Aus dem Institut für Virologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Development of a vectored equine herpesvirus type 1 (EHV-1) vaccine against pandemic influenza A virus (09/H1N1)

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Dedication

Dedicated to my Father´s soul, to my Mother, to my brothers, to my dearest wife, to my lovely daughter and to Dr. Nabila S. Degheidy.

Abdelrahman

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Abbreviations

BAC	Bacterial artificial chromosome
BGH	Bovine growth hormone
BSA	Bovine serum albumin
CT	Cycle threshold
CTL	Cytotoxic T lymphocyte
DIVA	Differentiating infected from vaccinated animals
DNA	Deoxyribonucleic acid
dpi	Day post infection
dpv	Day post vaccination
dpc	Day post challenge
ECL	Enhanced chemoluminscence
E.coli	Escherichia coli
EGFP	Enhanced green fluorescence protein
EHV-1	Equine herpsvirus type 1
EMEM	Earle's minimum essential medium
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FW	Forward
gM	Glycoprotein M
gp	Glycoprotein P
HA	Heamagglutinin
HBsAg	Human hepatitis B surface antigen
HCMV	Human cytomegalovirus
HI	Hemagglutination inhibition test
hpi	Hour post infection
HSV	Herpes simplex virus
IgA	Isotype immune globulin A
IgG	Isotype immune globulin G
IM	Intra-muscular
IN	Intra-nasal
IR_S	Internal repeat short
IR_L	Internal repeat long

KDa	Kilodalton
LB	Luria-Bertani
MAb	Monoclonal antibody
MDBK	Madin-darby bovine kidney
MEM	Modified eagle's medium
m.o.i	Multiplicity of infection
MLV	Modified live virus vaccine
MP	Matrix protein
NA	Neuraminidase
NEP	Nuclear export protein
NP	Nucleoprotein
NS	Non structural protein
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PB	Polymerase protein
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PFU	Plaque forming units
PVDF	Polyvinylidene fluoride
RFLP	Restriction fragment length polymorphism
RIPA	Radioimmunoprecipitation assay
RK13	Rabbit kidney cells
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
RV	Reverse
SDS	Sodium dodecyl sulfate
SI	Swine influenza
SIV	Swine influenza virus
SNT	Serum neutralizing assay
S-OIV	Swine-origin H1N1 influenza A virus

SQ	Subcutaneous
ssRNA	Single strand ribonucleic acid
TR_L	Terminal repeat long
TR_s	Terminal repeat short
UL	Unique long
US	Unique short
VNT	Virus neutralizing test
vRNP	Viral ribonucleoprotein
WHO	World Health Organization

Zusammenfassung

Entwicklung eines Impstoffes gegen das pandemische Influenzavirus A (09/H1N1) basierend auf einem equinen Herpesvirus Typ 1 (EHV-1)-Vektor

Die Influenza der Schweine (SI) ist eine hoch ansteckende Erkrankung, die durch milde bis schwere Krankheitsverläufe gekennzeichnet ist und unter bestimmten Umständen zum Tod des Tieres führen kann. Die Erkrankung wird durch das SI-Virus ausgelößt, welches innerhalb der Familie *Orthomyxoviridae* zu dem Genus Influenza A gezählt wird. Das neuauftretende, pandemische Influenzavirus A(H1N1)09 scheint das Resultat eines genetischen Rekombinationsvorganges zwischen der klassischen "Schweinegrippe" H1N1, dem humanen H3N2-Virus, aviären Influenzaviren sowie eurasischen Influenzaviren von Schweinen zu sein. Dadurch unterscheidet sich das Virus genetisch sowie antigenetisch von dem saisonal zirkulierenden H1N1-Influenzavirus A. Das SIV ist dabei nicht nur ein wichtiges Pathogen des respiratorischen Traktes der Schweine sondern stellt auch eine potentielle Bedrohung für den Menschen dar. Diesbezüglich sollte ein effektiver Impfstoff die industrielle Schweinezucht auf der einen Seite vor ökonomischen Verlusten bewahren und auf der anderen Seite die Entwicklung neuer Virusvarianten, welche die menschliche Gesundheit bedrohen können, unterbinden. Die Immunisierung mit inaktivierten Todimpfstoffen war für lange Zeit die Hauptmaßnahme zur Prävention von Influenzainfektionen. Aber auch für andere Impfstrategien konnte die Auslösung einer protektiven Immunantwort gegen die virale Infektion gezeigt werden. Im Vordergrund stehen hierbei rekombinante Vektoren, welche für Glykoproteine der Virushüllmembran des H1N1-Virus kodieren. Daher zielt diese Studie auf das Design, die Validierung und Entwicklung eines Produktionsprozesses zur Herstellung von rekombinaten Influenza-Hämagglutinin-Vakzinen.

