleavage sites to facilitate isolation of the respective ORFs. 5µg of each plasmids DNA were cleaved with 10U EcoRI in a final volume of 50µl containing 5µl 10x reaction buffers NEB2 or 10xTA, respectively. The reaction mixtures were incubated for 1,5 hours at 37°C. Correct cleavage was controlled by agarose gel electrophoresis.

5.1.4. Blunt ending of sticky ends with Klenow enzyme

Cleavage with restriction enzyme SmaI results in blunt ends at the restriction sites. Because cleavage with EcoR1 results in 5' overhanging ends of the DNA fragments (sticky ends), the synthetic ORFs of RHDV-2-VP60 meant to be integrated into the transfer vector pFBD-P10Uhis-ieGFP, cleaved with SmaI, had to be blunt ended by the Klenow fragment of the E.coli DNA polymerase I which lacks the 5‘ to 3‘ exonuclease activity and refills overhanging 5‘ ends by DNA polymerase activity. 5µg of DNA was resuspended with 5µl 10x TA buffer and 42µl aqua dest. Then 2µl dNTP-Mix (10mM) and 5U Klenow polymerase were added. After incubation for 30min at room temperature the reaction was stopped by adding 1µl EDTA (0,5M, pH 7,5).

5.1.5. Dephosphorylation of cleaved transfer vectors

To avoid religation of the linearized vectors, calf intestinal phosphatase (CIP) was used to dephosphorylate their 5‘ends. After mixing of 5µg appropriately cleaved vector DNA with 25µl 10x phosphatase buffer and aqua dest. ad 250µl, 1µl CIP (20U/µl) was added and then incubated at 37°C for 30min. After a second addition of 1µl CIP, the mixture was incubated for further 30min at 56°C. 50µl 60mM EGTA was added, followed by incubation at 65°C for 15min to stop the reaction. The phosphatase was digested by incubation for 30min at 56°C with 30µl 10% SDS and 1µl protein kinase K (10mg/ml).

5.1.6. Cleaning of transfer vector DNA

Following dephosphorylation, the 330µl vector DNA solution was mixed 1:1 v/v with phenol and centrifuged at 14000rpm in an Eppendorf centrifuge for 2min at room temperature. The upper phase was then mixed 1:1 (v/v) with 50% phenol/ 50% chloroform- isoamylalcohol (24:1). After thorough mixing, the upper phase was added to 1ml chloroform- isoamylalcohol (24:1) and mixed again. After adding of 1/10 volume 3M Na- acetate (pH 7) and 2.5 to 3 volumes 100% ethanol, the DNA was precipitated by incubation at -80°C for 30min and harvested by centrifugation with 14000rpm for 15min at room temperature with an Eppendorf centrifuge. The pellet was washed with 1ml 70% ethanol and centrifuged again with 14000rpm for 5min at room temperature. After drying, the pellet was resuspended in TE buffer by incubation at 56°C for 5min and shaking at room temperature for 15min afterwards. Recovery of DNA was controlled by agarose gel electrophoresis using 1µl of each sample.

5.1.7. Purification of DNA by phenol extraction of agarose gels

The respective DNA preparations were size separated by agarose gel electrophoresis in presence of ethidium bromide. DNA fragments were visualized by long wave UV light and excised. After mincing the gel slices in an Eppendorf tube with a glass rod, an equal amount of phenol was added. After mixing, the samples were frozen in liquid nitrogen for 20sec and centrifuged immediately afterwards with 14000rpm for 30min at room temperature with an Eppendorf centrifuge. The upper phase was added to 1ml of chloroform- isomylalcohol (24:1) followed by mixing and centrifuged as above for 2min. The upper phase transferred into a new reaction tube and 1/10 volume 3M Na- acetate (pH7) and 2.5 to 3 volumes 100% ethanol were added. After incubation at -80°C for 30min, precipitated DNA was pelleted by centrifugation with 14000rpm at room temperature for 15min. The pellet was washed with 1ml 70% ethanol and centrifuged with 14000rpm at room temperature for 5min. The pellet was then dried at 56°C and resuspended in 50µl TE buffer by incubation at 56°C for 5min and shaking at room temperature for 15min. 1µl of each sample was size separated by agarose gel electrophoresis to control recovery.

5.1.8. Ligation

For ligation, a ligation buffer was prepared, consisting of 5µl of each 10x TA buffer, ATP (10mM), DTT (100mM) and BSA (500µg/ml). Because blunt ends do not ligate as easily as sticky ends, blunt ended inserts were used in a ratio of 2:1 (µg/µg) with vector pFBD-P10Uhis-ieGFP whereas inserts with sticky ends, meant for vector pCAGGS-PHGFP, were used 1:1. As controls, vector DNAs alone were treated accordingly. To each reaction mixture 1µl T4-DNA-ligase was added with a concentration of 1U/µl for blunt end ligation and 0,1U/µl for sticky end ligation and filled with aqua dest. ad 50µl. The reaction mixture was incubated at 37°C for 5min, followed by incubation at 25°C for 1 hour and at 4°C overnight. As ligation control, 5µl of each sample were size separated by agarose gel electrophoresis.

5.1.9. Transformation and transposition

5.1.9.1. Transformation

10µl of each ligation mixture was incubated with 50µl transformation competent E.coli C600 on ice for 20min, at 42°C for 2min and again on ice for 5min. Afterwards 200µl LB^+ medium were added, followed by incubation for 1 hour at 37°C. Since the cloning vectors encode ampicillin resistance, the mixture was plated on LB-agar plates with ampicillin with 100µg/ml ampicillin and incubated at 30°C overnight. The next day, colonies were picked and cultivated in 3ml LB medium with 100µg/ml ampicillin overnight at 37°C and shaking at 300rpm. Clones containing transfer vectors with the respective RHDV-2-VP60 ORF in the correct orientation were identified by restriction enzyme cleavage of rapid-test plasmid DNA.

5.1.9.2. Transposition

1µl of bacterial plasmid DNA was incubated with 100µl transformation competent DH10Bac E.coli on ice for 20min, at 42°C for 2min and again on ice for 5min. After adding 900µl SOC-medium, incubation for 4 hours at 37°C and shaking at 300rpm using an Eppendorf thermomixer 5432 followed. A dilution series till 10^{-3} with 1ml of the bacterial suspension in SOC medium was incubated at 37°C and 300rpm overnight. The next day, 500µl dilutions till 10^{-5} were created from the 10^{-3} dilution. After further incubation at 37°C and 300rpm for 2 hours using an Eppendorf thermomixer, 200µl of dilutions 10^{-3} to 10^{-5} were plated on agar plates containing IPTG, X-Gal and antibiotics gentamycin, kanamycin and tetracycline. The plates were incubated at 37°C and at room temperature afterwards for 24 hours each time. After that time blue-stained and unstained (white) colonies could be differentiated on the plates. 4 white colonies that harbour baculovirus bacmid DNA with the target sequences from the transfer plasmids, were picked and each colony was incubated at 37°C overnight in 3ml LB selection medium.

5.1.10. Isolation of nucleic acids

5.1.10.1. Rapid-test, small scale purification of plasmid DNA and baculovirus bacmid DNA

After transformation of the ligation mixture and after transposition, 1ml of overnight bacterial cultures was centrifuged with 7000rpm for 30sec at room temperature with an Eppendorf centrifuge. The pellet was then shaken shortly at room temperature with an Eppendorf mixer 5432 before adding of 100µl solution I. After thorough mixing 100µl solution II was added and agitated shortly before adding 150µl of solution III. After incubation for 60min on ice, a centrifugation at 14000rpm at room temperature was performed. Supernatant was then mixed with 1ml 100% ethanol and incubated at -70°C for 15min. After another centrifugation step at 14000rpm and room temperature for 10min, the pellet was washed with 1ml 70% ethanol and centrifuged again with 14000rpm at room temperature for 5min. The pellet was then dried at 56°C and resuspended in 40µl TE buffer with RNase A (50µg/ml) at 56°C for 5min and shaking at room temperature for 15min.

For DNA prepared after transposition, RNase incubation was done at 37°C for 30min. Baculoviral bacmid DNA concentration was measured by spectrophotometry and regarded as pure when a 260nm/280nm ratio of approximately 2,0 was obtained.

For identification of E.coli clones containing the envisaged plasmid, 10µl bacterial plasmid DNA was added to 2,5µl NEB 3 buffer, 0,3µl NcoI and 12,2µl aqua dest. and incubated at 37°C for 1 hour. Cleavage products were analyzed by agarose gel electrophoresis.

5.1.10.2. Purification of bacterial plasmid DNA by Qiagen Plasmid Midi-Kit

To obtain larger quantities of pure plasmid DNA, 1µl plasmid DNA was added to 50µl transformation competent E.coli 600, treated as described above (3.1.9.1.) incubated at 37°C for 1 hour in 1ml LB medium and then in LB medium with ampicillin with 100 µg/ml overnight at 37°C while shaking at 300rpm. DNA was purified with a Qiagen Plasmid Midi Kit according to the manufacturer's protocol. Purified plasmid DNA was resuspended in 125µl TE buffer. The DNA concentration was determined by spectrophotometry. For verification of the identity, 500ng plasmid DNA were cleaved with 0,5µl NcoI, 2,5µl buffer NEB3 and aqua dest. ad 25µl at 37°C for 1 hour, followed by an agarose gel electrophoresis with 90V.

5.1.11. Photometric measurement of DNA concentration

Concentration of DNA was measured with a photometer at absorption of 260nm or 280nm in a dilution of 1:100 with aqua dest. 260nm is correlated to 50µg/ml dsDNA.

5.1.12. Cell cultures

5.1.12.1. Cultivation of insect cell lines

SF9 (*Spodoptera frugiperda*) cells were cultivated in Grace's supplemented insect cell medium with 10% FCS, 100U penicillin per ml, and 100µg streptomycin per ml (ZB15). High V cells were cultivated in Insectomed SF express-medium (Biochrome) (High V medium). Both cell lines were kept at 27°C in humidified atmosphere containing 2,5% CO_2.

Every 3-4 days the cells were passed. Old medium was removed, fresh medium was added, then cells were detached by hitting the bottom of the flasks and split in a ratio of 1:4 into new flasks.

5.1.12.2. Cultivation of rabbit kidney cell line

RK13 (rabbit kidney) cells were cultivated in MEM (Earl's and Hank's salts 1:1) supplemented with non-essential amino acids, 10% FCS, 100U penicillin per ml and 100µg streptomycin per ml (ZB5) at 37°C in humidified atmosphere with 2,5% or 5% CO_2. Cells were passed every 3-4 days. At first old medium was removed and cells were detached by trypsination at 37°C. Cells were then centrifuged for 2min at 500xg at room temperature. The pellet was washed once with medium ZB5 and centrifuged again. Cells were split in a ratio 1:4 into new flasks.

5.1.13. Transfection of recombinant bacmid DNA in High V cells

Circa 10^6 High V cells were seeded in a 6well plate with 2ml per well High V medium and incubated at 27°C for 1 hour. A transfection mix with 5µg DNA, 6µl Fugene^R HD and aqua dest. ad 100µl was prepared and incubated at room temperature for 40min before diluting it with 900µl High V medium. After washing the cells with High V medium, 1ml of the same medium was added. The diluted transfection mix was then carefully dropped on the cells and incubation for 5 hours at 27°C followed. Afterwards the culture supernatant was removed and replaced by 2ml High V medium with 100 U penicillin/ml and 100 µg streptomycin/ml per well before incubation at 27°C for 3 days. Then cells and supernatants were collected and frozen at -80°C. Replication of baculoviruses could be detected by GFP autofluorescence and cell lysis.

5.1.14. Isolation of recombinant baculoviruses by plaque assay

Into each well of 6 well plates circa 10^6 SF9 cells/well were seeded in 2ml ZB15 medium and incubated for 30min. A dilution series of the transfected High V cells supernatants from 10^0 till 10^{-2} was prepared and 100µl of each dilution was pipetted into the 6 wells. After incubation for 1 hour at 27°C, supernatants were removed and cultures were overlaid with an agarose overlay. After incubation at 27°C for 3 days, autofluorescent plaques were detected with the fluorescence microscope due to GFP expression of the recombinant baculoviruses in the insect cells. Cells within plaques were picked and resuspended in 1ml of ZB15 medium each. After shaking for 30min at room temperature with 600rpm, each plaque was transferred into flasks with 10^5 SF9 cells. After 5-7 days at 27°C, supernatants were frozen at -80°C in 2ml Eppendorf tubes.

5.1.15. Cultivation and titration of recombinant baculoviruses by endpoint dilution assay

For cultivation of baculovirus recombinants, SF9 cells were infected with an MOI of 0,1 and incubated for 7 days at 27°C. Infection progress was monitored by GFP autofluorescence and cell lysis. Cells and supernatants were harvested and aliquoted at -80°C.

For titration, supernatants of each picked plaque or aliquoted cell suspensions were diluted from 10^{-1} to 10^{-8} in ZB15 medium after thawing and treating by ultrasound (40W, 20sec). 100µl virus dilution was pipetted into the wells of a 96well plate in quadruplicate. Then $6x10^4$ SF9 cells/well were added. After 5-7 days at 27°C, the number of autofluorescence positive wells were counted and virus titers were calculated as endpoint dilution assay $TCID_{50}$:

$$\mathbf{TCID_{50} = D^{(n/p+0,5)} \times 1/\text{sample volume (ml)}}$$

D=dilution factor

n= number of positive wells

p= number of parallel values

5.2. Infection and transduction of cells with recombinant baculoviruses

5.2.1. Infection of SF9 cells with recombinant baculoviruses

For RHDV-2-VP60 production or generation of recombinant baculoviruses, SF9 cells were infected in suspension with ZB15 medium at the MOIs and for the times given in the results section and seeded into appropriate cell culture plates or flasks. At the indicated times the cells were detached and pelleted at 300g for 2 to 10min. Cell pellets were washed once with PBS. For protein analyses cells were lysed with lysis buffer directly and stored at -20°C until use. For further processing, cell pellets were adjusted to yield a 20% (weight per volume) suspension with PBS and stored at -80°C until further processing.

5.2.2. Transduction of RK13 cells with recombinant baculoviruses

For RHDV-2-VP60 production confluent RK13 cell cultures were trypsinized, washed with ZB5 and cells were seeded into appropriate cell culture vessels and transduced 24h later with the respective recombinant baculoviruses. Before transduction, cells were washed once with PBS^+ (with calcium and magnesium). Recombinant baculoviruses were added in PBS^+ at the MOIs and for the times given in the results section. Cells were incubated at 26°C either by shaking on a gyratory shaker with 300rpm for 5 hours or by shaking for 1,5 hours with 300rpm followed by 1 hour of centrifugation at 600g. After transduction, the inoculum was replaced by cell culture medium ZB5 containing 5mM butyrate for 24 hours to increase gene expression and the cells were incubated at 37°C. If applicable, further incubation continued in normal culture medium ZB5 at 37°C. At the indicated times, cells were either lysed directly in lysis buffer and stored at -20°C for protein analyses or, for further processing, they were detached by trypsinization, pelleted at 300g for 2 to 10min, washed with PBS, adjusted to yield a 20% (weight per volume) suspension with PBS and stored at -80°C until use.

5.3. Gel electrophoresis

5.3.1. Agarose gel electrophoresis

The appropriate amount of agarose was melted by boiling in water. After cooling to 56°C, TA buffer to a 1x final concentration and 0.1 µg/ml ethidium bromide (in 20mM Tris pH 8,0) were added. The mixture was poured into gel electrophoresis chambers of different sizes. After the gel had solidified, running buffer, which consisted of 1x TA buffer with 0,1 µg/ml ethidium bromide, was added. Electrophoresis was done at 90V- 135V, depending on the gel size. As molecular weight standard an 1kb DNA ladder was used. DNA fragments were visualized by UV light at 256nm or 366nm and documented by photography.

5.3.2. SDS-polyacrylamide gel electrophoresis

Protein samples were separated by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE). They consisted of a 10% separating gel and a 4,5% stacking gel mounted into a vertical gel electrophoresis chamber (Mini Protean® Tetra Cell, Bio-Rad). Once the gels were solidified, 1x running buffer was added. Protein samples were thawed and treated with ultrasound (ultrasonic waterbath, Branson) for 2x 20sec at 40W. If not already done at time of harvesting, samples were then mixed with sample buffer. After adding of 4% 2-mercaptoethanol, incubation at 85°C for 5min and short centrifugation, 20µl of samples were loaded into the wells of the stacking gel. 6µl of Prestained Protein Marker Page Ruler™ served as size marker. Electrophoresis was done at 200V for 45min.

5.4. Western Blot

5.4.1. Transfer of protein samples to nitro cellulose membrane

After separation of the protein samples by SDS-polyacrylamide gel electrophoresis, proteins were transferred onto a nitro cellulose membrane (Whatman®). In a semi dry western blot apparatus (Trans-Blot®-SD Semi-Dry Transfer Cell, Bio-Rad) 3 layers of Whatman® 3MM paper, which were soaked with transfer buffer, were placed. On top of those, the wet nitrocellulose membrane followed by the polyacrylamide gel and three more soaked layers of Whatman® 3MM papers were laid. Transfer was done at 20V for 45min.

5.4.2. Chemiluminescence

The nitro cellulose membrane was washed with PBS after blotting and then incubated in PBS with 6% skim milk powder for 60min and incubated at 4°C overnight afterwards. The next day the membrane was washed once with PBS/0,1% Tween20 and then incubated with antibodies αVP60_1 IgG (1:10000) or αGFP IgG (1:50000) in PBS/0,1% Tween20 for 1 hour by shaking at room temperature in the dark. After incubation it was washed 3 times with PBS/0,3% Tween20 and then incubated for 15min again by shaking at room temperature in the dark. 3 more washing steps were performed with PBS/0,1% Tween20, followed by incubation for 5min as described. Anti-rabbit IgG POD conjugate was diluted 1:20000 in PBS/0,1% Tween20 and added to the membrane. After incubating for 1 hour, the washing steps were repeated as describe above. Chemiluminescent substrates (Clarity™ Western ECL Substrate, Bio-Rad) were added as recommended by the supplier and chemiluminescent signals were recorded by a Bio-Rad VersaDoc™ Imaging System, using the software QuantityOne.

5.5. VLP purification

For VLP purification after transduction, RK13 cells in T162 flasks were incubated with the respective recombinant baculoviruses at an MOI of 25 in 20ml PBS^+ on a gyratory shaker at 26°C with 300rpm for 5 hours. The inoculum was replaced by culture medium ZB5 with 5mM butyrate and cells were incubated at 37°C and 5% CO_2 for 24h. The medium was then replaced by normal cell culture medium and cells were harvested by trypsinization and low speed centrifugation 1 day later. The pellets were washed once with PBS and resuspended in PBS to yield a 20% weight per volume suspension.

For VLP purification from SF9 cells, cultures in T162 flasks were infected with the respective recombinant baculoviruses at an MOI of 1 and harvested 3 days pi. After harvesting and low speed centrifugation, the cells were resuspended in PBS to yield a 20% weight per volume suspension.

After one freeze (-80°C)/ thaw cycle the suspensions were sonicated twice for 20 seconds in Branson ultrasonic water bath at 40W. Cell debris was removed by centrifugation with 5000rpm at 4°C for 30 minutes using a Heraeus Christ Minifuge. The supernatants were extracted with one third volume of chloroform by vortexing for 2 minutes and centrifuging again with 5000rpm at 4°C for 30min. The aqueous phase was laid on a 20% sucrose cushion made in 0,2M Tris-HCl, pH 6,8 and centrifuged with 30000rpm at 4°C for 2 hours using a Beckman SW 32 rotor. The pellet was resuspended in 3,5ml $CsCl_2$ solution and centrifuged in a Beckman SW 60 rotor with 48000rpm for 65h at 20°C. The visible band with accumulated VLPs was aspired and dissolved in PBS. VLPs were pelleted with a Beckman SW 32 rotor with 30000rpm at 4°C for 2h and resuspended in 400µl PBS for examination by electron microscopy.

5.6. Transmission electron microscopy

VLP samples were analyzed by Dr. K. Franzke, Head of the laboratory for Electron Microscopy at FLI- Insel Riems. For transmission electron microscopy the purified particles were adsorbed to formvar-coated nickel grids for 7min, stained with 1 % phosphotungstic acid (pH 6.0) and analyzed with a FEI Tecnai- 12 Spirit transmission electron microscope at an accelerating voltage of 80kV.

5.7. Evaluation of viral load

5.7.1. Liver homogenate

To determine the viral load in rabbit liver tissue after challenge with RHDV-2 different methods were used comparatively.

About 200mg liver samples were homogenized after adding 1,5ml of medium ZB12 using Qiagen Tissue Lyser II for 2 minutes at 30HZ. The lysed tissue suspension was then centrifuged with 14000rpm with an Eppendorf centrifuge at 4°C for 5 minutes and supernatant was immediately used or stored at -80°C until further analysis.

5.7.2. RNA purification

RNA was purified from the liver supernatant samples using the QIAamp Viral RNA Mini Kit (Qiagen) according to manufacturer’s protocol. For each sample 5µl of an internal process control RNA (“IC-RNA”; Hoffmann et. al, 2006; kindly provided by Dr. G. Strebelow, FLI-Insel Riems) was added as internal purification efficancy control. For up to 6 samples a sample of RNAse free water as RNA isolation control was additionally purified. Purified RNA was stored at -80°C until further use.

5.7.3. Quantitative real time RT-PCR

To verify the presence of RHDV-RNA in liver supernatant samples, 5µl of purified RNA were analyzed by an established and validated qRT-PCR method (Gall et.al. 2007) using the SuperScript^{TM} III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen). A no template control (“NTC”) with RNase free water served as negative control, purified RHDV-2 RNA at a previously determined threshold cycle value (ct) of 33 or standard RHDV-1 RNA with $2x10^6$ copies/µl served as positive control (“PC”). The primer/ probe mix “RHDV-2 Mix” was used to detect RHDV-2 RNA in the liver samples, the primer/probe mix “RHDV-1 Mix” to detect RHDV-1 RNA and the primer/probe mix “IC-Mix” for the validation of IC-RNA (Tab. 2). As reference dye ROX was used. To 20µl of master mix, 5µl sample were added. Preparation of the master mix and adding of samples were performed on ice. After reverse transcription for 30min at 50°C, the inactivation of the reverse transcriptase and activation of the taq polymerase was done for 2min at 94°C. The PCR consisted of 42 cycles with denaturation for 30sec at 94°C, annealing for 45sec at 55°C and elongation for 45sec at 68°C. The real time RT-PCR was analyzed using the real time PCR cycler MX3005P and the software program “MxPro” measuring the channels FAM (liver samples, “NTC” and “PC”), HEX (“IC”) and ROX (reference dye).

5.7.4. Antigen-ELISA

For the determination of the RHDV-2-VP60 antigen content in liver samples the commercial ELISA Kit “Ingezim RHDV DAS R.17.RHD.K2” (Ingenasa, Spain) was used according to manufacturer’s protocol. Briefly, 100µl of each liver sample in duplicates were incubated for 1h at 37°C in coated 96 well plates and washed 3 times with washing buffer. Then 100µl conjugate was added to each well and incubated for 1h at room temperature. After another 3 washing steps, 100µl substrate solution was added to each well and incubated for 5min at room temperature. The reaction was stopped with 100µl/well stop solution. Positive and negative controls samples provided within the kit served as internal controls. A liver sample from a rabbit infected with RHDV-2 “Werne” with a predetermined RHDV-2 antigen content served as external positive control. Absorbance was measured at 450nm with an ELISA reader (Spectra, Tecan) and E.A.S.Y Win software.

5.7.5. Hemagglutination assay (HA)

The hemagglutination assay (HA) was performed according to OIE standard procedure. In a 96well plate (U-shaped) 50µl of isotonic phosphate buffer were added per well. Then 50µl of each liver supernatant sample were titrated in two-fold steps and incubated with 50µl/well of a 1% dilution of blood group 0 human erythrocytes in isotonic phosphate buffer at 4°C for 90min. A RHDV-2 strain “Werne” liver homogenate with predetermined titer was used as positive control and isotonic phosphate buffer as negative control. All samples were run in duplicates. The HA titer was expressed as the value of the highest dilution resulting in complete hemagglutination assessed by visual observation.

5.8. Purification of RHDV-2 antigen for antibody-ELISA

A 10% suspension of ground infectious RHDV-2 liver material in medium ZB12 was centrifuged with 3000rpm at 4°C for 30min with a Heraeus Christ centrifuge. Supernatant was then extracted with 15% chloroform by shaking the mixture with 220rpm at room temperature. After adding of 4% binary ethylenimin to the supernatant, incubation at 4°C overnight followed. The next day 20% Na-thiosulfate was added and another centrifugation step at 4°C with 3000rpm for 30min was performed. Supernatant was precipitated with 10% PEG over 2 hours at room temperature and then incubated at 4°C overnight. After centrifugation at 4°C with 4000rpm for 50min the pellet was resuspended 1/20 volume TEN buffer (pH 7,5) and incubated at 4°C overnight. After centrifugation with 4000rpm at 4°C for 20min, supernatant was ultracentrifuged on a 17% sucrose/TEN cushion with a ratio of 4:1. Ultracentrifugation was performed using a Beckman SW 32 rotor with 25000rpm at 4°C for 2h. After drying, the pellet was resuspended in 5ml TEN buffer and stored at -80°C. The concentration of purified antigen was determined using reference sera.

5.9. Generation of RHDV-2 challenge virus

A 10% suspension of ground infectious RHDV-2 liver material in medium ZB12 was centrifuged with 3000rpm at 4°C for 10min with a Heraeus centrifuge. Supernatants were then lyophilized in aliquots of 1ml. The titer of the challenge virus was determined by hemagglutination assay.

5.10. Measurement of RHDV specific serum antibodies

Serum samples from all trials were analyzed in an indirect ELISA for the presence of RHDV-1 or RHDV-2 specific antibodies. 96well ELISA plates (Microlon® 200 96W Microplate, Greiner, Germany) were coated with 100 µl/well of purified RHDV-1 or RHDV-2 antigen respectively in coating buffer Tris-NaCl at 4°C overnight. After 3 times washing with PBS/0,05% Tween20 using a microplate washer (HydroFlex Tecan) the rabbit sera were two-fold diluted in PBS/0,05% Tween20 with 5 % horse serum. 100µl/well were shaken for a short time and then incubated for 1 hour at 37°C. After further 3 times washing as described 100µl anti-rabbit IgG POD conjugate diluted 1:20000 in PBS/0,05% Tween20 + 5% horse serum were added per well. Plates were shaken for a short time and again incubated at 37°C for 1 hour. After three more washing steps 100µl/well substrate solution was added and incubated for 30 minutes at room temperature in the dark. The reaction was stopped by adding 50µl 4M H_2SO_4 per well and absorbance at 492nm was measured with an ELISA reader (Spectra, Tecan) and E.A.S.Y Win Software.

5.11. Flow cytometric analysis (FACS)

FACS analysis of EDTA blood samples were performed by the laboratory of Dr. B. Köllner at the FLI- Insel Riems. Blood leukocytes were prepared by density gradient centrifugation. 1ml of EDTA blood was diluted 1:4 v/v with PBS, 0,01% 1mM EDTA. The cell suspensions were laid on 3 ml of Pancoll (1,077g/ml) and centrifuged for 30min with 1800rpm in an Eppendorf centrifuge. The cells at the interface were collected, resuspended with PBS, 0,01% 1mM EDTA, centrifuged again for 6min with 1600rpm and resuspended in 2ml of PBS, 0,01% EDTA. 2 x 10^5 cells/well were then incubated in a U-bottom 96well plate with combinations of different monoclonal antibodies specific for leukocyte differentiation markers (Tab. 3) at 4°C for 30min. Plates were again centrifuged with 1000rpm for 3min with an Eppendorf centrifuge and supernatants were then discarded. After washing with 100µl/well PBS, 0,01% 1mM EDTA the labelled cells were incubated with 50µl/well isotype specific fluorochrome (FITC or PE) antibody conjugates for 30min at 4°C. Another centrifugation with 1000rpm for 3min was performed. After discarding of supernatants, cells were washed with 100µl/well PBS, 0,01% 1mM EDTA once. After final washing the cells were resuspended in 300µl PBS, 0,01% 1mM EDTA and analyzed in FACScalibur (Becton Dickinson).

5.12. Generation of vaccine candidates

SF9 cells were seeded and immediately infected with the respective recombinant baculovirus stocks at an MOI of 1 and incubated for 72h at 27°C, 2,7 % CO_2 in T162 flasks. After 3 days the cells were detached and centrifuged with 1500rpm for 20min at 4°C with a Heraeus Christ centrifuge. The pellets were frozen at -80°C till further processing. After thawing, the pellets were resuspended in PBS, pooled and sonicated for 5x 20 seconds at 40W with a Branson ultrasonic water bath for disintegration of cells.

RK13 cells were seeded in T75 flasks. After 24 hours they were transduced with the respective recombinant baculoviruses at an MOI of 25 and then incubated in 10 ml PBS^+ on a gyratory shaker with 300rpm at 26°C for 5 hours. The inoculum was replaced by culture medium ZB5 with 5 mM butyrate and cells were incubated at 37°C and 5% CO_2 for 24h. The medium was then replaced by cell culture medium ZB5 and cells were harvested by trypsinization, low speed centrifugation and washing with PBS 1 day later. The pellets were resuspended in PBS, pooled and frozen at -80°C till further processing. After thawing, the cells were treated with ultrasound for 5x 20 seconds at 40W for disintegration of cells.

The recombinant vaccine candidates derived from SF9 were designated as “recRHDV2-vacc; BacBac-A” or “recRHDV2-vacc”. The recombinant vaccine candidate derived from RK13 cells was named “recRHDV2-vacc; BacMam-A”.

The recombinant vaccine candidates were used in comparison to a conventionally prepared vaccine using a liver homogenate from RHDV-2 (strain “Werne”) infected rabbits inactivated with BEI (referred to as “convRHDV2-vacc”; kindly provided by Dr. H. Schirrmeier, FLI-Insel Riems).

As a negative control preparation, SF9 cells were infected with recombinant baculovirus CO107 Baculop10GFP (kindly provided by C. Klopfleisch) at an MOI of 1. This recombinant expresses GFP but not VP60 (referred to as “recbacGFP-vacc”). Infected SF9 cells were processed the same way as for the “recRHDV2-vacc” vaccine.

Hemagglutination activity of rec-RHDV-2-VLPs in the obtained vaccine stocks was determined by hemagglutination assay and amount of RHDV-2-VP60 protein was confirmed using an indirect ELISA Kit (Ingenasa).

The candidate vaccine preparations were mixed with aluminum hydroxide following the standard operation procedure for the proprietary RHDV-1 vaccine “Cunivak RHD” by IDT Biologika (Riems, Germany)

5.13. Animal experiments

All animal trials received prior approval from the Federal state Ethical Committee for Animal Experimentation (LALLF-7221.3-1-025/15) and were performed following the acquirements of the EU directive 2010/63 and the EG recommendation 2007/526/ and the German animal welfare act.

5.13.1. Animals

For all trials 10-20 weeks old mix breed “Zimmermann”- rabbits from a commercial rabbit farm were used and randomly distributed into the groups. All rabbits were vaccinated twice against *Pasteurella multocida* before. The different trials started earliest 7 days after arrival of the rabbits to ensure that the animals were healthy and adapted to the housing conditions. All animals were clinically examined and the absence of antibodies against RHDV-2 was verified in an ELISA as described above.

The rabbits were fed with commercial rabbit food (ssniff-Spezialdiäten GmbH, Germany) and water ad libitum.

5.13.2. Blood sampling of rabbits

From all rabbits 1ml blood was sampled within 36 or 72 hours after vaccination or challenge infection from ear veins into EDTA pretreated tubes (Sarstedt) for isolation of leukocytes and 200µl blood was sampled in weekly or monthly time intervals before and after vaccination into non-treated tubes for serum collection (Becton, Dickinson).

5.13.3. Immunization of rabbits

For trials, groups of 4, 8 or 10 animals were used. Vaccination was done into the *musculus quadriceps femoris* of the left hind leg with 1ml of the recombinant vaccines “recRHDV2-vacc; BacBac-A”, “recRHDV2-vacc; BacMam-A”, as well as “convRHDV2-vacc” and 0,5ml of the commercial vaccine “Cunivak RHD” (recommended dose for RHDV-1 protection by manufacturer) for trial 1 to test immunogenic properties of VLPs. In the following trials, rabbits were vaccinated with 0,5ml of the “recRHDV2-vacc” or the “convRHDV2-vacc”. Respective HU contents for every trial are specified in the results section. A group of 4 non-vaccinated rabbits served as negative control group in each trial. Additionally, a group of 9 rabbits was vaccinated with 0,5ml of the “recbacGFP-vacc”. After vaccination, the animals were observed and checked for clinical signs.

5.13.4. Challenge infection

The challenge infection in all trials was done by injection into *musculus quadriceps femoris* of the left hind leg with 2560 HUs of challenge virus RHDV-2 strain “Werne” or RHDV-1 strain “Eisenhüttenstadt”. After challenge, the health status of all rabbits was monitored at least twice a day and rectal body temperature was taken twice a day over two weeks.

Blood was sampled as described until the rabbits were euthanized in a moribund stage or died. 14 days after challenge all remaining animals were euthanized in accordance with animal welfare and blood and organ samples were taken and prepared for further analysis.

5.13.5. Pathological observation and organ sampling

Postmortem macroscopic and histopathological analysis was performed by Dr. R. Ulrich at FLI- Insel Riems. All rabbits underwent complete necropsy under biosafety level 2 conditions according to FLI internal standard guidelines. Samples from heart, lung, spleen, liver, kidney, intestine and brain were fixed in 10% neutral buffered formalin, embedded in paraffin wax using a Leica ASP 300S fully enclosed tissue processor (Leica Biosystems, Nussloch, Germany), sectioned at 2-4µm thickness using a Microm HM 340E electronic rotary microtome, mounted on glass slides, and stained with hematoxylin and eosin (Mulisch and Welsch, 2010). Histopathological changes were assessed using a Zeiss Axio Scope.A1 microscope equipped with 5x, 10x, 20x, and 40x N-ACHROPLAN objectives. A selection of macroscopic and/or light microscopic morphological changes frequently occurring in RHD were recorded as being present (1) or not (0) in a spreadsheet for evaluation (Suppl. 1-6).

6. Results

6.1. Generation of recombinant baculoviruses

In order to obtain high yields of recombinant RHDV-2-VP60, two expression cassettes within the baculovirus transfer plasmids were constructed. In both, the sequence of RHDV-2-VP60 was optimized based on the codon usage (CU) of AcMNPV or glycoprotein B of bovine herpesvirus 1. These two RHDV-2-VP60 open reading frames had a nucleotide sequence identity of 76.2% among each other and 74.5% (BHV-1-CU) and 74.6% (AcMNPV-CU) identity to the authentic RHDV-2-VP60 sequence (GenBank accession number FR819781).

Both synthetic RHDV-2-VP60 ORFs were inserted into transfer vectors pFBD-P10Uhis-ieGFP and pCAGGS-PHGFP. This approach resulted in four different recombinant plasmids: (1) pFBD_RHDV-2_VP60_AcMNPV, (2) pFBD_RHDV-2_VP60_BHV1, (3) pMBCAGGS-RHDV-2_VP60_AcMNPV and (4) pMBCAGGS-RHDV-2_VP60_BHV1. The expression of recombinant RHDV-2-VP60 in the first two plasmids is controlled by the very late baculoviral P10 promotor for use in insect cells whereas gene expression in the second two plasmids is controlled by the hybrid CAG(GS) enhancer/promotor element for use in mammalian cells. GFP expression cassettes controlled by HCMVie promotor in plasmids pFBD_RHDV-2_VP60_AcMNPV and pFBD_RHDV-2_VP60_BHV1 or by the baculoviral polyhedrin promotor in plasmids pMBCAGGS-RHDV-2_VP60_AcMNPV and pMBCAGGS-RHDV-2_VP60_BHV1 were used to facilitate isolation and titer determination of the respective baculovirus recombinants in insect cells (Fig. 8).

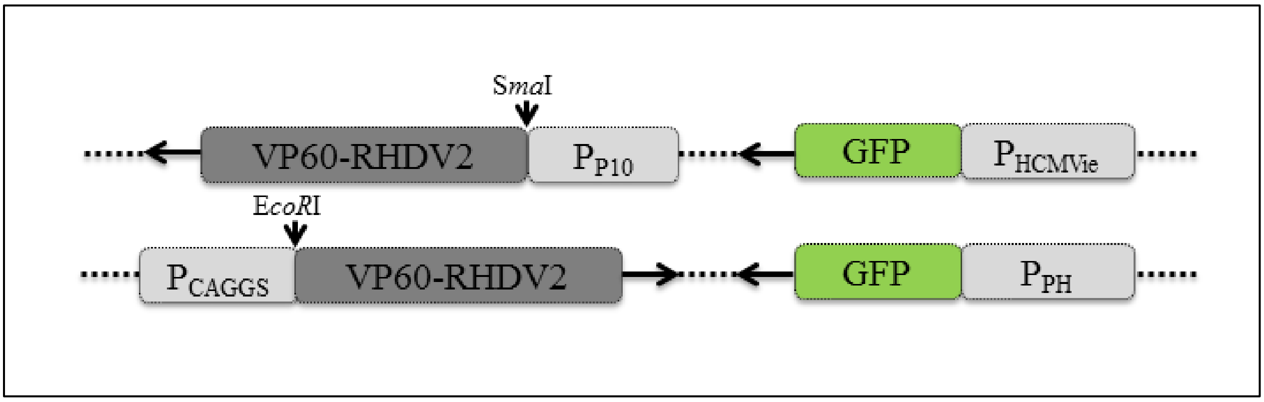

Fig. 8. Diagram of the arrangement of expression cassettes within the baculovirus transfer plasmids
Only relevant details are depicted (not to scale)
P_{PH}: polyhedrin promoter; P_{P10}: p10 promoter; P_{HCMVie}: human cytomegalovirus immediate-early enhancer/promoter; P_{CAGGS}: CAG(GS) enhancer/promotor element
Arrows indicate the transcription directions of the respective genes. Positions of relevant restriction enzyme cleavage sites are indicated.

After transposition of the GFP and RHDV-2-VP60 expression cassettes into the baculovirus bacmid DNA contained in E.coli, recombinant baculovirus DNA was isolated and used for transfection of High V cells. Two recombinant baculoviruses for infection of insect cells named BacBacVP60-2/AcMNPV (further referred to as BacBac-A) and BacBacVP60-2/BHV1 (further referred to as BacBac-B) and two for transduction of mammalian cells designated BacMamVP60-2/AcMNPV (further referred to as BacMam-A) and BacMamVP60-2/BHV1 (further referred to as BacMam-B) were generated and propagated on SF9 cells for further characterization. The resulting virus stocks reached $TCID_{50}$ titers of 1,8 x 10^9 for both „BacBac“ stocks and $TCID_{50}$ titers of 3,2 x 10^9 for both „BacMam“ stocks.

6.2. RHDV-2-VP60 expression levels were significantly influenced by the used promotors but only slightly by the codon usage of synthetic VP60

The resulting expression of RHDV-2-VP60 analyzed after infection of insect or transduction of vertebrate cells using the above described four recombinant baculoviruses (BacBac-A or -B; BacMam-A, -B) respectively is shown in figure 9. The expression kinetics in infected insect-derived SF9 cells indicated an increase over time which was shown also for kinetics in transduced rabbit kidney-derived RK13 cells but with a slight decrease from day 5 after transduction. After infection of SF9 cells using BacBac-A or BacBac-B at an MOI of 1 a comparable expression level of the RHDV-2-VP60 was determined by immunoblotting.

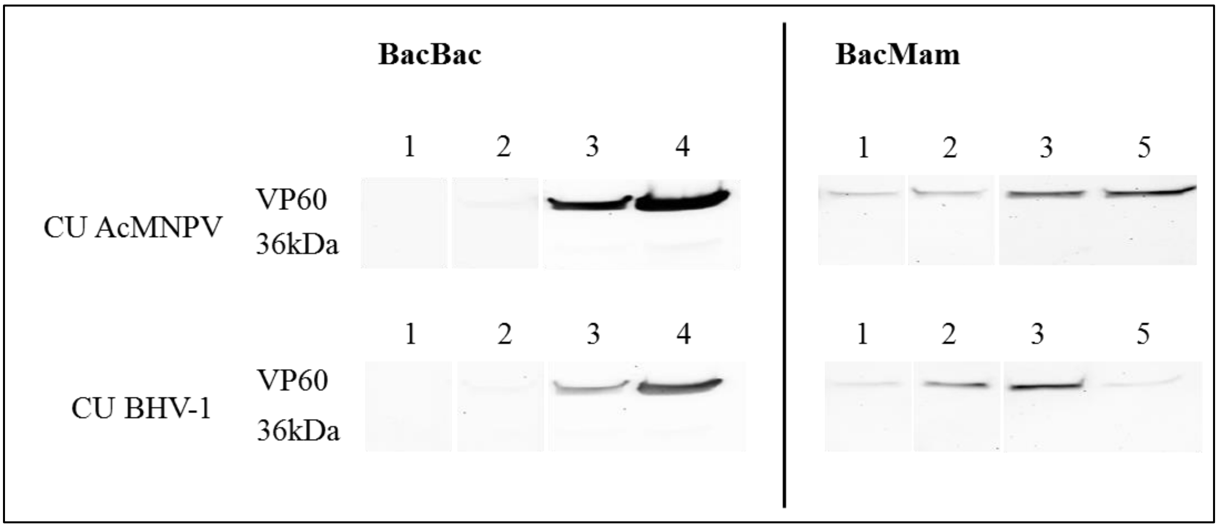

Fig. 9. Comparative kinetics of RHDV-2-VP60 expression

Left: after infection of insect-derived SF9 cells with BacBac-A (CU AcMNPV) or BacBac-B (CU BHV-1), MOI 1 or

Right: after transduction of rabbit kidney-derived RK13 cells with BacMam-A (CU AcMNPV) or BacMam-B (CU BHV-1), MOI 25.

Numbers above western blot bands represent the days after infection or transduction, respectively.

Non-infected SF9 or non-transduced RK13 cells showed no signals in Western Blots (not shown).

This was also true after transduction of RK13 cells with BacMam-A or BacMam-B, both at an MOI of 25 (Fig. 9).

Protein expression was found to be dependent on the amount of recombinant baculovirus with MOI 0,1 to 1 in SF9 cells and MOI 5 to 25 in RK13 cells (Fig. 10).

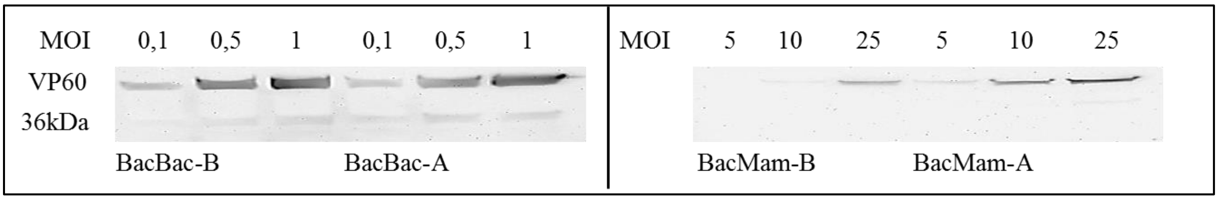

Fig. 10. Comparative kinetics of RHDV-2-VP60 expression dependent on MOI of recombinant baculoviruses

Left: 3 days after infection of insect-derived SF9 cells with BacBac-A (CU AcMNPV) or BacBac-B (CU BHV-1) or

Right: 1 day after transduction of rabbit kidney-derived RK13 cells using BacMam-A (CU AcMNPV) or BacMam-B (CU BHV-1)

To verify the kinetics of RHDV-2-VP60 protein expression in mammalian cells, RK13 cells were also transduced with BacMam-A together with a different recombinant baculovirus which expresses only GFP (BacMam-ieGFP) as a second indicator for target protein content progression (Fig. 11). The GFP autofluorescence images indicate that GFP content increases until 72h after transduction (p.a.tr.) (Fig. 11A) and then appears to remain largely unchanged until day 6 after transduction when a slight decrease was seen and confirmed by immunoblotting (Fig. 11B).

Similar findings were seen after infection of SF9 cells with BacBac-A. Due to baculoviral GFP activity in insect cells, no second indicator was needed. Like for RK13 cells GFP autofluorescence increases steadily until at least 90h post infection (data not shown). The same applies to the recombinant RHDV-2-VP60, from day 3 post infection degradation bands are occurring, though (Fig. 9 + 10). Non-infected SF9 or non-transduced RK13 cells showed no signals in Western Blots (not shown).

Previous experiments with GFP expressing "BacBac-recombinants" indicated that SF9 cells became successively fragile during progression of the infection at an MOI of 1 which results in leakage of soluble proteins into the extracellular media during cell harvest (data not shown).

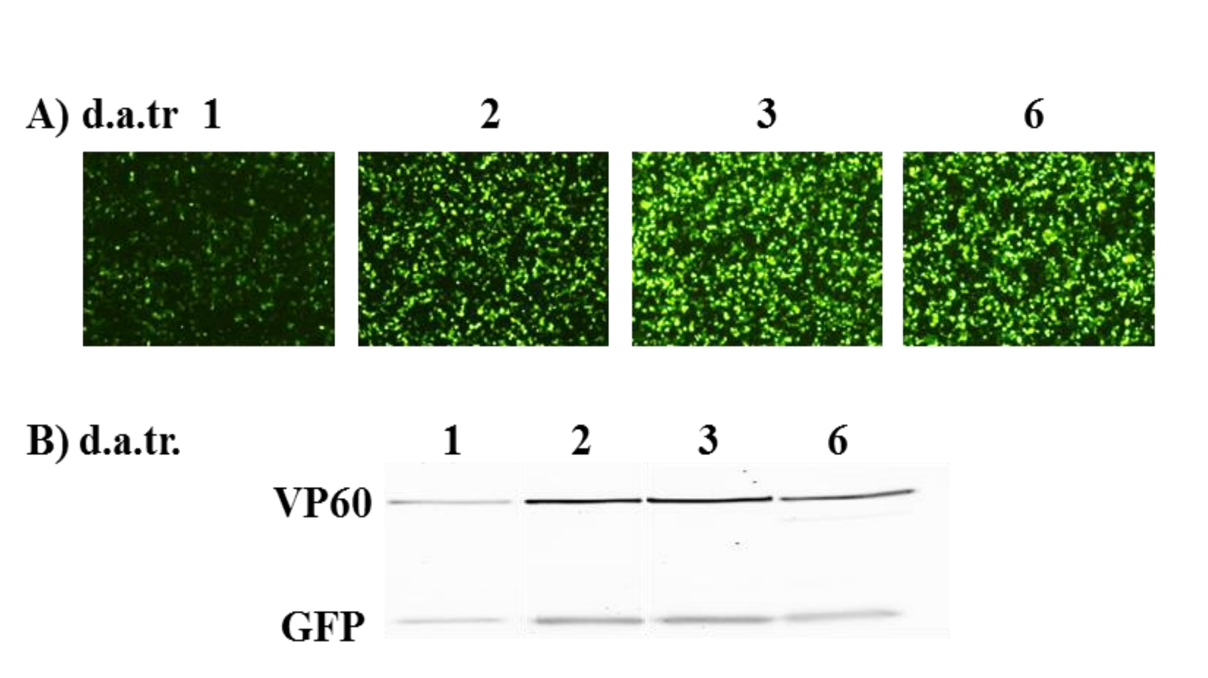

Fig. 11. Time course of RHDV-2-VP60 protein progression in rabbit kidney-derived RK13 cells
Incubation for more than 3 days after transduction (d.a.tr.) does not improve RHDV-2-VP60 protein expression levels.
RK13 cells were transduced with BacMam-A (CU AcMNPV), MOI of 25 together with BacMam-ieGFP, MOI of 10.
A) GFP after transduction determined by autofluorescence.
B) Protein expression estimated by immunoblotting. The position of VP60 and GFP is indicated.

6.3. Baculovirus-expressed RHDV-2-VP60 assembled to VLPs

To elucidate whether the RHDV-2-VP60 molecules synthesized in transduced RK13 cells (using BacMam-A, -B) and infected SF9 cells (using BacBac-A, -B) assemble to VLPs, cell pellets from both cell lines were processed as described in the materials and methods section. The visible turbid virion band was collected after density gradient centrifugation, resuspended in PBS and analyzed by electron microscopy.

In all four preparations VLPs, which resemble typical RHDV virions, were detected (only one picture for the BacMam- and BacBac recombinants, respectively, is shown) (Fig. 12).

RHDV virions have the ability to agglutinate human erythrocytes by binding to histo-blood group antigens on the cell surface. To prove that the recombinant expressed VLPs had assembled to particles which had a comparable biological activity as RHDV-2 virions, the hemagglutination (HA) activity of these VLPs was compared to a native RHDV-2 preparation. VLPs purified from RK13 cells transduced with both recombinant baculoviruses resulted in HA titers of 2^{11} while VLPs generated in SF9 cells resulted in HA-titers of 2^{13} for both CUs.