Das virale Oberflächenprotein Hämagglutinin (HA) stellt eines der entscheidenen Antigene in der Ausbildung der Immunantwort des Wirts dar weil entsprechende neutralisierende Antikörper die Infektion abschwächen oder gänzlich verhindern können. Zur Expression des rekombinanten H1 des pandemischen Influenzastammes H1N1 wurde das equine Herpesvirus-1 (EHV-1) als modifizierter Lebendvektor (MLV) verwendet. In Publikationen konnte kürzlich gezeigt werden, dass EHV-1 ein vielversprechendes Vehikel für die intrazelluläre Produktion fremder Antigene darstellt.

Das zweite und dritte Kapitel der Promotionsarbeit befasst sich mit der Konstruktion eines equinen Herpesvirus-1, welches das H1 des pandemischen Influenzavirus A(H1N1)09 (rH_H1) exprimiert, sowie der Beurteilung seiner Effizienz als Impfstoff im Mausmodell der

Influenzainfektion und Infektionen des Schweins. Die Expression des rekombinanten Proteins konnte durch indirekte Immunofluoreszenzmikroskopie sowie Western Blot Analyse nachgewiesen werden. Zudem wies der modifizierte Lebendvektor im Vergleich zum parentalen Wildtypvirus keine Unterschiede bezüglich seiner Replikation *in vitro* auf.
Die Immunisierung von Mäusen mit dem rH_H1 induzierte eine Influenzavirus-spezifische Antikörperantwort. Der Schutz von geimpften Mäusen und Schweinen nach einer *challenge*-Infektion konnte durch Verminderung der klinischen Symptome nachgewiesen werden. Auch die Virusreplikation in geimpften Tieren war beinträchtigt, wie durch eine reduzierte, nasale Ausscheidung und schnellere Beseitigung des Virus gezeigt werden konnte.
Zusammenfassend implizieren die Ergebnisse der vorliegenden Arbeit, dass ein rekombinantes, H1-exprimierendes EHV-1 eine vielversprechende Alternative zur Immunsierung von Schweinen gegen das pandemische Influenzavirus A (H1N1)09 oder anderer Influenzaviren darstellt.

Hauptergebnisse der Promotionsarbeit

► Ein rekombinates, equines Herpesvirus-1 ist zur robusten Expression des Hämagglutinin des pandemischen H1N1 (Influenzavirus der Schweine) befähigt.
► Mäuse und Schweine sind nach Immunisierung mit dem Impfstoff gegen die Influenzainfektion geschützt.
► Das Vakzine kann ohne vorherige Aufreinigung oder Zugabe von Adjuvanz verwendet werden.
► Die virale Belastung ist in geimpften gegenüber ungeimpften Tieren reduziert.

Schlüsselwörter

Rekombinante Vakzine, EHV-1, Vektor, pandemische Influenza H1N1, Hemagglutinin, Mäuse, Schweine.

Summary

Development of a vectored equine herpesvirus type 1 (EHV-1) vaccine against pandemic influenza A virus (09/H1N1)

Swine influenza (SI) is a highly contagious viral infection in pigs and is characterized by mild to severe illness, which can lead to death under certain circumstances. The disease is caused by SIV, which belongs to the influenza A virus genus in the *Orthomyxoviridae* family. The newly emerged pandemic influenza A(H1N1) 2009 virus appears to be a result of reassortment of classical swine H1N1, human H3N2, avian and Eurasian swine influenza viruses. The newly emerging virus is genetically and antigentically different from circulating seasonal H1N1 influenza A virus. SI is not only an important respiratory pathogen in pigs but also a potential threat to human health. An effective vaccine may protect the pork industry from economic losses and curb the development of new virus variants that may threaten public health. Immunization with inactivated vaccines has long been the main strategy for the prevention of influenza infections. However, approaches other than conventional vaccines have also been found to induce protective immunity against infection, the most prominent being recombinant containing the envelope glycoproteins of the H1N1 virus. Therefore, this study aimed at the design, validation and development of a production process for a recombinant hemagglutinin (HA) influenza vaccine for the prevention of pandemic influenza A(H1N1)09 infection. The viral surface protein HA is the key antigen in the host response to influenza virus since neutralizing antibodies directed against HA can mitigate or prevent infection. The equine herpesvirus type 1 (EHV-1) was selected as modified live vector (MLV) for the synthesis of recombinant H1 of pandemic influenza A(H1N1). EHV-1 has recently been demonstrated to be a promising alternative viral vehicle for delivery of foreign antigens.