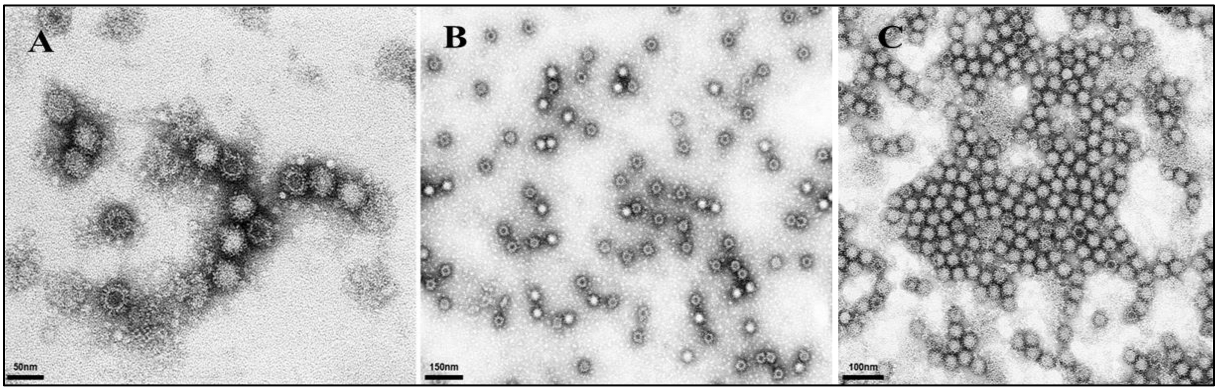

Fig. 12. Baculovirus-expressed RHDV-2-VP60 assembled to VLPs

A) RHDV-2-VP60-VLPs derived from RK13 cells transduced with BacMam-A (CU AcMNPV)

B) RHDV-2-VP60-VLPs derived from SF9 cells infected with BacBac-B (CU BHV-1)

C) RHDV particles prepared from a liver of a RHDV infected rabbit for comparison (photograph taken by Dr. H. Granzow, FLI- Insel Riems)

Cells were processed as described in the material and methods section and VLPs were visualized with electron microscopy (kindly provided by Dr. K. Franzke, FLI- Insel Riems).

6.4. Animal experiments

6.4.1. Immunogenicity of recombinant RHDV-2-VP60-VLPs - Proof of principle

Since the in vitro analyses did not reveal significant differences of the RHDV-2-VP60-VLP expression levels between different codon usages used in the four generated recombinant baculoviruses (see 6.2.), only the recombinant RHDV-2-VP60-VLPs prepared from BacBac-A and BacMam-A were chosen to test their immunogenicity as well as their protective capacity against lethal RHDV-2 infections (proof of principle). This was analyzed comparatively in a vaccination-challenge trial using crude extracts prepared from SF9 cells infected with BacBac-A at an MOI of 1 or RK13 cells transduced with BacMam-A at an MOI of 25 as described in the materials and methods section. The resulting recombinant RHDV-2-VP60-VLP vaccines will be referred to as “recRHDV2-vacc; BacBac-A or BacMam-A”, respectively, in the following section.

Non-vaccinated rabbits as well as rabbits vaccinated with the “convRHDV2-vacc” served as negative and positive controls. Rabbits immunized with the commercial anti-RHDV-1 vaccine “Cunivak RHD” were used as heterologous vaccine controls.

Groups of 4 rabbits were vaccinated with 1ml crude extract of “recRHDV2-vacc; BacBac-A” or “recRHDV2-vacc; BacMam-A”; or 512 HU/dose of “convRHDV2-vacc” or “Cunivak RHD”, respectively. Two rabbits of each group received a booster immunization 14 days later. A fifth group served as non-vaccinated control group. After vaccinations as well as after challenge with RHDV-2 (at day 35 after first vaccination) all animals were observed for the development of any RHD related clinical signs.

None of the vaccinated or non-vaccinated rabbits displayed any sign of disease till challenge infection 35 days after the first vaccination. However, within 36h after challenge with RHDV-2 all non-vaccinated rabbits developed typical clinical symptoms and died. They displayed severe pathological alterations such as necrotizing hepatitis, lung edema and hemorrhages in different organs (Tab. 4) as detected by necropsy for RHD related pathological changes in inner organs.

Two rabbits once vaccinated with the anti-RHDV-1 vaccine "Cunivak RHD" developed fever >40°C. One rabbit died after 36h with typical clinical symptoms and displayed similar pathological alterations as the non-vaccinated animals. The other animal survived and recovered 4 days later and only slight pathological alterations were found in inner organs at day 14 after challenge.

All "convRHDV2-vacc" rabbits survived the challenge infection. Although not showing any clinical symptoms, focal necrotizing hepatitis or hemorrhages in kidneys were found in 3 of 4 rabbits of the "convRHDV2-vacc" group but with less severity than in non-vaccinated rabbits. In contrast, all "recRHDV2-vacc; BacBac-A" and "recRHDV2-vacc; BacMam-A" vaccinated rabbits survived without any clinical symptoms or pathological alterations (Tab. 4).

Tab. 4. Clinical and pathological findings in rabbits vaccinated with different RHDV-vaccines after challenge with RHDV-2

vaccine	**Cunivak RHD**			**BacBac-A recRHDV2-vacc**		**MamBac-A recRHDV2-vacc**		**convRHDV2-vacc**		**non-vacc**
vaccination	**2x**	**1x**	**1x**	**2x**	**1x**	**2x**	**1x**	**2x**	**1x**	**-**
clinical outcome										
no. of animals	2	2		2	2	2	2	2	2	4
survived	2	1		2	2	2	2	2	2	
died			1							4
mean survival time, h	336	336	36	336	336	336	336	336	336	36
clinical symptoms/ pathological findings										
fever > 40°C	0	1	1	0	0	0	0	0	0	4
necrotizing hepatitis	0	0	1	0	0	0	0	1	1	4
lung edema	0	0	1	0	0	0	0	0	0	4
hemorrhages	0	1	1	0	0	0	0	2	0	4

In the liver samples of the deceased rabbits high viral RNA loads (q-RT-PCR), high amounts of RHDV-2 viral antigen (ELISA) and viral particles (HA titers) were detected. In the liver of the surviving „Cunivak RHD" once vaccinated rabbit low amounts of virus genome but neither RHDV-2 antigen (ELISA) nor RHDV-2 viral particles (HA titers) were found. In the livers of all "recRHDV2-vacc; BacBac-A", "recRHDV2-vacc; BacMam-A" and "convRHDV2-vacc" rabbits as well as of the prime-boost anti-RHDV-1 vaccine "Cunivak RHD" rabbits no RHDV-2 RNA, antigen or particles were detected (Tab. 5).

Tab. 5. Comparison between clinical outcome and viral load in liver of rabbits vaccinated with different RHDV-vaccines after challenge with RHDV-2

Note the differences of viral load between vaccinated and non-vaccinated animals and also between the surviving "Cunivak RHD" one-time vaccinated rabbit and all the other surviving vaccinated rabbits.

vaccine	**Cunivak RHD**			**BacBac-A recRHDV2-vacc**		**MamBac-A recRHDV2-vacc**		**convRHDV2-vacc**		**non-vacc**
vaccination	**2x**	**1x**	**1x**	**2x**	**1x**	**2x**	**1x**	**2x**	**1x**	**-**
clinical outcome										
no. of animals	2	2		2	2	2	2	2	2	4
survived	2	1		2	2	2	2	2	2	
died			1							4
mean survival time, h	336	336	36	336	336	336	336	336	336	36
viral load										
RNA, q-RT-PCR; 2^e	6,4	17,5	31,6	2,9	0	3,8	2	0	1,3	31,9
viral particle, HA, 2^e	0	0	12	0	0	0	0	0	0	12
VP60, ELISA, OD	0,08	0,07	1,02	0,07	0,06	0,09	0,12	0,06	0,07	0,94

In blood serum samples (taken weekly after first vaccination) all rabbits that received the recombinant or conventional RHDV-2 vaccines developed protective antibody titers against RHDV-2 which increased from day 0 until day 14 (Fig. 13). The anti-RHDV-2 antibody titers increased faster after the rabbits received a second immunization 3 weeks after the first one. But, after the challenge infection a-RHDV-2 antibody titers were always higher than 1:25600, independent whether the rabbits were vaccinated one or two times. However, the antibody response after vaccination with "recRHDV2-vacc; BacBac-A" and "recRHDV2-vacc; BacMam-A" was less intense than after vaccination with the "convRHDV2-vacc". After a single vaccination with the commercial anti-RHDV-1 vaccine "Cunivak RHD" the anti-RHDV-2 antibody titers were not high enough to prevent disease in one animal which died after challenge infection. However, after a prime-boost immunization the RHDV-2 specific antibody titers reached protective levels also against a challenge infection with RHDV-2 (Fig. 13B).

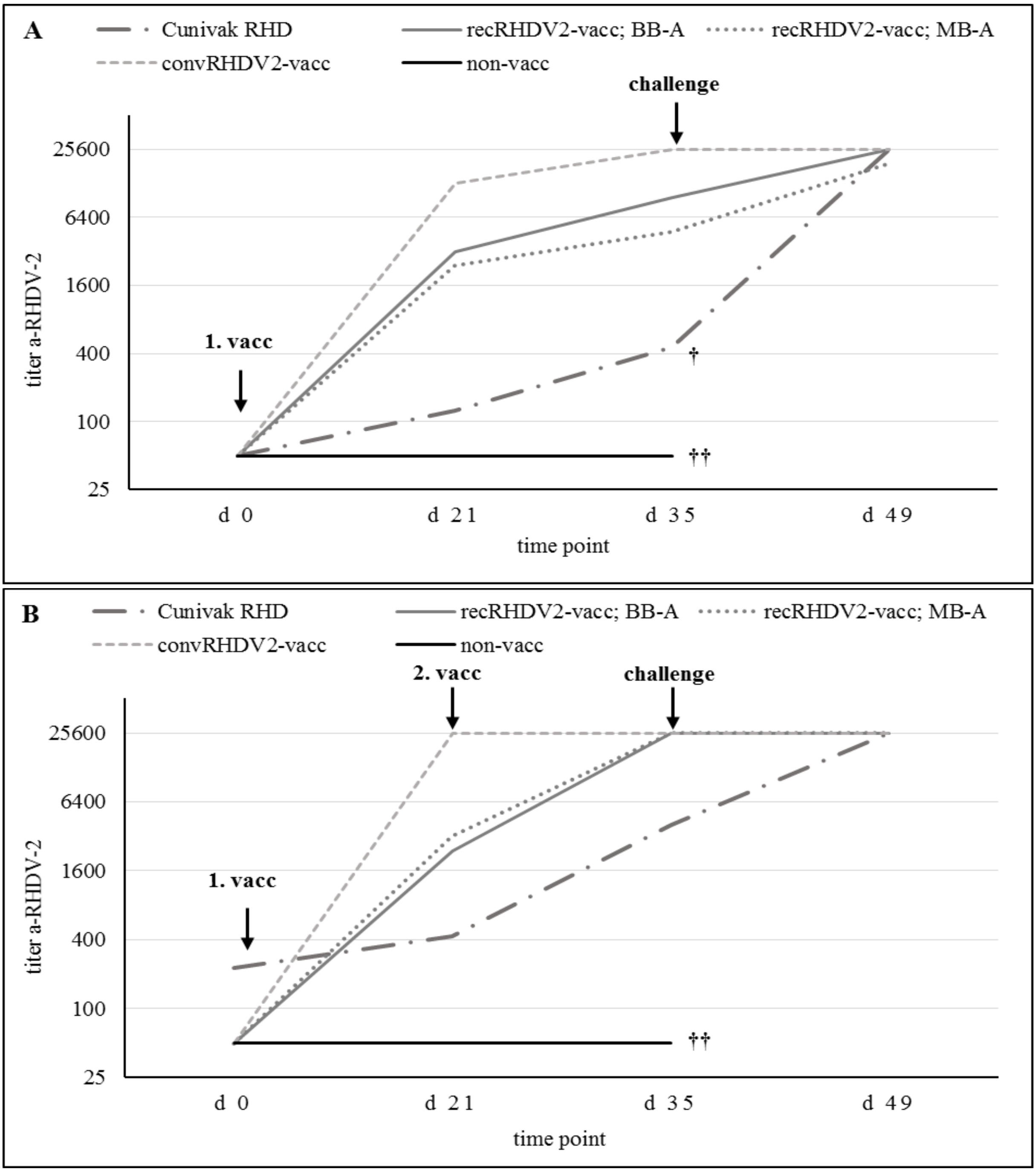

Fig. 13. Development of specific anti-RHDV-2 antibody titers in the sera of vaccinated and non-vaccinated rabbits (A) after a single or (B) after booster immunization with commercial anti-RHDV-1 vaccine "Cunivak RHD", recombinant baculovirus-derived vaccines and "convRHDV2-vacc"
d 0 = day of 1. vaccination; d 21 = day of 2. vaccination; d 35 = day of challenge; d 49 = end f trial
†: One of the two "Cunivak RHD" once vaccinated-rabbits died shortly after challenge infection
††: Death of the two non-vaccinated rabbits after challenge infection

6.4.2. Naïve rabbits or rabbits vaccinated with "recbacGFP-vacc" displayed only very limited natural resistance

The comparative analysis of all vaccination-challenge trials confirms, that almost all non-vaccinated rabbits (21 of 24; 87,5%) died after infection (Tab. 6).

Tab. 6. Overview about clinical signs and pathological changes in non-vaccinated rabbits or rabbits vaccinated with "recbacGFP-vacc" after challenge with RHDV-2

vaccine	**non-vaccinated**		**recbacGFP-vacc**
challenge with	**RHDV-2**		**RHDV-2**
clinical outcome			
no. of animals	24		9
survived	3		0
died		21	9
mean survival time, h	336	41	48
clinical symptoms/ pathological findings			
fever > 40°C	2	13	7
necrotizing hepatitis	0	21	9
lung edema	0	21	9
hemorrhages	0	21	9

Before death all animals showed poor general condition and reduced food intake. 57,1% of those developed high fever over 40°C. In autopsy hepatitis, lung edema and hemorrhages were the main pathological alterations (Tab. 6; Fig. 14a, b). But also other findings that are often described in literature were seen such as bloody nasal discharge, congested conjunctivae or splenomegaly (Fig. 14a, b, c). Livers usually appeared swollen and fragile after RHDV-2 infection and histopathological examination of livers confirmed hepatitis with signs of apoptosis of hepatocytes like pyknosis and karyorrhexis as is discussed in literature (Fig. 14b).

All of the 3 surviving non-vaccinated animals displayed clinical symptoms like reduced food uptake and apathy, while only 2 of those 3 developed fever >40°C for 4 days and recovered. No pathological alterations were found (Tab. 6).

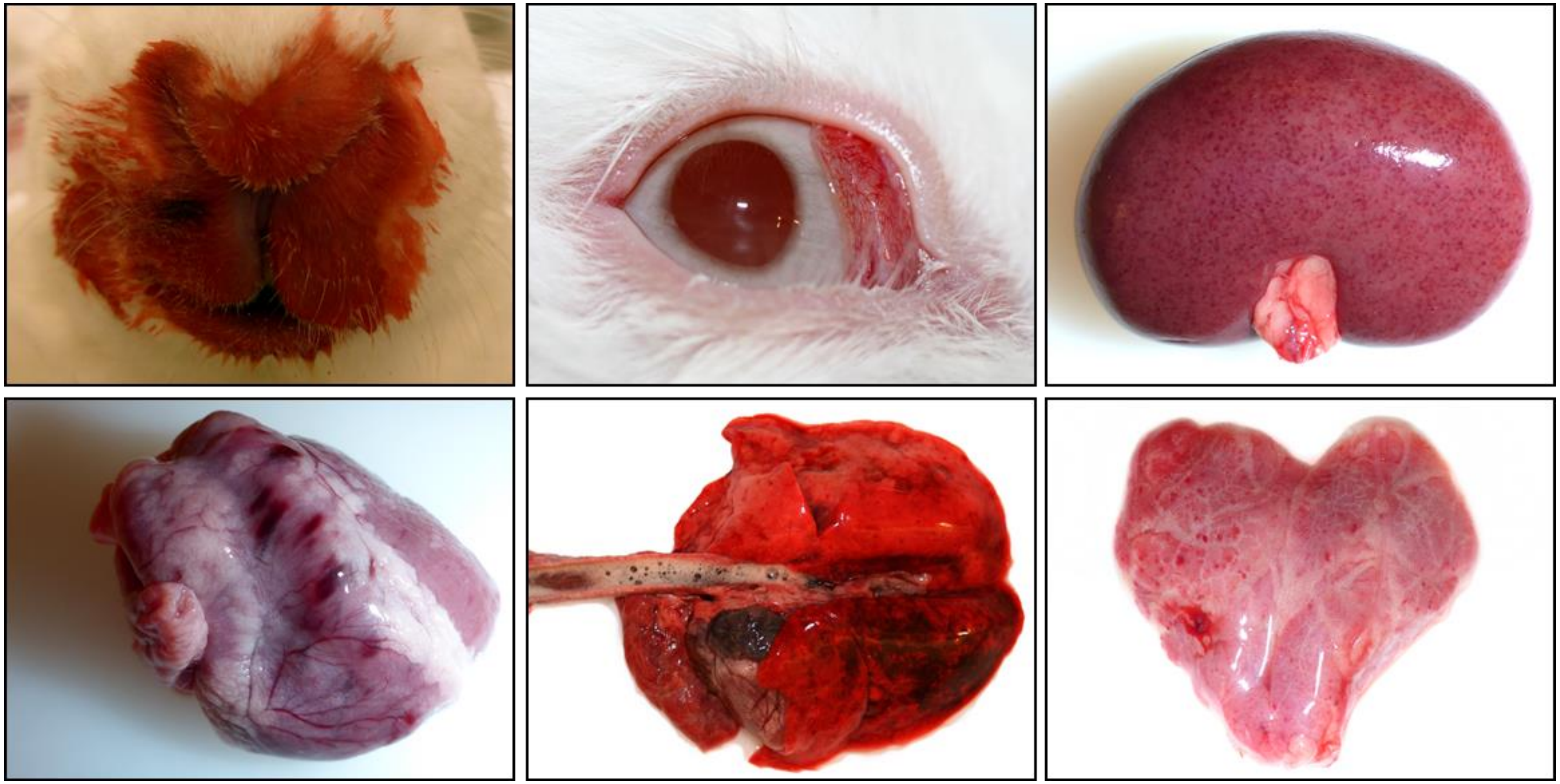

Fig. 14a. Gross lesions in non-vaccinated rabbits that died after challenge with RHDV-2 strain "Werne"

Upper row: nares, epistaxis (left); conjunctiva: congested vessels (middle); kidney: renal petechiae (right)
Lower row: heart: epicardial ecchymoses (left); lung: tracheal and alveolar edema, petechiae, ecchymoses (middle); thymus: petechiae and ecchymoses (right)

(Photos kindly provided by Dr. R. Ulrich, FLI- Insel Riems)

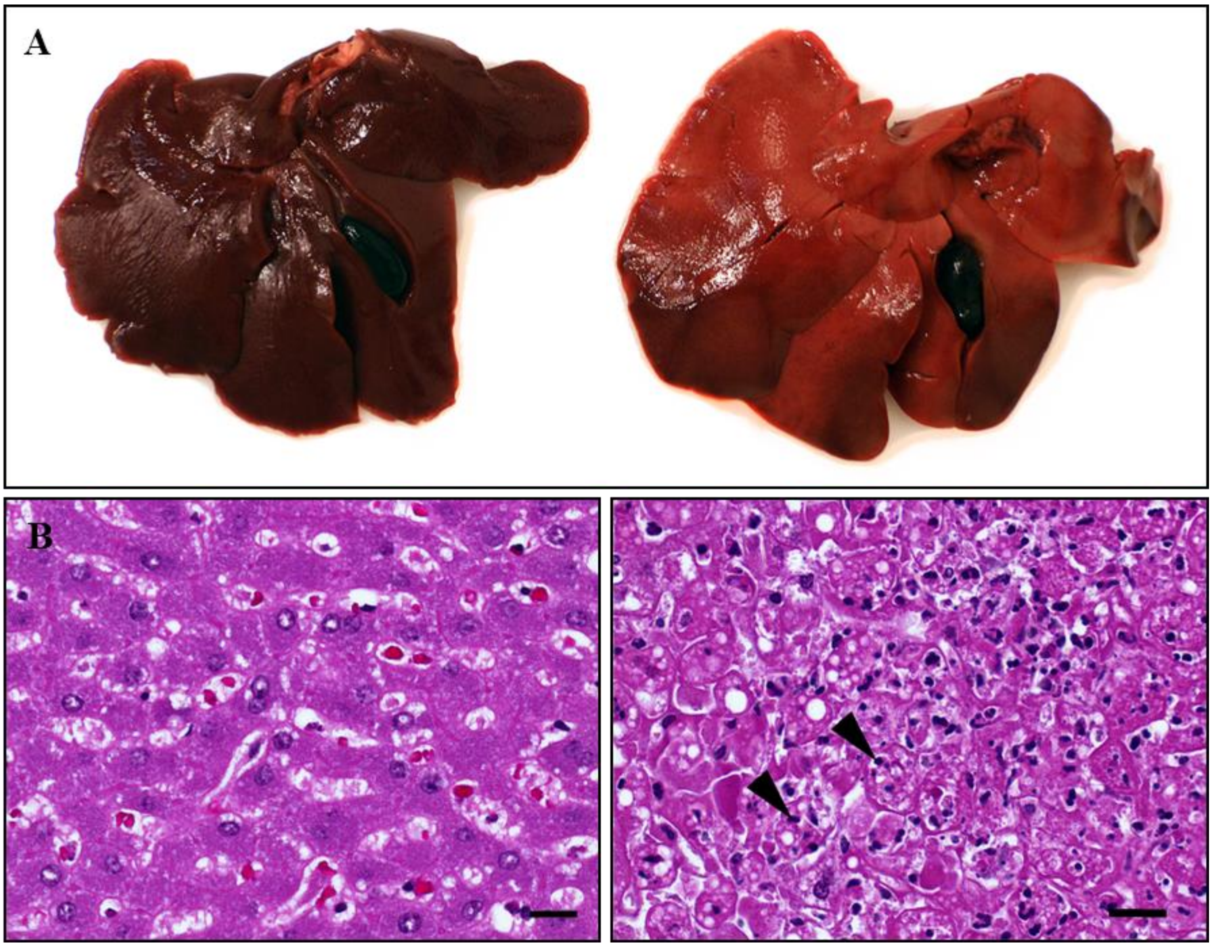

Fig. 14b. Pathological alterations in the liver from a non-vaccinated rabbit that died after challenge with RHDV-2 strain "Werne" in comparison to a liver from a healthy untreated control rabbit

A) Comparison of normal liver (left) and cinnabar red, swollen, friable, diffusely necrotic liver (necrotizing hepatitis) after RHDV-2 infection (right)

B) Histopathological image of normal liver from an uninfected rabbit (left) in comparison to necrotic liver from an RHDV-2 infected rabbit (right). Arrows indicate pyknotic and karyorrhectic hepatocellular nuclei (apoptosis and/or necrosis), H.E., bars = 20µm

(Photos kindly provided by Dr. R. Ulrich, FLI- Insel Riems)

Fig. 14c. Splenomegaly after infection with RHDV-2 strain "Werne" (left) in comparison to a normal sized spleen (right)

(Photos kindly provided by Dr. R. Ulrich, FLI- Insel Riems)

In post mortem liver samples of rabbits that succumbed to the RHDV-2 challenge infection, a high amount of viral RNA was detected between 36 and 96h post challenge by q-RT-PCR which was up to 2^{20} times higher than in the three survivors. Viral antigen and particles were also measured in high amounts in deceased rabbits (Tab. 7; Fig. 17).

Tab. 7. Overview about clinical outcome and viral load in non-vaccinated rabbits as well as rabbits vaccinated with "recbacGFP-vacc" after challenge with RHDV-2

vaccination	**non-vaccinated**		**recbacGFP-vacc**
challenge with	**RHDV-2**		**RHDV-2**
clinical outcome			
no. of animals	24		9
survived	3		0
died		21	9
mean survival time, h	336	41	48
viral load			
RNA, q-RT-PCR; 2e	5,1	29,9	31,5
viral particle, HA; 2e	0,3	11,8	11,3
VP60, ELISA; OD	0,04	1,01	0,98

Interestingly, infection with RHDV-2 induced a strong decrease of the absolute numbers of $CD4^+$ as well as $CD8^+$ T-cells shortly after infection in non-vaccinated rabbits. In some rabbits, 36 hours post infection nearly no $CD8^+$ T-cells were detectable in blood (Fig. 15).

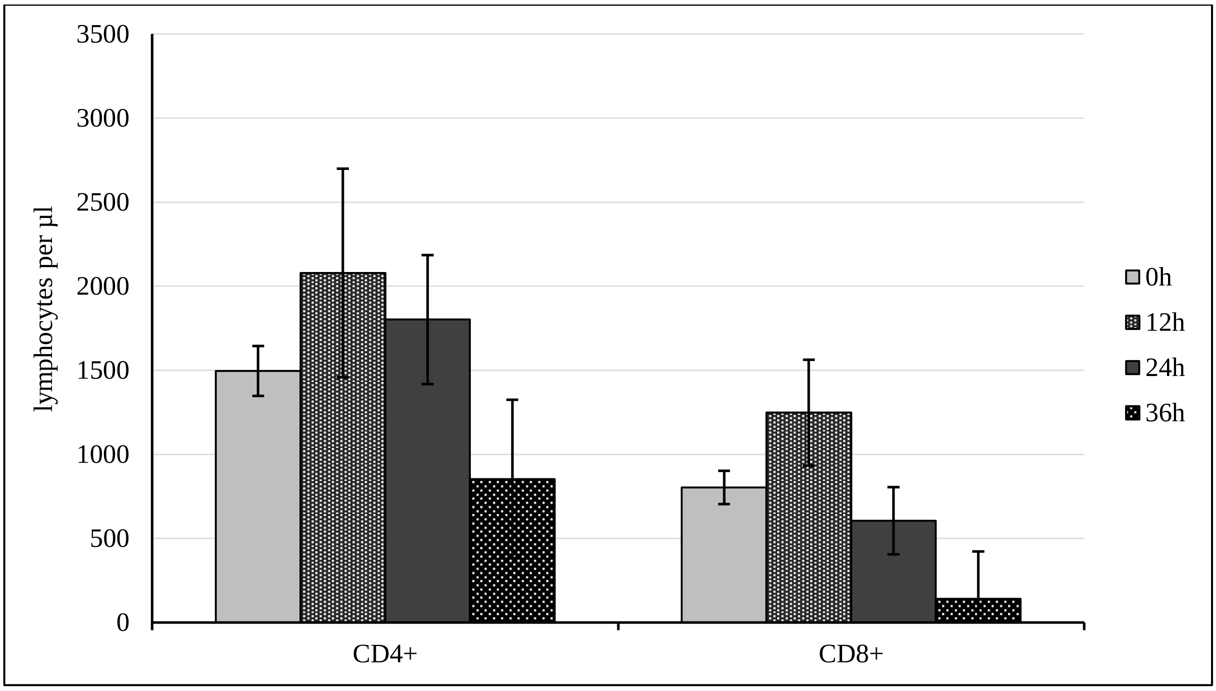

Fig. 15. Kinetics of $CD4^+$ and $CD8^+$ T-cells in blood of non-vaccinated rabbits after infection with RHDV-2 (Data kindly provided by Dr. B. Köllner, FLI- Insel Riems)

In surviving non-vaccinated rabbits an increase in $CD4^+$ and $CD8^+$ T-cells (Fig. 16) was observed as well as a significant increase of antibody titers after challenge infection.

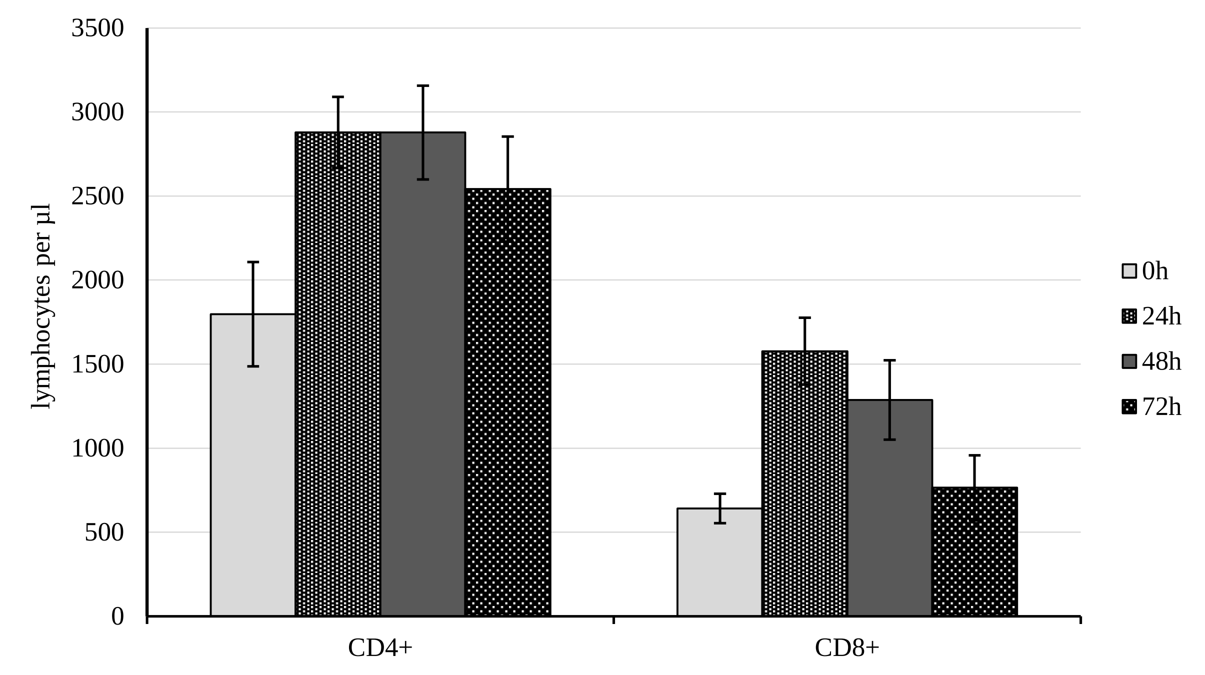

Fig. 16. Kinetics of $CD4^+$ and $CD8^+$ T-cells in blood of non-vaccinated rabbits which survived after infection with RHDV-2 (Data kindly provided by Dr. B. Köllner, FLI- Insel Riems)

As a further negative control a group of 9 rabbits was vaccinated with recombinant baculovirus expressing GFP but not RHDV-2-VP60 (“recbacGFP-vacc”) for evaluation of the unspecific immune response after vaccination. Blood serum samples were taken at day 7 and 14 after vaccination. At day 14 post vaccination this group was also challenged with RHDV-2. After challenge infection, animals were observed for the development of any RHD related clinical signs and evaluated by autopsy for RHD related pathological changes in inner organs after death.

All 9 animals vaccinated with “recbacGFP-vacc” died between 30 and 125h after challenge infection. 7 out of 9 rabbits developed high fever over 40°C and, like the non-vaccinated control group, all of them showed poor general condition before death. In necropsy the same pathological alterations as in the non-vaccinated control group occurred (Tab. 6; Fig. 14a, b, c). In post mortem liver samples of these rabbits a similar high viral load was detected between 36 and 125h post challenge (Tab. 7; Fig. 17) as in non-vaccinated rabbits. No specific anti-RHDV-2 antibody titers were measured in serum before challenge infection.

The kinetics of mortality in comparison to viral load of RHDV-2 in liver samples from both groups is shown in Fig. 17.

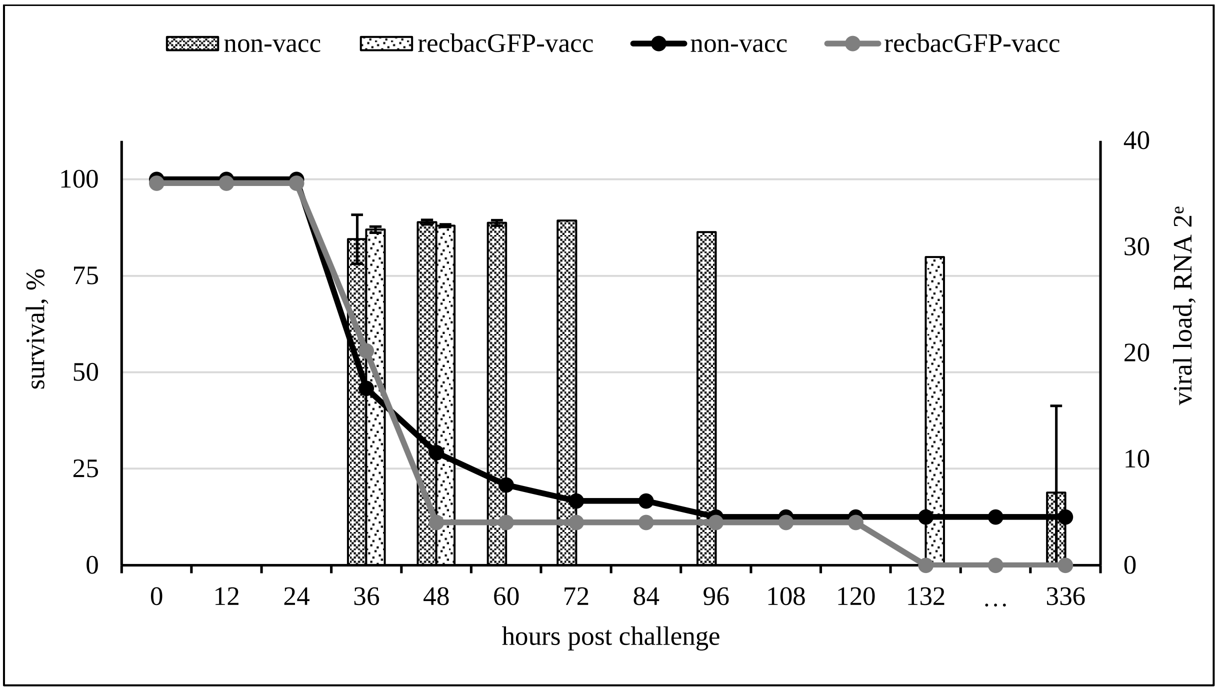

Fig. 17. Cumulative mortality (lines) of differentially treated rabbits after infection with RHDV-2 and corresponding viral load (columns) in liver samples taken from these rabbits
Rabbits were vaccinated with "recbacGFP-vacc" and compared to non-vaccinated rabbits.
Note the high viral load in rabbits that died after infection before end of trial in comparison to survivors at 336 hours post challenge.

6.4.3. An immunization with RHDV-2 vaccine formulation provided protection against RHDV-2 induced disease

After the proof of principle trial, the induction of protective immunity by vaccination with the newly established recombinant "recRHDV2-vacc" was further evaluated in comparison to a vaccination with the conventional "convRHDV2-vacc" by intramuscular challenge infection of immunized rabbits with RHDV-2. In the following trials only the newly established recombinant vaccine prototype "BacBac-A" prepared in SF9 cells infected with MOI 1 was further used. It will be referred to as "recRHDV2-vacc" in the following sections.

In total, 97,6% (40 of 41) of rabbits vaccinated with the newly developed "recRHDV2-vacc" survived after RHDV-2 challenge infection. 37 did not show any RHD specific clinical symptoms or pathological alterations in inner organs (Tab. 8). 4 animals displayed rectal body temperatures over 40°C after challenge infection but only at single time points in the 2 weeks after challenge. In the challenge experiment performed 14 months after vaccination 3 rabbits displayed mild clinical symptoms (low food intake and apathy) for 2 days but finally survived. One rabbit developed the typical severe RHD symptoms (apathy, no food intake) but no fever and died 34h after challenge infection with RHDV-2. This is in detail described in 6.4.6.

The control rabbits vaccinated with the “convRHDV2-vacc” survived to 100% (23 of 23) after RHDV-2 challenge infection without RHD specific clinical symptoms (Tab. 8).

Tab. 8. Overview about clinical signs and pathological changes in rabbits vaccinated with the newly established “recRHDV2-vacc” in comparison to “convRHDV2-vacc” after challenge with RHDV-2

vaccine	**recRHDV2-vacc**		**convRHDV2-vacc**
challenge with	**RHDV-2**		**RHDV-2**
clinical outcome			
no. of animals	41		23
survived	40		23
died		1	0
mean survival time, h	336	34	336
clinical symptoms/ pathological findings			
fever > 40°C	4	0	2
necrotizing hepatitis	0	1	2
lung edema	0	1	0
hemorrhages	0	1	2

2 animals displayed rectal body temperatures over 40°C after challenge infection but only at single time points in the 2 weeks after challenge. Slight pathological alterations were found in 3 animals like focal necrotizing hepatitis and renal hemorrhages (Tab. 8; Suppl. 1).

Tab. 9. Summarized overview about clinical outcome and viral load in RHDV-2 vaccination/challenge trials of rabbits vaccinated with the newly established “recRHDV2-vacc” in comparison to “convRHDV2-vacc”

vaccine	**recRHDV2-vacc**		**convRHDV2-vacc**
challenge with	**RHDV-2**		**RHDV-2**
clinical outcome			
no. of animals	41		23
survived	40		23
died		1	
mean survival time, h	336	34	336
viral load			
RNA, q-RT-PCR; 2^e	0,3	27,8	0,11
viral particle, HA; 2^e	0	12	0
VP60, ELISA; OD	0,05	0,72	0,06

In liver samples of all surviving animals vaccinated with the recombinant or the conventional vaccine candidate no RHDV-2 was detected. In the single “recRHDV2-vacc” immunized animal, which died 34h post challenge, a high amount of RHDV-2 was detected (RNA, viral antigen and viral particles) (Tab. 9).

The induction of a protective humoral immunity after vaccination was combined with a stimulation of both $CD4^+$ and $CD8^+$ T-cells in the blood but with a different pattern: whereas “recRHDV2-vacc” induced a strong increase of both T-cell populations, “convRHDV2-vacc” induced only a $CD4^+$ increase (Fig. 18).

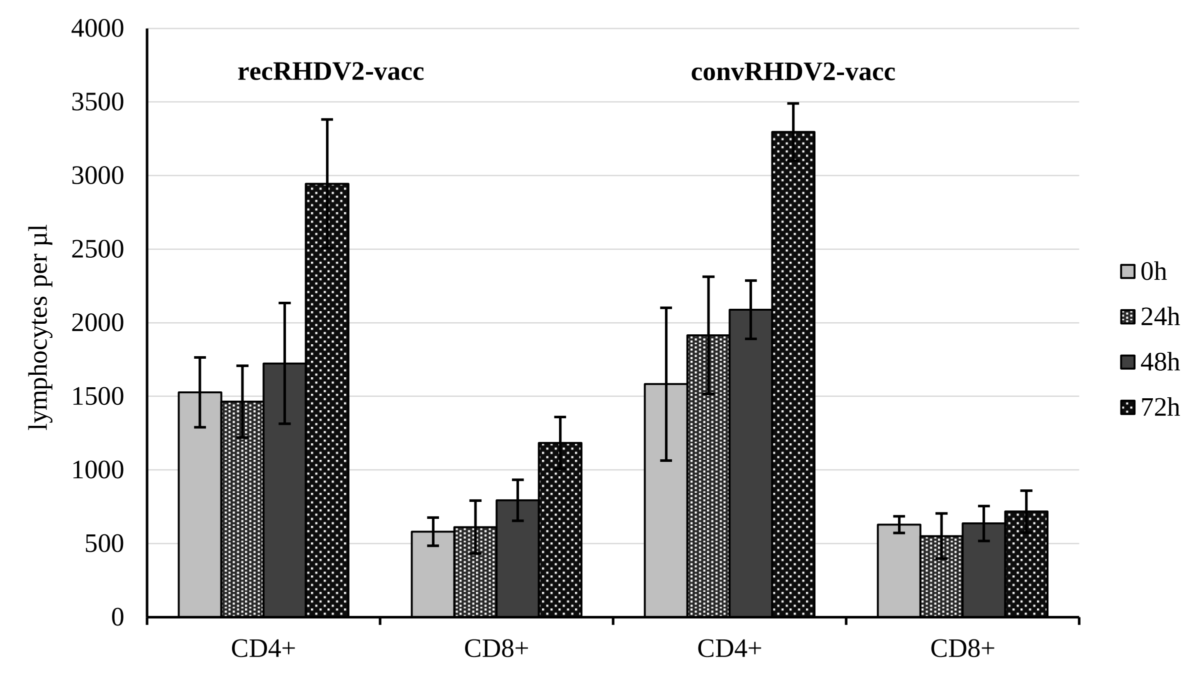

Fig. 18. Kinetics of $CD4^+$ and $CD8^+$ T-cells in blood of rabbits vaccinated with “recRHDV2-vacc” or “convRHDV2-vacc”
(Data kindly provided by Dr. B. Köllner, FLI- Insel Riems)

The vaccination of rabbits with the newly established “recRHDV2-vacc” or with the “convRHDV2-vacc” induced high titers of RHDV-2-VP60 specific antibodies in serum within 14 days post vaccination (for details see 6.4.4.; 6.4.7). In contrast, no RHDV-2 specific antibodies were measured in non-vaccinated or rabbits vaccinated with the “recombinant baculovirus-GFP” preparation.

6.4.4. A low dose of “recRHDV2-vacc” induced protection against RHDV-2 and protective anti-RHDV-2 antibody titers

To determine whether a protective immunity could be induced by vaccination with low doses of the recombinant vaccine, three different doses of “recRHDV2-vacc” with 256, 512 and 1024 HU, respectively, were used to immunize rabbits in comparison to rabbits that received 512 HU of “convRHDV2-vacc” and non-vaccinated rabbits as control. The animals were challenged 14 days post vaccination with RHDV-2. After vaccination blood serum samples were taken weekly for 4 weeks. All surviving rabbits were euthanized two weeks after challenge infection for pathological observation and organ sampling as described.

A protective immune response could be induced already with the lowest dose of “recRHDV2-vacc” of 256 HU 2 weeks after a single immunization. All rabbits vaccinated either with “recRHDV2-vacc” or the “convRHDV2-vacc” survived the homologous challenge with virulent RHDV-2 without any clinical signs of RHD and pathological alterations in inner organs. Neither viral RNA nor viral VP60 or viral particles were detected in livers of vaccinated rabbits at the end of the trial. In contrast, 3 of 4 non-vaccinated rabbits died within 50h after challenge with severe clinical signs, pathological alterations in inner organs and high viral load in the liver (Tab. 6 + 10). The surviving non-vaccinated rabbit developed clinical signs with fever over 40°C but recovered after 4 days. No pathological alterations were found in the liver of that rabbit but viral RNA and even a very low amount of viral capsid (HA titer 1) was still detected 14 days after challenge infection.

Tab. 10. Clinical outcome and viral load in rabbits vaccinated with different doses of “recRHDV2-vacc” in comparison to rabbits vaccinated with “convRHDV2-vacc” and non-vaccinated rabbits after challenge with RHDV-2

vaccine	**recRHDV2-vacc**			**convRHDV2-vacc**	**non-vacc**	
HU per dose	**1024**	**512**	**256**	**512**	**-**	
clinical outcome						
no. of animals	4	4	4	4	4	
survived	4	4	4	4	1	
died	0	0	0	0		3
mean survival time, h	336	336	336	336	336	41
viral load						
RNA, q-RT-PCR; 2^e	0	0	0	0	18,3	32,2
viral particle, HA; 2^e	0	0	0	0	1	12,7
VP60, ELISA; OD	0,06	0,06	0,06	0,07	0,08	1,01

The vaccination with different dosages of “recRHDV2-vacc” induced low titers of RHDV-2 specific antibodies which did not correlate to the dose used for vaccination and which are lower than after vaccination with “convRHDV2-vacc”. These RHDV-2 specific titers increased significantly after challenge with RHDV-2 in all vaccinated rabbits.

In contrast, in sera of non-vaccinated rabbits no RHDV-2 specific antibodies could be detected prior challenge. After challenge, the surviving, non-vaccinated rabbit developed also a high RHDV-2 specific antibody titer comparable to the vaccinated rabbits (Fig. 19).

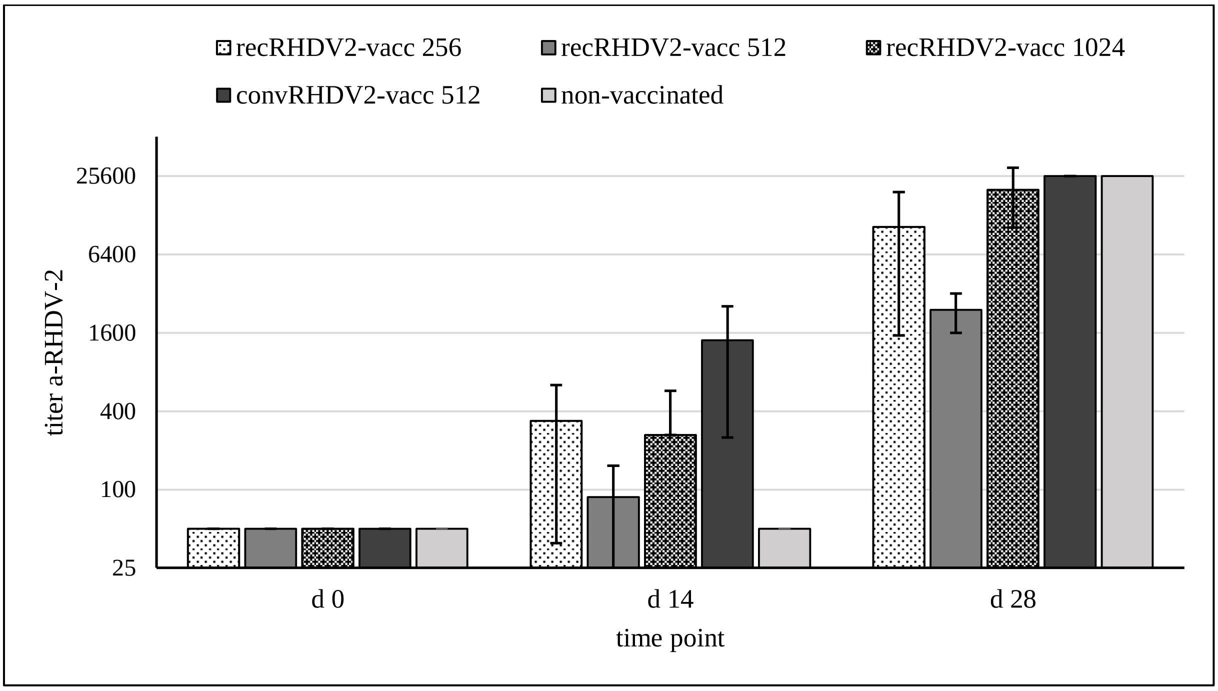

Fig. 19. Anti-RHDV-2 antibody titers in sera of rabbits vaccinated with different dosages of "recRHDV2-vacc" in comparison to rabbits vaccinated with "convRHDV2-vacc" and non-vaccinated rabbits
d 0 = day of vaccination; d 14 = day of challenge; d 28 = end of trial

6.4.5. The protective immune response against RHDV-2 infection was induced already 7 days post vaccination

To elucidate the onset of protective immunity, rabbits were immunized with 1024 HU of "recRHDV2-vacc" or 512 HU of "convRHDV2-vacc" and challenged with RHDV-2 seven days post vaccination. No viral load in liver samples from surviving rabbits was examined after challenge infection as these rabbits were kept for long-term antibody titer observations to determine if an early infection with RHDV-2 shortly after vaccination has an impact on duration of immunity (see 6.4.6.). Blood serum samples were taken weekly for 4 weeks, then monthly.

All rabbits immunized one times either with "recRHDV2-vacc" or with "convRHDV2-vacc" survived the challenge infection with RHDV-2 and developed no clinical signs of RHD.

The 4 non-vaccinated rabbits died between 40 and 64h with severe clinical signs of RHD, pathological alterations in inner organs and comparable high viral load as in the other trials before (Tab. 6 + 11).

Tab. 11. Clinical outcome and viral load in rabbits challenged with RHDV-2 already 7 days post vaccination with “recRHDV2-vacc” in comparison to “convRHDV2-vacc” and non-vaccinated rabbits
n.d. = not determined after the first challenge infection

vaccine	**recRHDV2-vacc**	**convRHDV2-vacc**	**non-vacc**	
clinical outcome				
no. of animals	4	4	4	
survived	4	4	1	
died	0	0		3
mean survival time, h				51
Mean survival time, mths	14	14	14	
viral load				
RNA, q-RT-PCR; 2e	n.d.	n.d.	n.d.	32,5
viral particle, HA; 2e	n.d.	n.d.	n.d.	11
VP60, ELISA; OD	n.d.	n.d.	n.d.	1,12

However, this protection was not correlated in all rabbits with high anti-RHDV-2 antibody titers in sera sampled before challenge. After challenge the titers of RHDV-2 specific antibodies increased significantly (Fig. 20).