In the second and third chapter of the thesis, we report on the construction of an equine herpesvirus 1 that expresses H1 of pandemic influenza A(H1N1)09 (rH_H1) and the evaluation of its efficacy as a vaccine in mouse model of influenza virus and in the natural host (pigs). Immunofluorescence and western blotting demonstrated expression of the recombinant protein and the *in vitro* growth properties of the modified live vector were found to be comparable to those of the parental virus. After immunization of mice, the rH_H1 vaccine induced an influenza virus-specific antibody response. Upon challenge infection, protection of vaccinated mice and natural host (pigs) could be demonstrated by reduction of clinical signs of the disease, reduction of virus replication as evidenced by decreased nasal

virus shedding and faster virus clearance. Taken together, our results indicated that recombinant EHV-1 encoding H1 of pandemic influenza H1N1 may be a promising alternative for protection of pigs against infection with pandemic influenza A(H1N1)09 or other influenza viruses.

Highlights points of the thesis

► Equine herpesvirus type 1 robustly expresses hemagglutinin of pandemic H1N1 (swine influenza virus).
► Mice and pigs are clinically protected against challenge infection after vaccination.
► Vaccine was utilized with no purification or adjuvant.
► Viral loads are reduced in vaccinated versus non-vaccinated animals.

Key word

Recombinant vaccine, EHV-1, Vector, Pandemic Influenza H1N1, Hemagglutinin, Mice, Pigs.

Chapter 1: General Introduction

1.1. Vaccine history

The history of modern vaccination dates back to Edward Jenner in the 18th century. Louis Pasteur furthered the concept through his pioneering work in microbiology. In 1798, Jenner used cowpox virus inoculations in an attempt to protect against smallpox infection (Jenner, 1798). Moreover, Jenner discovered that cowpox could be transmitted from person to person, thereby enabling collective protection against fatal outbreaks (Jenner, 1798). Louis Pasteur generalized Jenner's idea when he discovered that alternative person-to-person vaccination strategies based on a similar concept of using a weakened form of disease agents to induce immunity (Pasteur, 1880). Pasteur's work was developed further in 1886 by Daniel Elmer Salmon and Theobald Smith who used heat to kill typhoid, cholera and plague organisms and proposed the resulting material as vaccines with increased safety because they were no longer infectious (Salmon and Smith, 1886). The development of new strategies and better understanding of the molecular biology and immunology of pathogens have accelerated vaccine development during recent years. Nowadays, It is possible to use only a small, defined part of the pathogen in a vaccine that results in the induction of protective immune responses, without the capacity of the whole pathogen to cause infection. As indicated below, there are many different types of routinely used vaccines and many more in various stages of development.

1.2. Types of vaccines

The development of vaccines has been one of the most important contributions to the health and welfare of humans and animals. Many approaches have been followed to design variant vaccines against microbes. These approaches are based on fundamental information about the microbe. Vaccination is a one of the most important methods, which enabled to induce protective immune responses that have resulted in reduction of the numbers of humans and animals suffering from infectious diseases and have prevented economic losses. In addition, vaccination has helped to limit or prevent transmission of diseases in animal reservoir hosts and from reservoir hosts to humans and animals. The existing vaccines can be divided into two general types:

(i) First generation vaccines (live attenuated vaccines and inactivated vaccines).

(ii) Second generation vaccines (subunit vaccines, gene-deleted vaccines, Deoxyribonucleic acid (DNA) vaccines and recombinant vector vaccines).

1.2.1. Live attenuated vaccines

Live attenuated vaccines are composed of attenuated organisms that replicate moderately *in vivo*, thus eliciting an immune response mimicking that induced by natural infection. Attenuation usually is obtained by (i) *in vitro* passages either in cell culture or in embryonated eggs (ii) selection of spontaneous or induced temperature-sensitive mutants and (iii) use of re-assortants obtained by co-infection of the same cell with two different viruses with segmented genomes. These vaccines are able to stimulate both the humoral and cellular arm of the immune system and can confer lifelong immunity with only one or two vaccine doses (Ellis, 1999; Plotkin and Plotkin, 2004). In spite of the advantages of live attenuated vaccines, there are some downsides. Live attenuated virus vaccine can potentially revert to virulence and sometimes lead to fatal disease in vaccinated animals or lack safety in pregnant or immuno-compromised animals (Plotkin and Plotkin, 2004). Another disadvantage of live attenuated vaccines is that they usually need to be refrigerated to maintain biological activity.