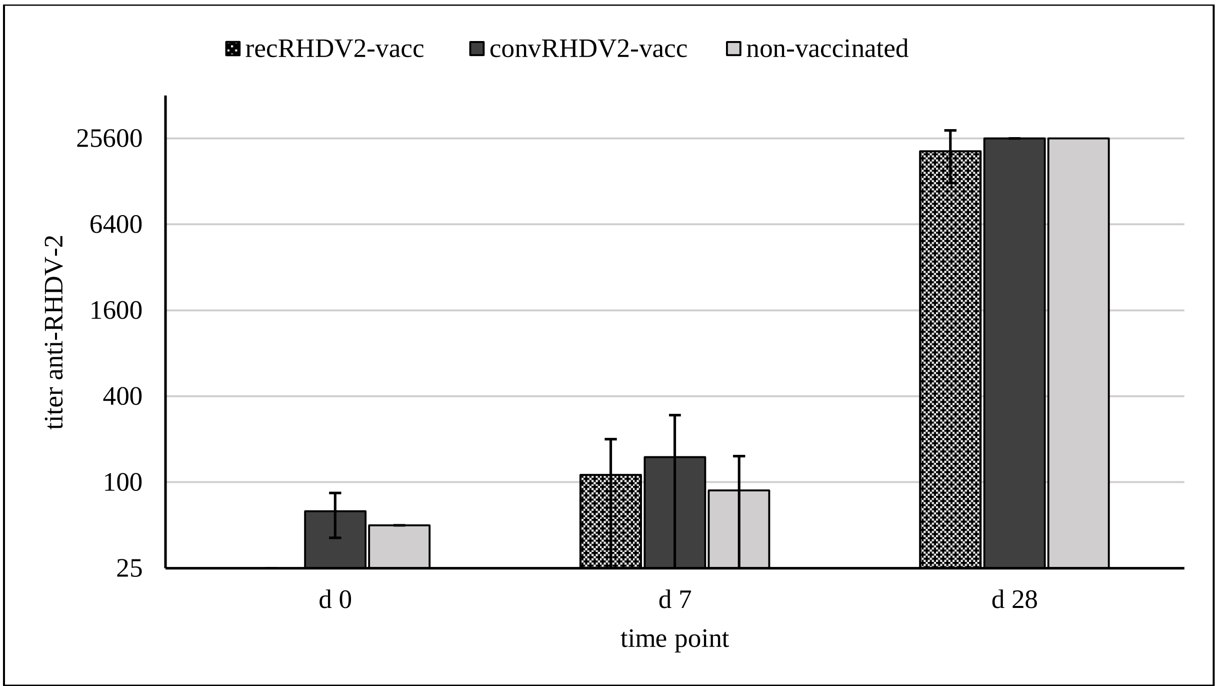

Fig. 20. Anti-RHDV-2 antibody titers in sera of rabbits challenged 7 days after vaccination with “recRHDV2-vacc” in comparison to rabbits vaccinated with “convRHDV2-vacc” and non-vaccinated rabbits
d 0 = day of vaccination; d 7 = day of challenge

6.4.6. A single immunization with the "recRHDV2-vacc" induced a long-lasting immunity against RHDV-2 infection

To evaluate the duration of immunity induced by vaccination with "recRHDV2-vacc", rabbits were immunized and challenged with RHDV-2 either 6 months or 14 months post vaccination. A group of 18 rabbits was vaccinated with 1024 HU of "recRHDV2-vacc", 21 days post vaccination 8 rabbits received a second vaccination with the same vaccine. A group of 10 rabbits vaccinated once with 512 HU of "convRHDV2-vacc" served as positive control and 8 non-vaccinated rabbits served as negative controls. From all rabbits blood serum was sampled weekly over 4 weeks and then monthly for measuring of antibody titers. 6 months post vaccination 4 rabbits of each group were challenged with RHDV-2. The remaining rabbits were challenged 14 months after vaccination. Blood serum samples were collected weekly after challenge infection. Two weeks after challenge infection the rabbits were euthanized for pathological observation and organ sampling as described.

6 months after vaccination all rabbits immunized either once or twice with "recRHDV2-vacc" as well as all rabbits immunized once with "convRHDV2-vacc" survived after challenge infection with RHDV-2 without any clinical signs or pathological alterations in inner organs. In liver samples from all vaccinated rabbits neither viral RNA nor viral proteins or viral particles were detected at the end of the trial (Tab. 12).

Tab. 12. Clinical outcome and viral load in rabbits challenged with RHDV-2 6 months after vaccination once or twice with "recRHDV2-vacc" in comparison to "convRHDV2-vacc" and non-vaccinated rabbits

vaccine	**recRHDV2-vacc**		**convRHDV2-vacc**	**non-vacc**	
vaccination	**1x**	**2x**	**1x**	**-**	
clinical outcome					
no. of animals	4	4	4	4	
survived	4	4	4	1	
died	0	0	0		3
mean survival time, h	336	336	336	336	32
viral load					
RNA, q-RT-PCR; 2^e	0	0,5	0	2,2	32,8
viral particle, HA; 2^e	0	0	0	0	13
VP60, ELISA; OD	0,05	0,05	0,05	0,05	0,96

In contrast, 3 of 4 non-immunized rabbits died within 32h post challenge with displaying typical clinical symptoms before death. Pathological alterations in inner organs (Tab. 6) and a similar high viral load were found as in other non-vaccinated rabbits after challenge infection. The surviving non-vaccinated rabbit displayed typical clinical symptoms but without fever. In the liver of this rabbit no viral load was detected (Tab. 12).

14 months after vaccination all rabbits immunized once with “recRHDV2-vacc” or with “convRHDV2-vacc” survived the homologous challenge infection with RHDV-2 with no clinical signs and pathological alterations in inner organs.
However, all 4 rabbits immunized a second time 21 days after the first vaccination developed clinical signs like apathy and low food uptake and one of them died 34h after challenge infection with RHDV-2 without fever. Typical pathological alterations in inner organs were detectable (Tab. 8). In the surviving twice-vaccinated rabbits, no typical pathological alterations were found and there was no indication for replication of RHDV-2 challenge virus as neither viral RNA nor viral VP60 or viral particles were detected in livers (Tab. 13). However, in liver samples of the vaccinated, deceased rabbit a high viral load (viral RNA, viral protein and particles) was measured (Tab. 13).
All 4 non-vaccinated rabbits died within 30-34h after challenge with severe clinical signs, pathological alterations and high viral load in the liver (Tab. 6 + 13).

Tab. 13. Clinical outcome and viral load in rabbits challenged with RHDV-2 14 months after vaccination once or twice with “recRHDV2-vacc” in comparison to “convRHDV2-vacc” and non-vaccinated rabbits
Note: Due to losses of rabbits during the year, at time of challenge only 4 two-time “recRHDV2-vacc” vaccinated rabbits and 3 “convRHDV2-vacc” immunized rabbits were left at time of challenge

vaccine	**recRHDV2-vacc**			**convRHDV2-vacc**	**non-vacc**
vaccination	**1x**	**2x**	**2x**	**1x**	**-**
clinical outcome					
survived	5	3		3	0
died	0		1	0	4
mean survival time, h	336	336	36	336	34
viral load					
RNA, q-RT-PCR; 2^e	0,8	0	27,8	0	27,3
viral particle, HA, 2^e	0	0	12	0	12
VP60, ELISA, OD	0,05	0,05	0,72	0,04	0,74

The kinetics of RHDV-2 specific antibody titers was measured in ELISA using the sera of these rabbits sampled over 6 or 14 months, respectively. In both “recRHDV2-vacc” groups and the “convRHDV2-vacc” group titers rose steadily over a time of approximately 2 months after the first vaccination. Between month 2 and 5 a slight decline of titers began which continued for the next 14 months (Fig. 21 + 22). Nonetheless, almost all rabbits of all three vaccinated groups still had protective antibody levels 6 months after the first vaccination and survived a challenge infection with RHDV-2. One exception was a rabbit that received a prime-boost vaccination and did not show antibody titers anymore at the time of challenge infection 6 months later (data not shown) but still survived without signs of RHD.

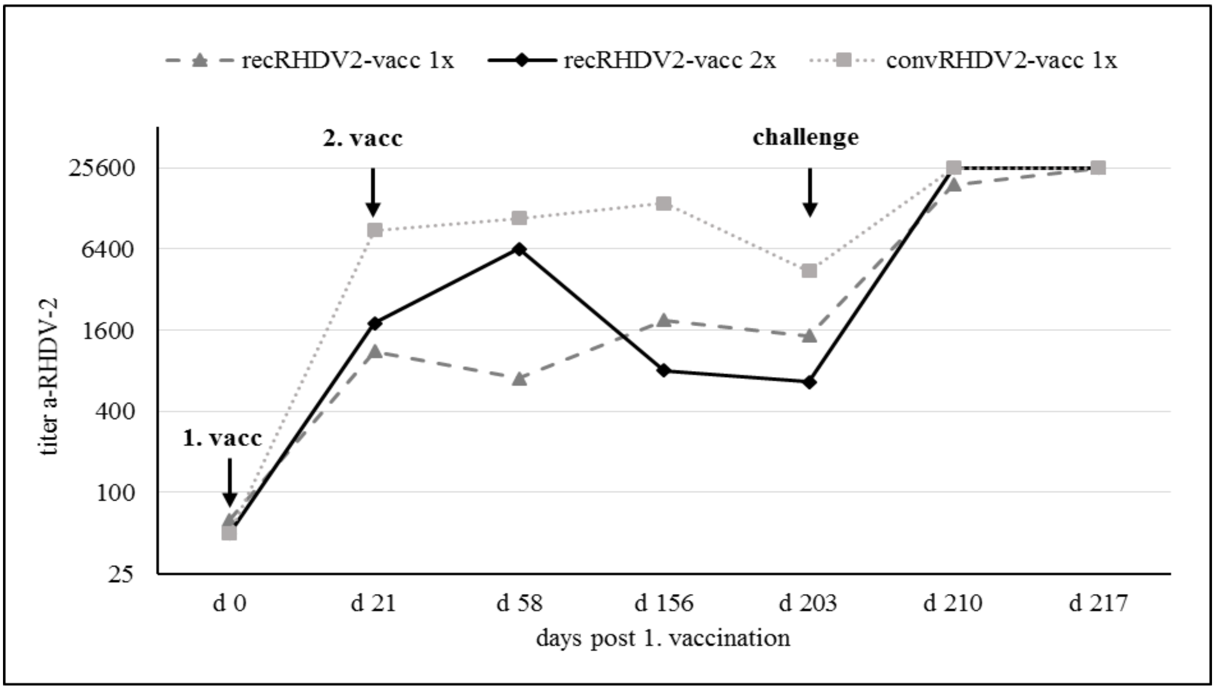

Fig. 21. Long-term observation over 6 months of anti-RHDV-2 antibody titers in sera of rabbits after vaccination once or twice with "recRHDV2-vacc" in comparison to rabbits vaccinated with "convRHDV2-vacc" once

d 0 + d 21 = vaccination; d 203 = challenge after 6 month observation; d 217 = end of trial

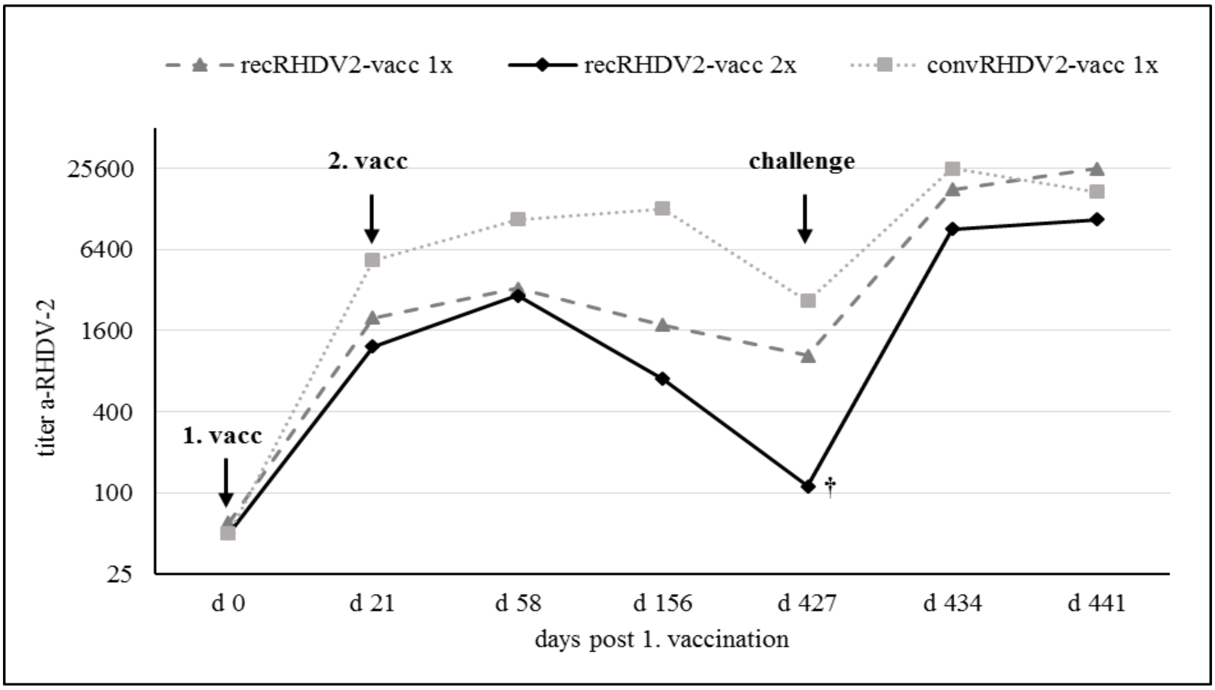

Fig. 22. Long-term observation over 14 months of anti-RHDV-2 antibody titers in sera of rabbits after vaccination once or twice with "recRHDV2-vacc" in comparison to rabbits vaccinated with "convRHDV2-vacc" once

d 0 + d 21 = vaccination; d 427 = challenge after 14 month observation; d 441 = end of trial

Note: One rabbit which received a prime-boost vaccination did not have any RHDV-2 specific antibody titers at day 427 before challenge and died after challenge with RHDV-2

†: death of one 2x "recRHDV2-vacc" vaccinated rabbit

Generally, in sera of twice “recRHDV2-vacc” immunized rabbits a stronger decline of RHDV-2 specific antibody titers was measured in comparison to once “recRHDV2-vacc” and “convRHDV2-vacc” immunized rabbits (Fig. 22). The “recRHDV2-vacc” vaccinated rabbit which did not survive the challenge infection after 14 months had no RHDV-2 specific serum antibodies at the time of challenge infection (data not shown).

These rabbits were compared with once “recRHDV2-vacc” or “convRHDV2-vacc” vaccinated animals that received an early infection with RHDV-2 seven days after vaccination (see 6.4.5). After a second RHDV-2 infection 14 months after the first, all rabbits survived without clinical symptoms and pathological alterations. No viral load was detected in the livers of those rabbits (Tab. 14).

Tab. 14. Clinical outcome and viral load in rabbits challenged with RHDV-2 14 months after a single vaccination with “recRHDV2-vacc” with first challenge infection 7 days after vaccination and a second challenge infection 14 months later in comparison to “convRHDV2-vacc” and non-vaccinated rabbits

vaccine	**recRHDV2-vacc**	**convRHDV2-vacc**	**non-vacc surv.**	**non-vacc**
clinical outcome				
no. of animals	4	4		4
survived	4	4	1	0
died	0	0		4
mean survival time, h	336	336	336	34
viral load				
RNA, q-RT-PCR; 2e	0	0	0	27,3
viral particle, HA; 2e	0	0	0	12
VP60, ELISA; OD	0,05	0,04	0,05	0,74

Rabbits of both vaccine groups developed high antibody titers. These titers did not decline from month 2. At the time of the second challenge infection all rabbits still had protective antibody titers (Fig. 23).

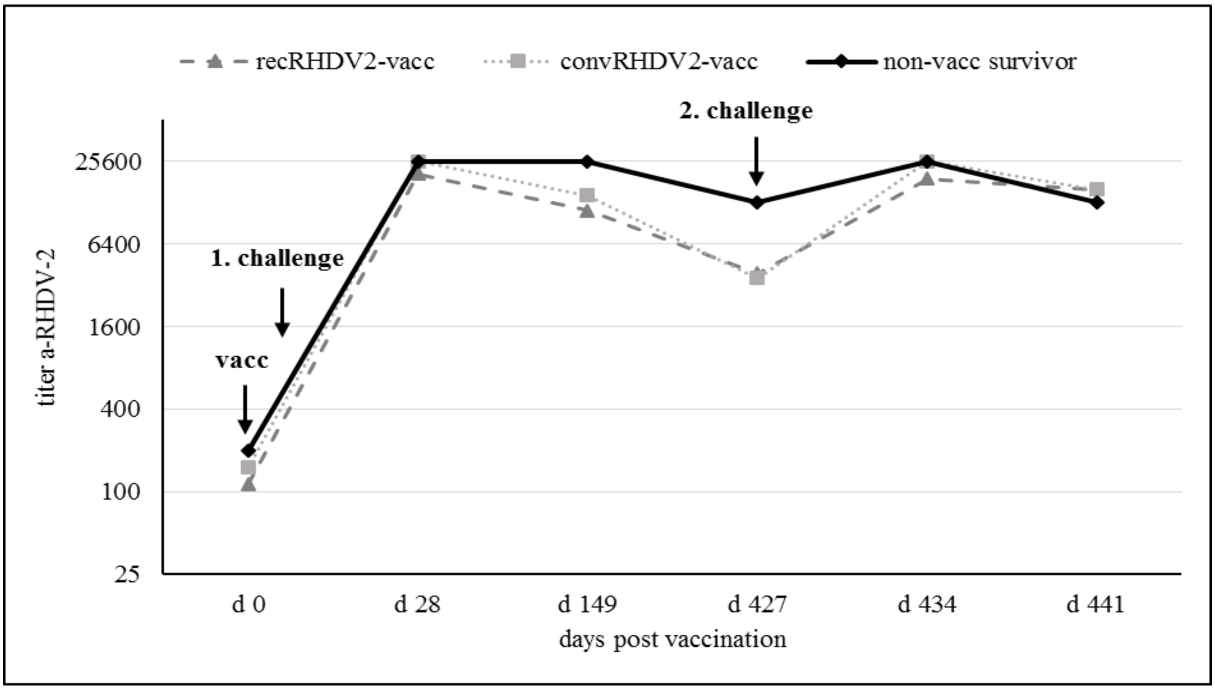

Fig. 23. Long-term observation over 14 months of anti-RHDV-2 antibody titers in sera of rabbits after a single vaccination with "recRHDV2-vacc" with first challenge infection 7 days after vaccination and second challenge infection 14 months later in comparison to rabbits vaccinated with "convRHDV2-vacc"
d 0 = vaccination; d 28 = 21 days after challenge infection with RHDV-2; d 427 = 2. challenge infection with RHDV-2 after 14 month observation; d 441 = end of trial

6.4.7. A limited cross-protection against heterologous RHDV-1 challenge was induced by a single vaccination with "recRHDV2-vacc"

To determine whether the "recRHDV2-vacc" provides a cross-protective immunity, two groups of 4 rabbits each were vaccinated with 1024 HU "recRHDV2-vacc" or 512 HU of "convRHDV2-vacc", respectively. Two groups of 4 non-vaccinated rabbits served as controls. After vaccination, blood serum samples were taken weekly over 4 weeks. Each vaccinated group and control group was challenged with either homologous RHDV-2 or heterologous RHDV-1, respectively, and the course of the disease was monitored over 14 days after challenge infection.

All 4 rabbits vaccinated either with the "recRHDV2-vacc" or with the "convRHDV2-vacc", survived the homologous challenge infection with RHDV-2 and developed no clinical signs or pathological alterations in inner organs. In contrast, only 2 rabbits of the "recRHDV2-vacc" group and 3 rabbits of the "convRHDV2-vacc" group survived the heterologous challenge infection with RHDV-1. Of the non-vaccinated rabbits all animals died after RHDV-2 infection within 90h and 3 of 4 rabbits died after RHDV-1 challenge infection within 52h.

All of the rabbits which did not survive the challenge infections developed severe clinical signs of RHD (fever, reduced food uptake, apathy) and displayed typical pathological alterations in inner organs whether they were vaccinated or not. In vaccinated rabbits that survived the heterologous challenge, clinical signs and pathological alterations in inner organs were recorded also, but with reduced severity. Whereas no viral load was measured after challenge infection with RHDV-2 in livers of all vaccinated rabbits, RHDV-1 RNA was detected in livers of vaccinated rabbits after heterologous challenge. However, viral particles or viral VP60 were only detected in rabbits which died after infection. In livers of the non-immunized rabbits high viral loads of RHDV-2 or RHDV-1, respectively, were found after the challenge infections (Tab. 15).

Tab. 15. Clinical outcome and viral load in rabbits after heterologous challenge with RHDV-1 or homologous challenge with RHDV-2 after vaccination with "recRHDV2-vacc" in comparison to "convRHDV2-vacc" and non-vaccinated rabbits

vaccine	**recRHDV2-vacc**			**convRHDV2-vacc**			**non-vacc**		
challenge with	**RHDV-2**	**RHDV-1**		**RHDV-2**	**RHDV-1**		**RHDV-2**	**RHDV-1**	
clinical outcome									
no. of animals	4	4		4	4		4	4	
survived	4	2		4	3		0	1	
died	0		2	0		1	4		3
mean survival time, h	336	336	48	336	336	42	52	336	45
viral load									
RNA, q-RT-PCR; 2e	0	9,5	32,3	0	8,1	33,1	31,9	8,8	32,5
viral particle, HA; 2e	0	0	12,5	0	0	13	10,8	0	12
VP60, ELISA; OD	0,05	0,07	1,72	0,06	0,07	1,61	1,33	0,06	1,4

As in the earlier experiments, in all sera of the vaccinated rabbits, high titers of specific anti-RHDV-2 antibodies were measured after vaccination (Tab. 24) and challenge infection with RHDV-2 (data not shown). However, after vaccination with "recRHDV2-vacc" as well as "convRHDV2-vacc", only low titers of RHDV-1 cross-reactive antibodies were measured. "ConvRHDV2-vacc" was able to induce slightly higher amounts of cross-reactive antibodies than "recRHDV2-vacc" (Fig. 24).

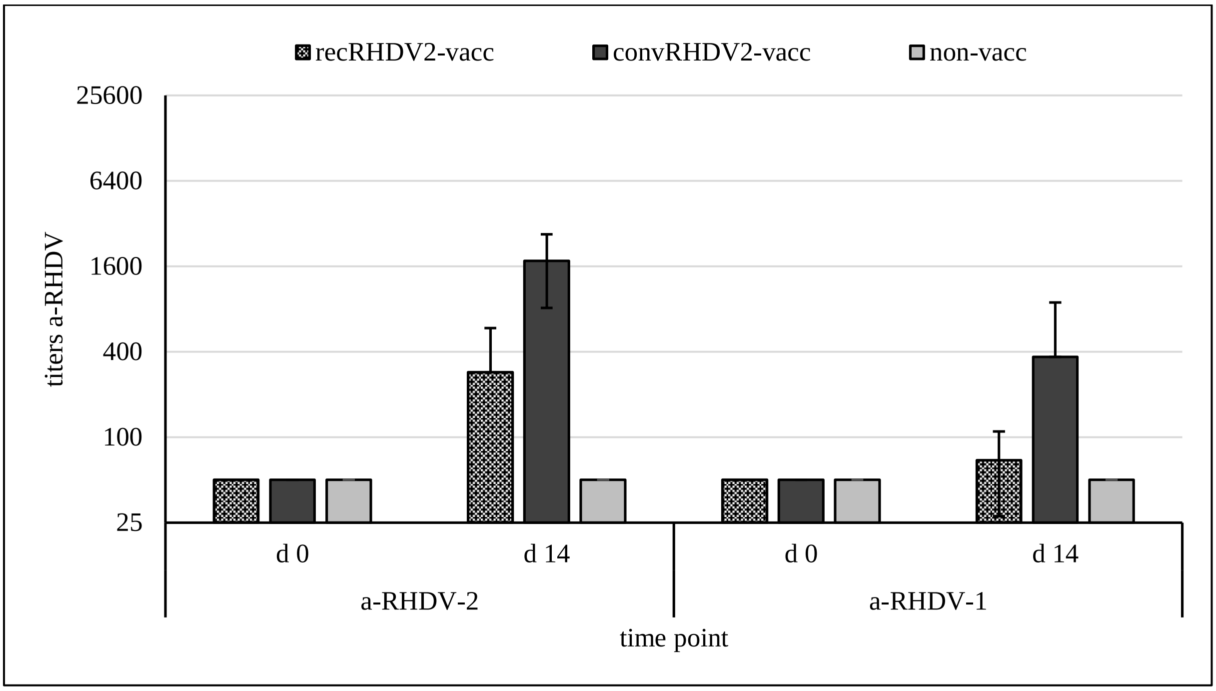

Fig. 24. Anti-RHDV-2 and anti-RHDV-1 antibody titers in sera of rabbits challenged with RHDV-2 14 days after vaccination with "recRHDV2-vacc" in comparison to rabbits vaccinated with "convRHDV2-vacc" or non-vaccinated rabbits

d 0: day of vaccination; d 14: day of challenge

7. Discussion:

Since in 1984 a newly emerging virus infection killed several millions of rabbits in commercial husbandries in China (Liu et al., 1984; Xu and Chen, 1989; Xu, 1991), it was clear that there is a strong need for effective vaccines to protect rabbits against this severe virus induced hepatitis with up to 100% mortality. However, all approaches to develop a cell culture based vaccine failed because the new Calicivirus could not be cultivated in cell culture (Granzow et al., 1989; Ohlinger et al., 1989; Ohlinger et al., 1990; Parra and Prieto, 1990; Meyers et al., 1991; Moussa et al., 1992). Therefore, vaccines were developed based on an inactivated virus suspension prepared from liver material of rabbits infected with RHDV-1. These vaccines were very effective and even the variability of RHDV-1 did not affect the success of these vaccines (Argüello-Villares, 1991; Smíd et al., 1991; Schirrmeier et al., 1999). However, this approach to infect and kill rabbits after induction of a severe hepatitis to produce a vaccine to protect other rabbits is not only a critical ethical issue but has also the disadvantage of the transfer of allogeneic material and of potentially remaining infectivity in the inactivated vaccine.

Since 1994 experimental vaccines based on recombinant VP60, the capsid protein of RHDV, have been developed and tested (Laurent et al., 1994). However, so far, only one recombinant anti-RHDV-1 vaccine based on a myxoma virus vector that expresses RHDV-1-VP60 is available on the market (“Nobivac Myxo-RHD”; Intervet International BV, Netherlands). The appearance of new strains of RHDV-1 with a different virulence and the emergence of the new variant RHDV-2 in 2010 in France (Le Gall-Reculé et al., 2013), which causes significant losses even in RHDV-1-vaccinated rabbits, underlines the requirement of further developments for improved vaccines. Therefore, the task of the presented thesis was (a) the development of an effective recombinant vaccine to protect rabbits against the new RHDV-2, (b) to characterize the onset, duration and possible cross-protection of the newly developed vaccine and (c) to evaluate some correlates of protection (RHDV-2 specific antibodies and cellular effectors) after vaccination with the newly developed vaccine.

7.1. Construction of recombinant RHDV-2-VP60

In all available RHDV vaccines the viral capsid protein VP60 is the main immunogenic component to induce a protective immune memory. This is proven by the kinetics of VP60 specific antibodies in sera of vaccinated rabbits which correlates with protection following challenge infection (Parra and Prieto, 1990; Laurent et al., 1994; Marín et al., 1995).

Since VP60 of classical RHDV-1 and its variants expressed by different vector systems like baculovirus (Laurent et al., 1994; Marín et al., 1995; Nagesha et al., 1995; Plana-Duran et al., 1996; Gromadzka et al., 2006; López-Vidal et al., 2015), E.coli (Boga et al., 1994; Guo et al., 2016), adenovirus (Fernández et al., 2011), vaccinia virus (Bertagnoli et al., 1996b), myxoma virus (Bertagnoli et al., 1996a; Bárcena et al., 2000), ORF virus (Rohde et al., 2011), yeast (Farnós et al., 2005), or Canarypox virus (Fischer et al., 1997) induced protective antibodies in vaccinated rabbits, in the present thesis a comparable cloning and expression strategy was used. Moreover, the advantage of the chosen baculovirus expression system is the disability of baculoviruses to replicate in mammalian cells (Hu, 2005) which increases the safety of recombinant vaccines in mammals. As shown for other capsid proteins of viruses that could not be cultivated in cell culture, like human papilloma virus or hepatitis C virus (Kost et al., 2005), the expression by recombinant baculoviruses can lead to self-assembly into highly immunogenic virus like particles (VLPs).

Another point for the decision to establish a baculovirus based expression system for the production of recombinant RHDV-2-VP60-VLPs was the need of a cost-effective vaccine to replace the ethically critical production of RHDV by infected rabbits. Such a vaccine approach was also used for recently established recombinant RHDV-1 vaccines (Gao et al., 2013; López-Vidal et al., 2015). Finally, recombinant baculovirus-expressed VLPs can be produced in high yields also for the use in diagnostic tests (Kost et al., 2005).

For the cloning and construction of the recombinant VP60, the sequence of the RHDV-2 strain 10-05 (GenBank FR819781) from the first outbreak in France in 2010 (Le Gall-Reculé et al., 2013), defined as reference strain, was used. Several other RHDV-2 isolates were shown to be very closely related with minimal sequence variation in the VP60 protein but quite different from RHDV-1 isolates (Le Gall-Reculé et al., 2013). These antigenic differences stress the need for a RHDV-2 vaccine.

To provide an optimal regulation of the RHDV-2-VP60 expression in different cell cultures, two promoter systems, the promotor P10 for VP60 expression in insect cells (SF9) and the CAG(GS) enhancer/promotor element for VP60 expression in rabbit kidney (RK13) cells were chosen with regard to a later possible commercial use of the vaccine, and also to ensure higher expression rates and to gain higher yields of VP60. The very late promotor P10 of baculoviruses is a commonly used promotor in baculovirus expression systems (van Oers et al., 2015) for protein expression in insect cells, and was proven to be very effective. The CAG(GS) enhancer/promotor element was developed for high yield protein expression in mammalian cells using recombinant baculoviruses (Shoji et al., 1997; Hu, 2005; Keil et al., 2009, 2016). The strategy used in this thesis resulted in the construction of four recombinant baculoviruses expressing RHDV-2-VP60 with no significant differences in baculovirus titers. Therefore, all four were tested in comparison for the quantitative and qualitative expression of RHDV-2-VP60 in the chosen cell systems.

7.2. Influence of the baculovirus construction on RHDV-2-VP60 expression

7.2.1. Influence of chosen promotors

Both promotors induced the expression of RHDV-2-VP60 in SF9 or RK13 cells, respectively. The expression of viral proteins as early as 24h after transduction was also shown for other viruses in different mammalian cells (Kost et al., 2007; Keil et al., 2009).

The comparative quantitative analysis of the two different promotors used for RHDV-2-VP60 expression in the respective cell system confirmed that expression under the promotor P10 in SF9 cells is more efficient than expression under the enhancer/promotor element CAG(GS) in RK13 cells. Additionally, the higher MOI necessary for the transfection of RK13 cells indicated a limited efficacy of VP60 expression. This is in accordance with the lower protein expression for capsid protein VP6 of rotaviruses after transfection of human embryonic kidney cells in comparison to infection of SF9 cells (Da Silva Junior et al., 2012).

7.2.2. Influence of the codon usage

To optimize protein expression rates, two different codon usages were tested. The classical baculovirus codon usage “AcMNPV” was chosen because native baculoviruses replicate with high efficiency in SF9 cells and it has been proven to be efficient regarding protein expression with recombinant baculoviruses in insect cells (Hu, 2005). The “BHV-1” codon usage was chosen because BHV-1 replicates efficiently in RK13 cells, the envisaged target cell line for transduction with RHDV-2-VP60 baculoviruses. Moreover, previous experience had shown that this CU adaptation leads to increased expression levels of proteins encoded by RNA viruses (Kühnle et al., 1998; Schmitt et al., 1999).

The comparative analysis of both codon usages for the ORFs of the RHDV-2-VP60 revealed no general influence on the expression of RHDV-2-VP60 in both cell systems (SF9, RK13) as increasing amounts of complete VP60 were detected. Therefore, both codon usages proved to be sufficient to gain high amounts of RHDV-2-VP60 in both cell systems. Similar observations were made in studies for the expression of BHV-1 glycoprotein D (Keil et al., 2009) and indicate that translation efficiency may not be solely dependent on the codon usage (Menzella, 2011).

The nature of minor bands of about 36kDa appearing 72h after infection irrespective of the codon usage in SF9 cells (see 6.2; Fig. 10) is not clear, although the polyclonal rabbit anti-RHDV-2-VP60 serum used for detection of VP60 in Western Blot analysis indicated that these fragments likely are VP60 related. The phenomenon of degraded VP60 in SF9 cells has been discussed earlier (Marín et al., 1995), but a negative effect in immunogenicity was excluded. Protein degradation using baculovirus expression systems was correlated with the lytic replication of baculoviruses in SF9 cells which negatively influences the correct expression and folding of recombinant proteins (Ho et al., 2004). The baculoviruses used in this thesis displayed a lysis of SF9 cells especially after longer incubation. This was not seen after transduction of RK13 cells most likely because baculoviruses are unable to lyse RK13 cells.

As all of these proteins were detected by a polyclonal RHDV-2-VP60 specific rabbit antiserum in Western Blot analysis one can assume that they still contain epitopes which also induce an anti-VP60 specific immune response. Hypervariable motifs in RHDV-VP60 are distributed over the whole protein and 7 regions (V1-V7) were identified as being important for immunogenicity (Wang et al., 2013b). Using RHDV type specific monoclonal antibodies antigenic differences between RHDV-1 and RHDV-2 but also possible overlapping epitopes could be defined (Le Gall-Reculé et al., 2013). This indicates that not just one part of VP60 is responsible for the induction of VP60 specific antibodies. Whether degraded VP60 proteins might interfere with the induction of a protective humoral immunity in rabbits after vaccination is unclear.

Since the expression level of RHDV-2-VP60 “AcMNPV” was slightly higher in SF9 (BacBac-A) as well as in RK13 (BacMam-A) cells, both preparations were selected for a “proof of principle” vaccination/challenge experiment to investigate the induction of a protective immune response against RHDV-2 infection.

7.2.3. Generation of RHDV-2-VP60-VLPs

For many capsid proteins e.g. from Norwalk-virus, Feline Calicivirus or Canine Parvovirus self-assembly to empty virus like particles (VLP), which do not contain viral genetic material, occurs spontaneously (Green et al., 1993; Di Martino et al., 2006; Jin et al., 2016). Moreover, in immunization experiments a higher immunogenicity of assembled compared to non-assembled capsid proteins was demonstrated (Grgagic and Anderson, 2006; Chen and Lai, 2013). Self-assembly to VLPs was also found for VP60 of RHDV-1 (Laurent et al., 1994;

Nagesha et al., 1995; Gromadzka et al., 2006) and RHDV-2 (Bárcena et al., 2015), which, however was not observed in all studies using recombinant baculoviruses (Marín et al., 1995).
As demonstrated by electron microscopy and analyzed quantitatively by HA tests, all four recombinant RHDV-2-VP60 assembled to VLPs. This indicates that the two different codon usages to generate the synthetic RHDV-2-VP60 ORF in combination with two different promotors for the expression in two different cell culture systems had no detectable influence on the self-assembly to VLPs. This revealed that the VP60 gene cassette within the different vectors was translated completely to the correct VP60, independent from the cell system. VLPs of RHDV-1 were shown to induce a protective immunity in rabbits after vaccination (Nagesha et al., 1995; Gao et al., 2013; Guo et al., 2016). Therefore, it was expected to find a similar induction of protective immunity after vaccination with the two recombinant baculoviruses expressing RHDV-2-VP60 (BacBac-A and BacMam-A).

7.3. Induction of protective immunity after vaccination with the newly established RHDV-2-VP60 vaccine

7.3.1. General findings after vaccination – proof of principle trial

As RHDV-2 is a highly virulent virus, beside strict hygiene management, vaccination of rabbits is the only tool to protect rabbits against the Rabbit hemorrhagic disease. Therefore, vaccines that induce a long-lasting immunity against RHDV-2 are desired (Le Gall-Reculé et al., 2013; Bárcena et al., 2015).
The protective capacity against RHDV-2 infection of the newly established recombinant vaccines was analyzed in rabbits immunized once or twice with the two recombinant vaccine candidates. Obviously, the vaccination with “recRHDV2-vacc” provides a protection against challenge infection with RHDV-2 proved by survival and absence of RHD specific clinical symptoms. This clinical outcome after vaccination with both recombinant RHDV-2 vaccines was comparable to the protection induced by a conventional liver-derived vaccine “convRHDV2-vacc”. The protective potential of recombinant VP60 was known from other recombinant vaccines generated with baculoviruses against classical RHDV-1 after prime or prime-boost vaccinations (Plana-Duran et al., 1996; Fernández-Fernández et al., 2001; Guo et al., 2016).
The increased body temperature measured in 4 of 41 rabbits vaccinated with “recRHDV2-vacc” and in 2 of 23 rabbits vaccinated with “convRHDV2-vacc” at single time points could not be correlated to other RHDV infection related clinical signs and was most presumably related e.g. to individual handling stress responses. Also mild histopathological alterations

detected in some vaccinated rabbits could not be associated with RHDV infection. In livers of 3 rabbits immunized with “convRHDV2-vacc”, sampled 14 days post challenge, more RHD typical pathological alterations were found, although these rabbits survived without visible clinical signs after challenge infection. Whether these pathological alterations in the liver were vaccination-induced, due to the RHDV challenge infection or even induced by unknown pathological processes, could not be verified. The fact that at the time of sampling two weeks after RHDV-2 challenge infection no viral loads (RHDV-2 RNA, viral protein or capsids) were detected in all of these animals might indicate that RHDV induced pathological changes in liver need a longer time to be completely healed. Furthermore, the absence of clinical signs (fever, apathy etc.) not necessarily excludes, that a viral infection of liver cells followed by pathological alterations happened before the RHDV is eliminated by the vaccine induced immunity. Continuing viral replication in RHDV-vaccinated or in RHDV-infected, but surviving rabbits have been reported. Severe clinical courses of the disease or even a RHDV infection induced mortality in vaccinated rabbits was rarely observed (Plana Duran et al., 1996; Guo et al., 2016).

The immunogenicity measured by the induced VP60 specific serum antibody titers showed no significant differences between both recombinant vaccines (BacBac-A or BacMam-A). The differences in the induction of specific anti-RHDV-2 serum antibodies by “convRHDV2-vacc” indicated a higher amount of either VP60 or of additional viral components. The comparable antigen titers in all vaccines determined by the HA test only quantifies the amount of VLP or viral particles in the vaccine. Further, VP60-derived antigenic structures could also induce VP60 specific antibodies after vaccination. Whether such antibodies provide an antiviral activity after infection is not clear. This was also measured in several other studies were a strong induction of RHDV specific serum antibodies was especially measured after vaccination with comparable conventionally prepared vaccines (Laurent et al., 1994; Plana-Duran et al., 1996; Fernández-Fernández et al., 2001).

The overall strong $CD8^+$ T-cell activation by the recombinant RHDV-2 vaccines could be an advantage for an early effective protection against RHDV-2, because surviving non-vaccinated rabbits display also a very strong $CD4^+$ and $CD8^+$ T-cell activation. T-cell activation was confirmed before for the recombinant RHDV-1-VP60 vaccine candidates as well as for liver-derived vaccines. However, no differentiation between $CD4^+$ and $CD8^+$ T-cells was made, whereas a general advantage of recombinant vaccine candidates over conventional vaccines in the stimulation of T-cell effector mechanisms also has to be further investigated (Guo et al., 2016).

As expected, the anti-RHDV-1 vaccine “Cunivak RHD” was not able to induce a sufficient protective immune response against RHDV-2 challenge in once immunized rabbits. The severe clinical symptoms and the induced mortality clearly showed that the induced anti-RHDV-1 antibodies were not able to efficiently neutralize RHDV-2. Furthermore, that vaccination did not appear to strengthen innate immune mechanisms. However, twice vaccinated rabbits survived without detectable clinical symptoms but with still measureable viral load (RHDV-2 RNA) in livers sampled 14 days post infection. This indicates that an only partial cross-protection was induced by prime-boost vaccination with the anti-RHDV-1 “Cunivak RHD” vaccine. The molecular basis of a possible cross-protection was analyzed after the emergence of the new RHDV-2 virus by comparative analysis of the VP60 of different RHDV strains. In 7 hypervariable regions distributed over the VP60 protein remarkable differences were detected. Using specific monoclonal antibodies against different RHDV variants, possible cross-reactive epitopes were also defined (Le-Gall Reculé et al., 2013). Very recently, it was found that the RHDV-1 strain K5 was able to break the immunity induced in wild rabbits that survived an infection with the Czech RHDV-1 strain V351 in Australia (www.pestsmart.org.au). This is a first hint of a necessary continued adaptation of anti-RHDV vaccines to recent circulating virus variants.

In summary, both newly developed recombinant RHDV-2 vaccine candidates (BacBac-A; BacMam-A) were able to induce an efficient protection against RHDV-2 infection. This reflects the high immunogenicity of these recombinant RHDV-2 vaccines due to the high content of self-assembled VLPs. Furthermore, it reassures the expectation from former studies that an immunization using recombinant RHDV-1-VP60 vaccines induced a protection, especially when a self-assembly of the expressed VP60 to VLPs was detected (Laurent et al., 1994; Plana-Duran et al., 1996; Gao et al., 2013).

7.3.2. General findings in “non-vaccinated” or in “recbacGFP” vaccinated rabbits

To assess the protective potential of the newly established vaccine in more detail a comparative analysis with non-vaccinated animals was necessary. For the recombinant RHDV-2-VP60 vaccines a further control was used, especially to determine the possible influence of recombinant baculovirus particles itself.

After analyzing the clinical course of the disease in non-vaccinated control animals (24 rabbits), the fast acting character of the disease was confirmed. The total mortality in this study with 21 of 24 (=88%) non-vaccinated, i.m. infected rabbits was even higher than described in a comparatively study earlier (Le Gall-Reculé et al, 2013). There in 3 experiments 5 of 12 (=42%) i.m. infected rabbits died. The high mortality in the present study might be induced by a higher challenge dose but also indicates the differences in virulence of different RHDV-2 strains.

The severe character of the induced RHD after infection with RHDV-2 is also proofen by the very short mean survival time of just 41 hours after infection, with displaying typical symptoms of RHD, prominent severe pathological alterations in inner organs, high viral loads in the liver and depletion of leukocytes in these 21 rabbits that died after infection. Similar findings are described for RHDV-1 or RHDV-2 in non-vaccinated rabbits (Prieto et al., 2000; Ferreira et al., 2006; Abrantes et al., 2012; Le Gall-Reculé et al., 2013). The survival time is too short to expect a humoral antibody response in sera of rabbits which died after infection very quickly. However, the cellular immune response or better the influence of the infection on the leukocytes was measured in different studies (Ferreira et al., 2005, 2006). Similar to the reported results a quick and severe depletion after an initial increase of both $CD4^+$ and $CD8^+$ T-cell populations were measured in this study in non-vaccinated rabbits which died after infection. Especially the $CD8^+$ T-cells were not measurable in about 50% of all investigated rabbits shortly before death, indicating (a) the impact of RHDV induced pathological processes of this cell population and (b) a possible involvement in protective immune responses in naïve rabbits which survive. The depletion of both T-cell populations was shown to be related to apoptosis and one reason of the very rapid fatal progress with high mortality after RHDV infection in naïve rabbits (Ferreira et al., 2006).

A completely different picture was seen in the three surviving, non-vaccinated rabbits. Although 2 of 3 reacted with high fever, all three finally survived after displaying mild clinical signs but with no pathological alterations in organs sampled 14 days post infection.

These rabbits displayed a more effective cellular immunity after challenge infection. The complete depletion of $CD8^+$ T-cells as found in moribund rabbits was not observed and especially $CD4^+$, $CD8^+$ T-cells, which display the regulatory phenotype, were increased in all three surviving animals. A similar response was reported in rabbits surviving a RHDV-1 infection which had significantly increased interferon (IFN) γ levels in the liver. Finally, an early activation of B- and T-cell and macrophages as well as pro-inflammatory cytokines like IFNα and IFNγ, as is seen in young RHDV-1 resistant rabbits (Ferreira et al., 2005; Marques et al., 2012) could have been induced also in naïve rabbits surviving the RHDV-2 challenge infection in this study. This would explain the significantly decreased viral load, especially the very low HA titers, indicating that the replication of RHDV-2 is blocked.

A further difference, which is discussed for resistant rabbits, is a different HBGA pattern resulting in a lower susceptibility of host cell populations in the liver. Therefore, the infection of such cells is less effective or impossible which would result in a much lower replication level in the whole organ (Nyström et al., 2011; Le Pendu et al., 2014). The lower infection pressure would allow activating necessary immune mechanisms and would result in decreased damage of liver tissue. This was exactly found in naïve rabbits surviving the infection.
Because of the similar induction of a protective humoral anti-RHDV-2 immune response, the "BacBac-A" SF9 cell-derived vaccine (further referred to as "recRHDV2-vacc") was used in all following experiments to characterize the onset, duration or cross-protection.

7.3.3. Determination of minimal protective vaccine dose

To determine the minimal protective dose three different doses of "recRHDV2-vacc" were used for single vaccinations of rabbits followed by a challenge infection 14 days later. The high potency of "recRHDV2-vacc" was demonstrated by the fact that even the rabbits vaccinated with the lowest dose of 256 HU developed high titers of RHDV-2-VP60 specific antibodies and survived the challenge infection without any clinical signs and without detectable viral replication in liver. This correlates with previous studies where rabbits vaccinated with comparable low doses of either inactivated RHDV virus or recombinant VP60 expressed by recombinant baculoviruses (Argüello-Villares, 1991; Smíd et al., 1991; Laurent et al, 1994; Nagesha et al., 1995) survived following challenge infections.
Whether even a lower dose would have induced similar protection was not tested. Interestingly, the induced titers of RHDV-2-VP60 specific antibodies did not correlate directly with the used vaccine dose as reported for RHDV-1-VP60 (Marín et al., 1995; Plana-Duran et al., 1996). As mentioned above, the increased number of $CD4^+$ as well as $CD8^+$ T-cells in the blood of immunized rabbits shortly after immunization indicated that also the cellular immune response was stimulated possibly explaining why also low vaccine doses are able to protect rabbits. The involvement of T-cells in the protective immunity against RHDV was recently confirmed in studies with mice after intranasal or intramuscular vaccination (Farnós et al., 2006), and in infection trials with rabbits (Guo et al., 2016) where an induction of IFNγ and IL-4 production has been shown as soon as 7 days post vaccination.
A dose of 256 HU of the "recRHDV2-vacc" was able to induce full protection against the RHDV-2 challenge infection, although the antibody response was comparably low. Therefore, to ensure that the antibody titer is high enough for the investigation of onset, duration and possible cross-protection a dose of 1024 HU "recRHDV2-vacc" was chosen. The dose of 512

HU of the “convRHDV2-vacc” was selected because this is also the dose used in the commercial anti-RHDV-1 vaccine “Cunivak RHD”.

7.3.4. Onset of protection after vaccination

Vaccination against pathogens with a rapid progress of the disease requires an early onset of protective immunity. This is especially necessary in case of epidemic spread of a virus in susceptible host populations (Elnekave et al., 2015; Piontkowski et al., 2016). To test the onset of protective immunity after a single vaccination, rabbits were infected with RHDV-2 seven days after vaccination. The survival of all vaccinated rabbits indicated that early protective immunity had been induced by “recRHDV2-vacc”. Furthermore, because no clinical signs were found in vaccinated rabbits after challenge infection in contrast to non-vaccinated rabbits, which died, this induced immunity seems to inhibit the productive infection of RHDV at this early time point. This was also seen after vaccination with “convRHDV2-vacc”. Such early protection after vaccination with recombinant VP60 was reported before after vaccination with a recombinant baculovirus-derived RHDV-1-VP60 vaccine as already 5 days after a single vaccination most rabbits were protected against RHDV-1 infection (Laurent et al., 1994).

Conventional RHDV-1 vaccines induce a humoral protective immune response from day 4-5 after vaccination which is claimed to be effective enough to protect rabbits from illness and death (Argüello-Villares, 1991; Smíd et al., 1991). Whether innate resistance related immune mechanisms like type I IFN-mediated antiviral activity are also induced by recombinant RHDV vaccines is unknown.

Interestingly, at the timepoint of challenge only low antibody titers were measured in vaccinated rabbits which indicates an involvement of other early immune mechanisms (like type I interferon or IFNγ induced resistance or early activation of T-cells) which was not measured in the present study. An induction of IFNγ and IL-4 has been shown as soon as 7 days post vaccination in rabbits immunized with RHDV-1-VP60-VLPs and liver-derived RHDV-1 vaccines (Guo et al., 2016). The resistance of young rabbits against RHDV seems to be correlated with elevation of pro-inflammatory cytokines (TNF-α, IL-1, IFN-α, IFN-γ, IL-6, IL-8) (Marques et al., 2012).

An induction of interferons after vaccination with baculovirus alone (as negative control for recombinant baculovirus-derived RHDV vaccines) has been discussed (Gronowski et al., 1999). However, the fatal outcome of RHDV challenge infection of rabbits vaccinated with

“recbacGFP-vacc” alone in this study does not indicate any influence of an innate, IFN-based unspecific resistance against RHDV in the liver as main target organ.
It is not yet clear whether early, so called natural antibodies of IgM isotype (Holodick et al., 2017) might be stimulated and involved in early protection against RHDV infection, but IgM was detected in young rabbits after infection with apathogenic RCV (Capucci et al., 1997).

7.3.5. Duration of anti-RHDV-2 immunity after vaccination

One very important parameter of a good vaccine is the induction of a long-lasting immunity without the need of repeated booster vaccination (Castellino et al., 2009).
In this study, all vaccinated rabbits were completely protected 6 months after vaccination, independent of a prime or prime-boost vaccination scheme, and neither clinical signs of RHD nor indications for viral replication were found. Interestingly, although about 5 weeks after the second vaccination the titers were much higher in prime-boost vaccinated than in just single-shot vaccinated rabbits. These higher RHDV-2 specific antibody titers did not last over 6 months. The influence of the time schedule for prime-boost vaccinations was investigated in detail in studies where rabbits served as models for human diseases. It was demonstrated that a too early second vaccination could end up in an unwanted reduction of serum antibody titers late after vaccination (Radaelli et al., 2003; Vaine et al., 2008).
A completely different outcome after challenge was seen 14 months after vaccination, where all prime vaccinated rabbits completely survived, but the prime-boost vaccinated rabbits displayed mild to severe clinical signs and one rabbit died after 36h. The boost vaccination 21 days after the prime vaccination seemed to interfere with the developing antibody response most presumably due to a “catching” of VP60 specific antibodies induced by the first vaccination. A premature second vaccination might influence the development of higher antibody titers or the formation of long-lasting B-cell memory (Radaelli et al., 2003; Vaine et al., 2008). A comparable effect was reported in rabbits vaccinated with a conventional vaccine where the induced anti-RHDV-antibody titers decrease already 3 months after vaccination (Argüello-Villares, 1991). Similar findings were reported for recombinant RHDV-1 vaccines where a booster vaccination three weeks after the first immunization did not induce an increase of RHDV specific antibodies (Farnós et al., 2009; Fernández et al., 2011).
Whereas the protection against RHDV-2 14 months after vaccination seems to be dependent on the presence of specific anti-RHDV-2 antibodies, the situation 6 months after vaccination seems to be different. One rabbit did not have detectable RHDV-2 specific antibody titers

6 months after prime-boost vaccination but survived a RHDV-2 infection without clinical symptoms. This survival could be a result of a quick activation of memory B-cells and by activation of $CD8^+$ T-cells followed by strongly elevated IFNγ levels resulting in resistance against the challenge infection (West and Calandra, 1996).

As expected, after challenge infection the antibody titers increased even further. The very effective biological activity of a humoral immune response induced by a RHDV infection was also seen in the group of rabbits which were challenged already after 7 days post prime vaccination. These rabbits were kept for 14 months after the vaccination/challenge infection to evaluate the impact of a RHDV-2 infection shortly after vaccination on long-term protection. After the second challenge infection they were also completely protected from disease and showed no sign of viral replication. In contrast to only vaccinated rabbits, these animals displayed a stronger increase of antibody titers after the first challenge. The sharp increase of RHDV specific antibodies after infection confirmed earlier studies with recombinant vaccines (Plana-Duran et al., 1996). However, for some conventional vaccines this was not the case (Argüello-Villares, 1991).

As seen in surviving, non-vaccinated rabbits, a RHDV-2 infection led to a strong stimulation of the cellular immune system, which should be also stimulated in vaccinated rabbits after challenge. Hence those rabbits were still protected after 14 months although the circulating antibodies induced by vaccination were apparently partly consumed already after the first challenge infection shortly after vaccination. A strong and reliable cellular immunity in combination with the formation of long-living B-memory cells that convey lifelong protection against RHD is also seen in rabbits that survived a RHDV infection (Patton, 1989; Ferreira et al., 2005; Marques et al., 2012).

In summary, the long-lasting immunity is mainly based on circulating RHDV specific antibodies. Whether the booster vaccination 3 weeks after the first one interferes negatively with the induced humoral immune response might also depend on the used adjuvants and on the vaccination route which was different in most cited studies.

Generally, the protective immunity after RHDV vaccination lasts at least 12 months. This was comparable after vaccination with “convRHDV2-vacc” and also seen in RHDV-1 vaccines (either for recombinant vaccine candidates or conventional liver-derived vaccines) after subcutaneous or intramuscular administration (Argüello-Villares, 1991; Farnós et al., 2009; Fernández et al., 2011).

7.3.6. Cross-protection against RHDV-1 after vaccination against RHDV-2

Since a vaccination with “recRHDV2-vacc” proved to be protective against RHDV-2, cross-protection against RHDV-1 was also tested. Rabbits infected with RHDV-1 two weeks after RHDV-2 vaccination were only partially protected as about 50% of the rabbits died. In liver samples of surviving rabbits the viral load (viral RNA, viral proteins and particles) was measured 14 days after challenge infection. Comparable results were seen in rabbits after vaccination with “convRHDV2-vacc”. Similar results were found after vaccination with conventional anti-RHDV-1 vaccine “Cunivak RHD”, where only a partial cross-protection against RHDV-2 was induced after a single vaccination. However, a prime-boost vaccination with “Cunivak RHD” conveyed full cross-protection against RHDV-2 (this thesis and Dr. M. Müller, IDT, personal communication).

Development of cross-protective anti-RHDV-1 antibodies against RHDVa after a single or double vaccination with baculovirus-derived RHDV-1-VP60 has been shown whereas vaccination with a conventional liver-derived vaccine could not always induce high anti-RHDVa titers (Farnós et al., 2009; Fernández et al., 2011). However, cross-protection of conventional RHDV-1 vaccines against RHDVa has been confirmed (Schirrmeier et al., 1999). This could be explained by the low genetic divergence between RHDV-1 and RHDVa whereas there is a greater genetic distance between RHDV-1 and RHDV-2 (Capucci et al., 1998; LeGall-Reculé et al., 2013). As is described, cross-protectivity occurs also under natural conditions between different RHDV variants. Non-virulent virus strains are able to convey cross-protection in rabbits, e.g. in Australia where the non-pathogenic Australian strain RCV-A1 induced partial cross-protection in rabbits against the virulent RHDV-1 which was released into the wild to decimate rabbit populations, and therefore RCV-A1 interfered with the reduction of rabbits (Strive et al., 2009). Because there is constant adaptation of RHDV strains it is likely that new RHDV variants will appear in the future. Thus, it may be necessary to constantly adapt RHDV vaccines.

In conclusion, the data shown in this present study confirm that the newly established recombinant vaccine based on RHDV-2-VP60 not only protects rabbits after a single vaccination against clinical signs and death caused by RHDV-2 but also reduces viral replication to a minimum level and therefore seems to restrict viral shedding. During these studies two phenomena occurred that still need clarification though. Rabbits that received a second immunization 3 weeks after the first were less protected against RHDV-2 14 months after vaccination than rabbits that received a single immunization. So, further characterization of the differences in the immune responses of those two groups needs to be done. Another question that needs to be addressed is the ability of the vaccine to protect rabbits younger than 12 weeks. As RHDV-2 affects rabbits from 4 weeks of age, it needs to be examined further if the vaccine is able to induce protection also in such young animals or if reactions of the innate immune system or maternal anti-RHDV-1 or anti-RHDV-2 antibodies transmitted by vaccinated mothers would interfere with the vaccine.

8. Summary

The calicivirus *Rabbit hemorrhagic disease virus* (RHDV) causes the Rabbit hemorrhagic disease in rabbits. RHDV emerged 1984 in angora rabbits in China. In the following years it spread to many parts of the world resulting in huge losses among wild rabbit populations and rabbits used in fur and meat industry. It is a fatal disease to which rabbits from age of 9 weeks are fully susceptible. After an incubation period of 1-3 days, animals often develop high fever (>40°C) and die by acute liver failure and internal bleeding due to blood coagulation disorders (Abrantes et al., 2012). 2010 a new virus variant, called RHDV-2, emerged in France and is spreading through Europe at the moment. It causes the same clinical symptoms and pathological alterations as classical RHDV but also more prolonged clinical courses are described. The most important difference is, however, the susceptibility of rabbits from 4 weeks of age, sometimes even younger, and susceptibility of different hare species. There is no cure and the only prevention of disease is vaccination of rabbits (Le Gall-Reculé et al., 2013; Puggioni et al., 2013). An ethical problem is that most currently available conventional RHDV vaccines contain inactivated liver material-derived from RHDV infected rabbits and many rabbits have to die for vaccine development and production (Argüello-Villares, 1991). Conventional vaccines developed against classical RHDV only induce a partial protection against RHDV-2, which leads to significant economic problems in the fur and meat industry. Therefore, development of new vaccines against RHDV-2 is urgently necessary. Recently, vaccines against RHDV-2 came to economical use. However, these vaccines are also derived from livers of RHDV-2 infected rabbits.

Thus, the goal of this study was to develop a vaccine candidate that protects rabbits against illness and death by RHDV-2 and to bypass the questionable use of liver material of infected rabbits for vaccine production at the same time. Therefore, the virus capsid protein VP60 of RHDV-2 was expressed in cell culture by recombinant baculoviruses which self-assembled to VLPs. A vaccine candidate against RHDV-2, containing VLPs consisting of RHDV-2-VP60, was generated, that after a single dose vaccination protects rabbits against RHDV-2. In detailed vaccination/challenge experiments the induction of a protective long-lasting humoral and cellular immune response with an early onset already 7 days after a single immunization and partial cross-protection against classical RHDV was confirmed.

9. Zusammenfassung

Das Calicivirus *Rabbit hemorrhagic disease virus* (RHDV) ruft die „Rabbit hemorrhagic disease” in Kaninchen hervor. RHDV ist das erste Mal 1984 bei Angorakaninchen in China aufgetreten. In den darauffolgenden Jahren verbreitete es sich weltweit und verursachte hohe Verluste in wilden Kaninchenpopulationen und bei Kaninchen in der Pelz- und Fleischindustrie. Es ist eine tödlich verlaufende Krankheit, für die Kaninchen ab der 9. Lebenswoche voll empfänglich sind. Nach einer Inkubationszeit von 1-3 Tagen entwickeln die Tiere oft hohes Fieber (>40°C) und sterben an akutem Leberversagen und inneren Blutungen aufgrund von Blutgerinnungsstörungen (Abrantes et al., 2012). 2010 tauchte eine neue Virusvariante, genannt RHDV-2, in Frankreich auf und verbreitet sich momentan in Europa. Es verursacht die gleichen klinischen Symptome und pathologischen Veränderungen wie die klassische RHDV Variante, allerdings sind auch langwierigere Verläufe beschrieben. Der größte Unterschied ist jedoch die Empfänglichkeit von Kaninchen ab der vierten Lebenswoche, manchmal sogar jünger, und von verschiedenen Hasenarten. Die Krankheit ist nicht heilbar und der einzige Schutz besteht darin, Kaninchen zu impfen (Le Gall-Reculé et al., 2013; Puggioni et al., 2013). Ein ethisches Problem ergibt sich aus der Verwendung von inaktiviertem Lebermaterial von mit RHDV infizierten Kaninchen für die Herstellung der meisten konventionell erhältlichen RHDV Vakzinen und dem Umstand, dass viele Kaninchen für die Impfstoffentwicklung und -herstellung sterben müssen (Argüello-Villares, 1991). Konventionelle Impfstoffe, entwickelt gegen klassisches RHDV, induzieren nur einen Teilschutz gegen RHDV-2, was zu erheblichen wirtschaftlichen Verlusten in der Pelz- und Fleischindustrie führt. Somit ist die Entwicklung von Impfstoffen gegen RHDV-2 dringend notwendig. Seit kurzem sind RHDV-2 Vakzinen auf dem Markt, welche jedoch ebenfalls mit Lebermaterial von infizierten Kaninchen hergestellt werden. Das Ziel der vorliegenden Arbeit war daher die Entwicklung eines Impfstoffkandidaten, der in der Lage ist, Kaninchen vor Erkrankung und Tod durch RHDV-2 zu schützen und gleichzeitig den fragwürdigen Einsatz von Lebermaterial infizierter Kaninchen in der Impfstoffherstellung zu umgehen. Daher wurde das Viruskapsidprotein VP60 von RHDV-2 in Zellkultur mithilfe rekombinanter Baculoviren exprimiert, welches sich dann selbstständig zu VLPs zusammenlagerte. Dieser rekombinante Impfstoff gegen RHDV-2, der VLPs aus RHDV-2-VP60 enthält, schützt Kaninchen gegen RHDV-2. In verschiedenen Immunisierungs- und Challenge-Versuchen wurde die induzierte langanhaltende humorale und zelluläre Immunantwort, die bereits 7 Tage nach einmaliger Impfung eintritt und auch eine partielle Kreuzprotektivität gegen die klassische RHDV Variante erzeugt, bestätigt.

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Suppl. 1. Macroscopic and histopathological findings in individual rabbits after vaccination and challenge; proof of principle trial

Rabbit No.	Vaccine	Survival	Macroscopic changes						Histopathologic changes						
		Yes/No	Liver: hepatitis, necrotizing	Trachea: congestion	Lung: alveolar edema	Spleen: splenomegaly	Kidney: congestion	Multiple organs: petechiae and ecchymoses	Liver: hepatitis, necrotizing	Liver: hepatitis, periportal, lymphohistio-cytic	Spleen: splenitis fibrino-necrotizing	Spleen: lymphoid depletion	Spleen: follicular lymphoid hyperplasia	Spleen: congestion	Kidney: glomerular thrombosis
1	Cunivak RHD 2x	Y	0	0	0	1	1	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	Cunivak RHD 2x	Y	0	0	0	1	1	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	Cunivak RHD 1x	N	1	1	1	1	1	1	1	0	1	1	0	1	0
4	Cunivak RHD 1x	Y	1	0	0	1	1		0	1	0	0	1	1	0
5	recRHDV2-vacc BacBac-A 2x	Y	0	0	0	0	1	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	recRHDV2-vacc BacBac-A 2x	Y	0	0	0	0	1	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7	recRHDV2-vacc BacBac-A 1x	Y	0	0	0	0	1	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9	recRHDV2-vacc BacBac-A 1x	Y	0	0	0	0	1	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	recRHDV2-vacc BacMam-A 2x	Y	0	0	0	0	1	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
11	recRHDV2-vacc BacMam-A 2x	Y	0	0	0	0	1	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12	recRHDV2-vacc BacMam-A 1x	Y	0	0	0	0	1	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
13	recRHDV2-vacc BacMam-A 1x	Y	0	0	0	1	1	0	0	1	0	0	1	1	0
14	convRHDV2-vacc 2x	Y	1 (focal)	0	0	0	0	1 (kidney)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15	convRHDV2-vacc 2x	Y	0	0	0	0	0	1 (kidney)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16	convRHDV2-vacc 1x	Y	1 (focal)	0	0	1	1	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
17	convRHDV2-vacc 1x	Y	0	0	0	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18	non-vaccinated	N	1	1	1	1	1	1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
19	non-vaccinated	N	1	1	1	1	1	1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20	non-vaccinated	N	1	1	1	1	1	1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
21	non-vaccinated	N	1	1	1	1	1	1	1	0	1	1	0	1	1

Note: In this and the following supplementary tables all macroscopic and histopathological changes of all rabbits in the vaccination/challenge trials are documented. All animals which died after challenge infection displayed the typical RHD related symptoms. In some individual vaccinated rabbits histopathological alterations were detected which could not be associated with RHD because neither viral RNA nor viral protein was measured in liver samples.

n.d. = not determined

Suppl. 2. Macroscopic and histopathological findings in individual rabbits after vaccination and challenge; determination of minimal protective dose

Rabbit No.	Vaccine	Survival	Macroscopic changes						Histopathologic changes						
		Yes/No	Liver: hepatitis, necrotizing	Trachea: congestion	Lung: alveolar edema	Spleen: splenomegaly	Kidney: congestion	Multiple organs: petechiae and ecchymoses	Liver: hepatitis, necrotizing	Liver: hepatitis, periportal, lymphohistio-cytic	Spleen: splenitis fibrino-necrotizing	Spleen: lymphoid depletion	Spleen: follicular lymphoid hyperplasia	Spleen: congestion	Kidney: glomerular thrombosis
22	recRHDV2-vacc 256	Y	0	0	1	0	0	0	0	0	0	0	0	0	0
51	recRHDV2-vacc 256	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
60	recRHDV2-vacc 256	Y	0	0	0	0	0	0	0	1	0	0	0	0	0
61	recRHDV2-vacc 256	Y	0	0	0	0	0	0	0	1	0	0	0	0	0
23	recRHDV2-vacc 512	Y	0	0	0	0	0	0	0	0	0	0	1	0	0
36	recRHDV2-vacc 512	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
62	recRHDV2-vacc 512	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
63	recRHDV2-vacc 512	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
37	recRHDV2-vacc 1024	Y	0	0	0	0	0	0	0	1	0	0	0	0	0
38	recRHDV2-vacc 1024	Y	0	0	0	0	0	0	0	0	0	0	1	0	0
64	recRHDV2-vacc 1024	Y	0	0	0	1	0	0	0	1	0	0	1	0	0
65	recRHDV2-vacc 1024	Y	0	0	0	0	0	0	0	1	0	0	0	0	0
39	convRHDV2-vacc	Y	0	0	0	0	0	0	0	1	0	0	0	0	0
48	convRHDV2-vacc	Y	0	0	0	0	0	0	0	0	0	0	1	1	0
66	convRHDV2-vacc	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
67	convRHDV2-vacc	Y	0	0	0	1	0	0	n.d.	0	0	0	1	1	0
49	non-vaccinated	N	1	0	1	1	0	1	1	0	0	0	0	1	1
52	non-vaccinated	N	1	1	1	0	0	1	1	0	0	0	0	1	0
68	non-vaccinated	Y	0	0	0	1	0	0	0	0	0	0	0	0	0
69	non-vaccinated	N	1	0	1	1	0	1	1	0	1	1	0	1	1

Suppl. 3. Macroscopic and histopathological findings in individual rabbits after vaccination and challenge; long-term duration of vaccination induced protection after 6 months

Rabbit No.	Vaccine	Survival	Macroscopic changes						Histopathologic changes						
		Yes/No	Liver: hepatitis, necrotizing	Trachea: congestion	Lung: alveolar edema	Spleen: splenomegaly	Kidney: congestion	Multiple organs: petechiae and ecchymoses	Liver: hepatitis, necrotizing	Liver: hepatitis, periportal, lymphohistio-cytic	Spleen: splenitis fibrino-necrotizing	Spleen: lymphoid depletion	Spleen: follicular lymphoid hyperplasia	Spleen: congestion	Kidney: glomerular thrombosis
1	recRHDV2-vacc 1x	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
2	recRHDV2-vacc 1x	Y	0	0	0	0	0	0	0	1	0	0	0	0	0
8	recRHDV2-vacc 1x	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
9	recRHDV2-vacc 1x	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
28	recRHDV2-vacc 2x	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
29	recRHDV2-vacc 2x	Y	0	0	0	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
30	recRHDV2-vacc 2x	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
31	recRHDV2-vacc 2x	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
12	convRHDV2-vacc 1x	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
32	convRHDV2-vacc 1x	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
45	convRHDV2-vacc 1x	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
46	convRHDV2-vacc 1x	Y	0	0	0	0	0	0	0	1	0	0	0	0	0
50	non-vaccinated	N	1	0	1	1	0	1	1	1	1	1	0	1	1
53	non-vaccinated	Y	0	0	0	0	0	0	0	1	0	0	0	0	0
94	non-vaccinated	N	1	0	1	1	0	1	1	0	1	1	0	1	1
96	non-vaccinated	N	1	0	1	1	1	1	1	0	1	1	0	1	1

Suppl. 4. Macroscopic and histopathological findings in individual rabbits after vaccination and challenge; long-term duration of vaccination induced protection after 14 months

Rabbit No.	Vaccine	Survival	Macroscopic changes						Histopathologic changes						
		Yes/No	Liver: hepatitis, necrotizing	Trachea: congestion	Lung: alveolar edema	Spleen: splenomegaly	Kidney: congestion	Multiple organs: petechiae and ecchymoses	Liver: hepatitis, necrotizing	Liver: hepatitis, periportal, lymphohistio-cytic	Spleen: splenitis fibrino-necrotizing	Spleen: lymphoid depletion	Spleen: follicular lymphoid hyperplasia	Spleen: congestion	Kidney: glomerular thrombosis
3	recRHDV2-vacc 1x	Y	0	0	1	0	0	0	0	1	0	0	0	0	0
4	recRHDV2-vacc 1x	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
6	recRHDV2-vacc 1x	Y	0	0	1	0	0	0	0	1	0	0	0	0	0
7	recRHDV2-vacc 1x	Y	0	1	1	1	0	0	0	1	0	0	0	0	0
10	recRHDV2-vacc 1x	Y	0	0	0	0	0	0	0	1	0	0	0	0	0
40	recRHDV2-vacc 2x	Y	0	0	1	0	0	0	0	1	0	0	0	0	0
41	recRHDV2-vacc 2x	N	1	1	1	1	1	1	1	0	1	1	0	1	1
42	recRHDV2-vacc 2x	Y	0	0	0	0	0	0	0	1	0	0	0	0	0
43	recRHDV2-vacc 2x	Y	0	0	0	0	0	0	0	1	0	0	0	0	0
34	convRHDV2-vacc 1x	Y	0	0	1	0	0	0	0	1	0	0	0	0	0
35	convRHDV2-vacc 1x	Y	0	0	1	0	0	0	0	0	0	0	0	0	0
44	convRHDV2-vacc 1x	Y	0	0	1	0	0	0	0	1	0	0	0	0	0
2-1	non-vaccinated	N	1	1	1	1	1	1	1	0	1	1	0	1	1
2-2	non-vaccinated	N	1	1	1	1	0	1	1	0	1	1	0	1	1
2-3	non-vaccinated	N	1	1	1	0	1	1	1	0	1	1	0	1	1
2-4	non-vaccinated	N	1	1	1	1	0	1	1	0	1	1	0	1	1

Suppl. 5. Macroscopic and histopathological findings in individual rabbits after vaccination and challenge; long-term duration of vaccination induced protection after challenge infection 7 days and 14 months after vaccination

Rabbit No.	Vaccine	Survival	Macroscopic changes						Histopathologic changes						
		Yes/No	Liver: hepatitis, necrotizing	Trachea: congestion	Lung: alveolar edema	Spleen: splenomegaly	Kidney: congestion	Multiple organs: petechiae and ecchymoses	Liver: hepatitis, necrotizing	Liver: hepatitis, periportal, lymphohistio-cytic	Spleen: splenitis fibrino-necrotizing	Spleen: lymphoid depletion	Spleen: follicular lymphoid hyperplasia	Spleen: congestion	Kidney: glomerular thrombosis
16	recRHDV2-vacc 1x	Y	0	1	0	1	0	0	0	0	n.d.	n.d.	n.d.	n.d.	0
17	recRHDV2-vacc 1x	Y	0	0	1	1	0	0	0	1	0	0	0	0	0
18	recRHDV2-vacc 1x	Y	0	0	0	0	0	0	0	1	0	0	0	0	0
19	recRHDV2-vacc 1x	Y	0	0	0	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0
13	convRHDV2-vacc 1x	Y	0	1	1	0	0	0	0	1	0	0	0	0	0
14	convRHDV2-vacc 1x	Y	0	1	0	0	0	0	0	1	0	0	0	0	0
15	convRHDV2-vacc 1x	Y	0	0	1	0	0	0	0	0	0	0	0	0	0
20	convRHDV2-vacc 1x	Y	0	0	1	0	0	0	n.d	n.d.	0	0	0	0	0
25	non-vaccinated survivor	Y	0	0	1	0	0	0	0	1	0	0	0	0	0
2-1	non-vaccinated	N	see above trial "long term 14 months"						see above trial "long term 14 months"						
2-2	non-vaccinated	N													
2-3	non-vaccinated	N													
2-4	non-vaccinated	N													

Rabbit No.	Vaccine	Survival	Macroscopic changes						Histopathologic changes						
		Yes/No	Liver: hepatitis, necrotizing	Trachea: congestion	Lung: alveolar edema	Spleen: splenomegaly	Kidney: congestion	Multiple organs: petechiae and ecchymoses	Liver: hepatitis, necrotizing	Liver: hepatitis, periportal, lymphohistio-cytic	Spleen: splenitis fibrino-necrotizing	Spleen: lymphoid depletion	Spleen: follicular lymphoid hyperplasia	Spleen: congestion	Kidney: glomerular thrombosis
24	non-vaccinated	N	1	0	0	0	1	1	1	1	0	0	0	0	1
26	non-vaccinated	N	1	0	0	1	1	1	1	0	1	1	0	0	0
27	non-vaccinated	N	1	0	0	1	1	1	1	0	0	0	0	1	1

Suppl. 6. Macroscopic and histopathological findings in individual rabbits after vaccination and challenge; determination of cross-protection

	Rabbit No.	Vaccine	Survival	Macroscopic changes						Histopathologic changes						
			Yes/No	Liver: hepatitis, necrotizing	Trachea: congestion	Lung: alveolar edema	Spleen: splenomegaly	Kidney: congestion	Multiple organs: petechiae and ecchymoses	Liver: hepatitis, necrotizing	Liver: hepatitis, periportal, lymphohistio-cytic	Spleen: splenitis fibrino-necrotizing	Spleen: lymphoid depletion	Spleen: follicular lymphoid hyperplasia	Spleen: congestion	Kidney: glomerular thrombosis
challenge infection with RHDV-2	70	recRHDV2-vacc	Y	0	0	1	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	71	recRHDV2-vacc	Y	0	0	0	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	72	recRHDV2-vacc	Y	0	0	0	0	0	0	0	1	0	0	0	0	0
	73	recRHDV2-vacc	Y	0	0	0	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	74	convRHDV2-vacc	Y	0	0	1	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	75	convRHDV2-vacc	Y	0	0	1	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	76	convRHDV2-vacc	Y	0	0	0	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	77	convRHDV2-vacc	Y	0	0	0	0	0	0	0	0	0	0	1	0	0
	78	non-vaccinated	N	1	1	1	1	0	1	1	0	1	1	0	0	0
	79	non-vaccinated	N	1	1	1	0	0	1	1	0	1	1	0	0	1
	80	non-vaccinated	N	1	1	1	0	0	1	1	0	n.d.	n.d.	n.d.	n.d.	1
	81	non-vaccinated	N	1	0	1	1	0	1	1	0	1	1	0	1	1
challenge infection with RHDV-1	82	recRHDV2-vacc	Y	1	1	0	1	1	1	1	0	1	1	0	1	1
	83	recRHDV2-vacc	N	0	0	0	0	0	0	0	0	0	0	1	0	0
	84	recRHDV2-vacc	N	1	0	0	0	0	0	1	1	0	0	1	0	0
	85	recRHDV2-vacc	Y	1	1	1	1	1	1	1	0	1	1	0	1	1
	86	convRHDV2-vacc	Y	0	0	1	0	0	0	0	0	0	0	0	0	0
	87	convRHDV2-vacc	Y	0	0	1	0	0	0	0	1	0	0	1	0	0
	88	convRHDV2-vacc	N	1	1	1	1	0	1	1	0	1	1	0	0	1
	89	convRHDV2-vacc	Y	0	0	0	0	0	0	0	1	0	0	1	0	0
	90	non-vaccinated	N	1	0	1	1	1	1	1	0	1	1	0	0	1
	91	non-vaccinated	N	1	0	1	1	1	1	1	0	1	1	0	1	0
	92	non-vaccinated	Y	0	0	0	0	0	0	0	1	0	0	1	0	0
	93	non-vaccinated	N	1	1	0	1	1	1	1	0	1	1	0	0	1

Suppl. 7. Gene sequences of the artificial genes of RHDV-2-VP60 with the different codon usage of “BHV-1” or “AcMNPV”

Name of the gene: RHDV-2_VP60_BHV1_Cod

```
                                                            BglII     EcoRI  NcoI
        CACTATAGGGCGAATTGAAGGAAGGCCGTCAAGGCCGCATAGATCTGAATTCCACCATGG
1       ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        GTGATATCCCGCTTAACTTCCTTCCGGCAGTTCCGGCGTATCTAGACTTAAGGTGGTACC

                                              BsmBI
        AGGGGAAGGCCCGCGCCGCGCCGCAGGGGGAGACGGCGGGCACGGCCACCACAGCCTCCG
61      ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        TCCCCTTCCGGGCGCGGCGCGGCGTCCCCCTCTGCCGCCCGTGCCGGTGGTGTCGGAGGC

         SmaI                                                 AatII
        TCCCGGGCACGACCACGGACGGGATGGACCCCGGCGTGGTCGCGACGACGTCGGTGGTGA
121     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        AGGGCCCGTGCTGGTGCCTGCCCTACCTGGGGCCGCACCAGCGCTGCTGCAGCCACCACT

                                                                          PflMI
                                                                              BspMI
                                 BtgZI                                  AarI
        CGACCGAAAATGCTTCCACCAGCATCGCGACCGCCGGTATCGGCGGCCCTCCCCAGCAGG
181     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        GCTGGCTTTTACGAAGGTGGTCGTAGCGCTGGCGGCCATAGCCGCCGGGAGGGGTCGTCC

                    BsmBI
        TGGACCAGCAGGAGACGTGGCGGACGAACTTCTACTACAACGACGTGTTCACTTGGAGCG
241     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        ACCTGGTCGTCCTCTGCACCGCCTGCTTGAAGATGATGTTGCTGCACAAGTGAACCTCGC

        TGGCAGACGCGCCAGGGAACATTCTGTACACTGTGCAGCACAGCCCTCAGAACAACCCGT
301     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        ACCGTCTGCGCGGTCCCTTGTAAGACATGTGACACGTCGTGTCGGGAGTCTTGTTGGGCA

        TTACGGCGGTCCTGTCGCAGATGTACGCTGGATGGGCCGGCGGGATGCAGTTCCGGTTTA
361     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        AATGCCGCCAGGACAGCGTCTACATGCGACCTACCCGGCCGCCCTACGTCAAGGCCAAAT

                                                          EagI        SmaI
        TCGTCGCGGGATCTGGCGTGTTTGGCGGACGCCTGGTGGCGGCCGTGATCCCGCCCGGGA
421     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        AGCAGCGCCCTAGACCGCACAAACCGCCTGCGGACCACCGCCGGCACTAGGGCGGGCCCT

        TTGAGATCGGCCCCGGCCTAGAGGTTCGGCAGTTCCCGCACGTGGTAATTGACGCCCGCA
481     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        AACTCTAGCCGGGGCCGGATCTCCAAGCCGTCAAGGGCGTGCACCATTAACTGCGGGCGT
```

```
                                      BspMI
        GTCTGGAGCCGGTGACGATTACGATGCCGGACCTGCGACCGAACATGTACCATCCGACGG
541     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        CAGACCTCGGCCACTGCTAATGCTACGGCCTGGACGCTGGCTTGTACATGGTAGGCTGCC

        GCAACCCTGGGCTGGTGCCCACCCTGGTGCTGTCCGTGTATAACAACCTGATTAACCCCT
601     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        CGTTGGGACCCGACCACGGGTGGGACCACGACAGGCACATATTGTTGGACTAATTGGGGA

                                           BsmBI
        TCGGAGGCAGTACCAGCGCCATCCAGGTGACGGTGGAGACGCGGCCCAGCGAGGACTTCG
661     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        AGCCTCCGTCATGGTCGCGGTAGGTCCACTGCCACCTCTGCGCCGGGTCGCTCCTGAAGC

                                           SalI
                                           HincII
                       ApaI                AccI
        AGTTTGTGATGATCCGGGCCCCGTCGAGCAAGACCGTCGACAGCATCAGCCCGGCGGACC
721     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        TCAAACACTACTAGGCCCGGGGCAGCTCGTTCTGGCAGCTGTCGTAGTCGGGCCGCCTGG

        TCCTGACGACGCCCGTGCTTACTGGGGTGGGGACGGACAACCGCTGGAACGGGGAGATTG
781     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        AGGACTGCTGCGGGCACGAATGACCCCACCCCTGCCTGTTGGCGACCTTGCCCCTCTAAC

        TGGGCTTGCAGCCCGTCCCTGGCGGTTTCTCGACATGCAACCGGCACTGGAACCTTAACG
841     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        ACCCGAACGTCGGGCAGGGACCGCCAAAGAGCTGTACGTTGGCCGTGACCTTGGAATTGC

         SalI
         HincII
         AccI           SacI                PvuI
        GGTCGACGTTTGGCTGGAGCTCCCCGCGCTTCGCTGCGATCGACCACGATAGGGGCAACG
901     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        CCAGCTGCAAACCGACCTCGAGGGGCGCGAAGCGACGCTAGCTGGTGCTATCCCCGTTGC

                     XhoI                  XhoI
        CCTCGTACCCTGGCTCGAGCAGCAGCAACGTCCTCGAGTTGTGGTACGCGAGCGCGGGGT
961     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        GGAGCATGGGACCGAGCTCGTCGTCGTTGCAGGAGCTCAACACCATGCGCTCGCGCCCCA

        EagI
        CGGCCGCCGACAACCCCATCTCTCAGATCGCCCCGGACGGCTTCCCGGATATGAGCTTTG
1021    ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        GCCGGCGGCTGTTGGGGTAGAGAGTCTAGCGGGGCCTGCCGAAGGGCCTATACTCGAAAC

        TGCCGTTCTCGGGGACAACGGTCCCGACGGCGGGCTGGGTTGGCTTCGGGGGCATCTGGA
1081    ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
        ACGGCAAGAGCCCCTGTTGCCAGGGCTGCCGCCCGACCCAACCGAAGCCCCCGTAGACCT
```

```
                BssHII
               AscI                                    BsiWI
     ACAGCAACAACGGCGCGCCGTTCGTCACCACGATGCAGGCGTACGAGCTGGGCTTTGCCA
1141 ---------+---------+---------+---------+---------+---------+
     TGTCGTTGTTGCCGCGCGGCAAGCAGTGGTGCTACGTCCGCATGCTCGACCCGAAACGGT

                                                         BssHII
     CTGGCGCACCTAGCAATCCCCAGCCCACGACCACCACGAGCGGCGCGCAGATCGTGGCCA
1201 ---------+---------+---------+---------+---------+---------+
     GACCGCGTGGATCGTTAGGGGTCGGGTGCTGGTGGTGCTCGCCGCGCGTCTAGCACCGGT

                                                     EagI
     AGAGTATCTACGGTGTGGCCACGGGGATCAACCAGGCGGCGGCCGGCTTATTCGTGATGG
1261 ---------+---------+---------+---------+---------+---------+
     TCTCATAGATGCCACACCGGTGCCCCTAGTTGGTCCGCCGCCGGCCGAATAAGCACTACC

     CGTCCGGCGTCATCTCTACGCCGAACTCGTCGGCCATCACGTACACGCCCCAACCGAACC
1321 ---------+---------+---------+---------+---------+---------+
     GCAGGCCGCAGTAGAGATGCGGCTTGAGCAGCCGGTAGTGCATGTGCGGGGTTGGCTTGG

                 SmaI             EagI
     GTATTGTGAACGCCCCGGGCACCCCGGCCGCCGCGCCCGTGGGCAAGAACACCCCAATCA
1381 ---------+---------+---------+---------+---------+---------+
     CATAACACTTGCGGGGCCCGTGGGGCCGGCGGCGCGGGCACCCGTTCTTGTGGGGTTAGT

     TGTTCGCGTCGGTCGTGCGGCGCACCGGGGACATCAACGCGGAGGCAGGCAGTGCCAACG
1441 ---------+---------+---------+---------+---------+---------+
     ACAAGCGCAGCCAGCACGCCGCGTGGCCCCTGTAGTTGCGCCTCCGTCCGTCACGGTTGC

     GTACGCAGTACGGCGCGGGCAGCCAGCCGTTGCCCGTGACCGTCGGGCTCTCGCTGAACA
1501 ---------+---------+---------+---------+---------+---------+
     CATGCGTCATGCCGCGCCCGTCGGTCGGCAACGGGCACTGGCAGCCCGAGAGCGACTTGT

                          SrfI
                           SmaI                              PvuII
     ATTACAGCTCCGCGCTCATGCCCGGGCAATTTTTCGTCTGGCAGCTGAACTTTGCCTCCG
1561 ---------+---------+---------+---------+---------+---------+
     TAATGTCGAGGCGCGAGTACGGGCCCGTTAAAAAGCAGACCGTCGACTTGAAACGGAGGC

                                                                NarI
                                                                KasI
     GGTTCATGGAATTGGGTCTATCGGTGGACGGGTACTTTTACGCAGGGACGGGCGCCAGCG
1621 ---------+---------+---------+---------+---------+---------+
     CCAAGTACCTTAACCCAGATAGCCACCTGCCCATGAAAATGCGTCCCTGCCCGCGGTCGC

                                   SalI
                                   HincII
                                   AccI             ApaI
     CAACGCTGATCGACCTCAGCGAGCTGGTCGACATCCGCCCCGTGGGCCCTCGCCCGAGCA
1681 ---------+---------+---------+---------+---------+---------+
     GTTGCGACTAGCTGGAGTCGCTCGACCAGCTGTAGGCGGGGCACCCGGGAGCGGGCTCGT
```

```
                                          KpnI                                         EcoRI
         CGTCCACGCTGGTGTACAATCTGGGCGGTACCACGAACGGGTTTAGCTACGTATAGAATT
1741     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
         GCAGGTGCGACCACATGTTAGACCCGCCATGGTGCTTGCCCAAATCGATGCATATCTTAA

          HindIII
         CAAGCTTCTGGGCCTCATGGGCCTTCCTTTCACTGCCCGCTTTCCAG
1801     ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
         GTTCGAAGACCCGGAGTACCCGGAAGGAAAGTGACGGGCGAAAGGTC
```

Name of the gene: RHDV-2_VP60_AcMNPV

```
                                                BglII EcoRI   NcoI
      CACTATAGGGCGAATTGAAGGAAGGCCGTCAAGGCCGCATAGATCTGAATTCCACCATGG
1     ---------+---------+---------+---------+---------+---------+
      GTGATATCCCGCTTAACTTCCTTCCGGCAGTTCCGGCGTATCTAGACTTAAGGTGGTACC

      AGGGCAAAGCCCGCGCGGCACCGCAAGGAGAAACGGCGGGTACGGCCACAACAGCGAGTG
61    ---------+---------+---------+---------+---------+---------+
      TCCCGTTTCGGGCGCGCCGTGGCGTTCCTCTTTGCCGCCCATGCCGGTGTTGTCGCTCAC

                                  SmaI
      TGCCTGGCACCACCACCGACGGTATGGACCCGGGAGTGGTGGCTACCACCTCGGTTGTAA
121   ---------+---------+---------+---------+---------+---------+
      ACGGACCGTGGTGGTGGCTGCCATACCTGGGCCCTCACCACCGATGGTGGAGCCAACATT

                                                          PflMI
                                                             BspMI
                 NheI                PvuII                   AarI
      CGACGGAAAACGCTAGCACTTCGATTGCCACAGCTGGTATTGGAGGACCGCCCCAGCAGG
181   ---------+---------+---------+---------+---------+---------+
      GCTGCCTTTTGCGATCGTGAAGCTAACGGTGTCGACCATAACCTCCTGGCGGGGTCGTCC

      TGGACCAGCAAGAAACTTGGCGAACGAATTTCTACTACAACGACGTATTTACTTGGTCAG
241   ---------+---------+---------+---------+---------+---------+
      ACCTGGTCGTTCTTTGAACCGCTTGCTTAAAGATGATGTTGCTGCATAAATGAACCAGTC

                               AccI
      TCGCGGATGCACCCGGCAACATATTGTATACAGTACAACACAGCCCTCAAAACAACCCCT
301   ---------+---------+---------+---------+---------+---------+
      AGCGCCTACGTGGGCCGTTGTATAACATATGTCATGTTGTGTCGGGAGTTTTGTTGGGGA

                                                 SphI
      TCACGGCAGTTTTATCGCAAATGTACGCTGGCTGGGCCGGTGGCATGCAATTTCGCTTTA
361   ---------+---------+---------+---------+---------+---------+
      AGTGCCGTCAAAATAGCGTTTACATGCGACCGACCCGGCCACCGTACGTTAAAGCGAAAT

           BspMI                      BsmBI
      TTGTCGCAGGTAGCGGCGTTTTTGGTGGTCGTCTCGTTGCAGCCGTCATTCCCCCAGGCA
421   ---------+---------+---------+---------+---------+---------+
      AACAGCGTCCATCGCCGCAAAAACCACCAGCAGAGCAACGTCGGCAGTAAGGGGGTCCGT

                SrfI
                 SmaI
              ApaI
      TTGAAATAGGGCCCGGGCTGGAAGTGCGACAATTTCCGCATGTGGTGATTGATGCACGAA
481   ---------+---------+---------+---------+---------+---------+
      AACTTTATCCCGGGCCCGACCTTCACGCTGTTAAAGGCGTACACCACTAACTACGTGCTT
```

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          GTTTGGAACCTGTAACGATCACTATGCCCGATTTACGCCCCAACATGTACCACCCCACAG
541       ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
          CAAACCTTGGACATTGCTAGTGATACGGGCTAAATGCGGGGTTGTACATGGTGGGGTGTC

                                                             PacI       PflMI
          GCAATCCTGGCCTTGTACCAACGTTGGTTTTATCTGTGTATAATAATTTAATTAACCCAT
601       ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
          CGTTAGGACCGGAACATGGTTGCAACCAAAATAGACACATATTATTAAATTAATTGGGTA

                        SpeI
          TTGGTGGCTCAACTAGTGCTATCCAAGTGACTGTAGAAACGCGACCTTCAGAAGATTTTG
661       ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
          AACCACCGAGTTGATCACGATAGGTTCACTGACATCTTTGCGCTGGAAGTCTTCTAAAAC

                     BclI
          AATTTGTGATGATCAGAGCCCCCTCCTCTAAAACCGTCGATTCCATAAGTCCAGCCGACT
721       ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
          TTAAACACTACTAGTCTCGGGGGAGGAGATTTTGGCAGCTAAGGTATTCAGGTCGGCTGA

                          ScaI
          TGCTGACAACACCAGTACTTACGGGGGTGGGTACTGATAATCGCTGGAATGGCGAGATCG
781       ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
          ACGACTGTTGTGGTCATGAATGCCCCCACCCATGACTATTAGCGACCTTACCGCTCTAGC

                     AgeI    SmaI
          TAGGATTACAACCGGTCCCGGGCGGATTTAGCACTTGTAATCGCCACTGGAATCTAAATG
841       ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
          ATCCTAATGTTGGCCAGGGCCCGCCTAAATCGTGAACATTAGCGGTGACCTTAGATTTAC

                                                    PvuI             SacII
          GCAGCACTTTTGGCTGGTCGAGTCCCAGATTTGCGGCGATCGACCATGACCGCGGAAATG
901       ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
          CGTCGTGAAAACCGACCAGCTCAGGGTCTAAACGCCGCTAGCTGGTACTGGCGCCTTTAC

          CGAGTTACCCCGGCTCTAGCTCCTCGAACGTGCTAGAATTGTGGTACGCTTCAGCCGGTA
961       ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
          GCTCAATGGGGCCGAGATCGAGGAGCTTGCACGATCTTAACACCATGCGAAGTCGGCCAT

          GTGCTGCGGACAACCCTATAAGTCAAATAGCTCCTGACGGCTTTCCTGATATGTCATTTG
1021      ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
          CACGACGCCTGTTGGGATATTCAGTTTATCGAGGACTGCCGAAAGGACTATACAGTAAAC

          TGCCCTTTTCGGGAACTACCGTTCCTACGGCAGGGTGGGTGGGATTCGGCGGCATTTGGA
1081      ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
          ACGGGAAAAGCCCTTGATGGCAAGGATGCCGTCCCACCCACCCTAAGCCGCCGTAAACCT

          ACTCTAACAACGGCGCTCCGTTTGTCACAACGATGCAAGCATACGAACTGGGCTTCGCCA
1141      ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
          TGAGATTGTTGCCGCGAGGCAAACAGTGTTGCTACGTTCGTATGCTTGACCCGAAGCGGT
```

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     CGGGCGCTCCCTCGAACCCCCAACCGACGACCACTACCTCTGGTGCCCAAATTGTTGCGA
1201 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     GCCCGCGAGGGAGCTTGGGGGTTGGCTGCTGGTGATGGAGACCACGGGTTTAACAACGCT

                         AgeI
     AAAGCATTTACGGCGTAGCGACCGGTATCAACCAAGCCGCTGCCGGCTTATTTGTTATGG
1261 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     TTTCGTAAATGCCGCATCGCTGGCCATAGTTGGTTCGGCGACGGCCGAATAAACAATACC

     CGAGTGGCGTGATTTCTACACCGAACAGTAGTGCGATAACGTACACTCCGCAACCGAACC
1321 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     GCTCACCGCACTAAAGATGTGGCTTGTCATCACGCTATTGCATGTGAGGCGTTGGCTTGG

          HincII                    PvuII
     GCATCGTTAACGCACCTGGTACGCCCGCAGCTGCCCCAGTTGGCAAAAACACTCCAATCA
1381 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     CGTAGCAATTGCGTGGACCATGCGGGCGTCGACGGGGTCAACCGTTTTTGTGAGGTTAGT

     TGTTTGCCTCGGTGGTGCGTAGAACCGGAGACATTAACGCTGAAGCCGGTAGCGCCAACG
1441 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     ACAAACGGAGCCACCACGCATCTTGGCCTCTGTAATTGCGACTTCGGCCATCGCGGTTGC

                                       BstEII
     GGACACAATACGGCGCTGGCTCTCAACCGTTGCCGGTGACCGTTGGACTTTCATTGAACA
1501 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     CCTGTGTTATGCCGCGACCGAGAGTTGGCAACGGCCACTGGCAACCTGAAAGTAACTTGT

     ATTATAGTTCCGCATTGATGCCGGGCCAGTTTTTTGTCTGGCAATTGAACTTTGCATCTG
1561 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     TAATATCAAGGCGTAACTACGGCCCGGTCAAAAAACAGACCGTTAACTTGAAACGTAGAC

                BsaI                              BspMI
     GTTTCATGGAATTGGGTCTCTCGGTGGATGGCTATTTTTATGCAGGTACAGGAGCCAGCG
1621 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     CAAAGTACCTTAACCCAGAGAGCCACCTACCGATAAAAATACGTCCATGTCCTCGGTCGC

     CTACCTTAATTGATCTATCGGAACTGGTGGACATTCGTCCAGTTGGACCCCGGCCTTCTA
1681 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     GATGGAATTAACTAGATAGCCTTGACCACCTGTAAGCAGGTCAACCTGGGGCCGGAAGAT

                              KpnI                BsiWI     EcoRI
     CATCGACTCTGGTTTATAATTTGGGCGGTACCACAAACGGGTTTTCGTACGTATAAGAAT
1741 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     GTAGCTGAGACCAAATATTAAACCCGCCATGGTGTTTGCCCAAAAGCATGCATATTCTTA

        HindIII
     TCAAGCTTCTGGGCCTCATGGGCCTTCCTTTCACTGCCCGCTTTCCAG
1801 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     AGTTCGAAGACCCGGAGTACCCGGAAGGAAAGTGACGGGCGAAAGGTC
```

12. Publications

Parts of the present study have been published or were presented at congresses and published as congress abstracts

Poster and oral presentation + Abstract

Claudia Müller, Horst Schirrmeier, Günther M. Keil, Kati Franzke. Baculovirus mediated generation of rabbit haemorrhagic disease virus variant b VLPs in SF9 insect cells and RK13 rabbit cells from codon usage modified VP60b open reading frames. ESVV congress & Epizone meeting. Montpellier, France, Aug 31th-Sept 3rd.

Keil GM, Pollin R, Müller C, Giesow K, Schirrmeier H: BacMam Platform for Vaccine Antigen Delivery. In: Methods in Molecular Biology, Vaccine Technologies for Veterinary Viral Diseases, Methods and Protocols. Edited by: Brun A. Springer: Humana Press 2016; 1349:105-19.

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