1.2.2. Killed or inactivated vaccines

Inactivating the disease-causing microbes with chemicals, heat, or radiation produces inactivated vaccines. Such vaccines are usually more stable and safer than live attenuated vaccines as the dead microbes cannot revert to their virulent form. On the other hand, there are several disadvantages associated inactivated vaccines including (i) generation of weaker immune responses relative to live attenuated vaccines because mainly the humoral immune system is stimulated; (ii) multiple rounds of vaccination are needed; (iii) highly antigenic mass and strong adjuvants are usually needed; (iv) lack of long-term of protection; (v) vaccinated animals exposed to infection can become asymptomatic carriers, and finally (vi) vaccines can contain traces of non-structural proteins making it difficult to distinguish between vaccinated and infected animals.

1.2.3. Subunit vaccines

Subunit vaccines are composed of purified antigens that best stimulate the immune system, but are not infectious and thus subunit vaccines contain only the antigens essential for induction of a protective immune response and not all the other molecules that make up the microbe, which may induce adverse side effects. However, the potential disadvantages of subunit vaccines are: (i) they require strong adjuvants to stimulate immune system response to the target antigen and (ii) duration of immunity is shorter than that induced by live attenuated

vaccines. Subunit vaccines are becoming the focus of the vaccine industry due to their safety profile and the ability to manufacture them recombinantly in large quantities in a reproducible fashion (Plotkin and Plotkin, 2004). Examples of purified subunit vaccines include the hemagglutinin (HA) subunit vaccines for influenza A and B (MacKenzie, 1977; Skowronski et al., 2012; Xuan et al., 2011).

1.2.4. Gene-deleted vaccines

Molecular technology can be used to achieve specific modifications or deletions in the genome of microorganisms, but this technology also allows a more targeted design of live vaccines with specific deletions of genes with known function. The target genes for these deletions are responsible for key metabolic processes that inhibit the spread of infection but allow the development of protective immune responses (Meeusen et al., 2007). The availability of full-length cDNA clones of various viruses and microbes, for example, equine arteritis virus (Glaser et al., 1998) or equine Venezuelan encephalitis virus (Paessler et al., 2006) has greatly facilitated the direct insertion of specific mutations in the genome and the development of gene-deleted live vaccines. Genetically engineered vaccines have also provided the basis for the development of assays that differentiate infected from vaccinated animals (DIVA). The corresponding vaccines, so-called DIVA vaccines, are generated by the introduction of deletions in non-essential genes encoding for immunogenic proteins.

1.2.5. DNA vaccines

Nucleic acid (DNA) immunization is a technique used to stimulate both humoral and cell-mediated immune responses to antigens. The direct injection of DNA into a living host causes a small amount of its cells to produce the desired gene products. This gene expression within the host has important immunological consequences, resulting in a great potential for reducing infectious disease by the specific immune activation in the host against the delivered antigen (Koprowski and Weiner, 1998). DNA vaccines have been generated for a number of viral, bacterial and parasitic models of disease. The basis for DNA immunization involves the direct introduction of plasmid DNA containing the gene encoding the antigen against which one wishes to induce cellular and humoral immune response. The gene encoding for the antigens of pathogens can be incorporated into plasmids under the control of eukaryotic promoters in order to get expression when used *in vivo* (Fig. 1.1). The potential advantages of DNA vaccines offer many of the potential benefits of live vaccines without the same inherent risks, such as reversion to virulence. Immunization of animals with DNA vaccines encoding

protective antigens promote the induction of both humoral and cellular immune responses after intracellular expression of the antigens (Robinson, 1999) and have the ability to overcome maternally derived immunity in neonates or young animals (Fischer et al., 2003). Furthermore, DNA vaccines are very stables and do not require a cold chain. Potential development of vaccines based on plasmid DNA in the stimulation of immune responses in animal models have been demonstrated using immunogenic genes from a variety of infectious agents including influenza virus (Kim and Jacob, 2009; Kodihalli et al., 1999), hepatitis B virus (Goilav et al., 1990), rabies virus (Kaur et al., 2010; Lodmell et al., 2000), West Nile virus (Ledgerwood et al., 2011), and malaria (Doolan and Hoffman, 2001).

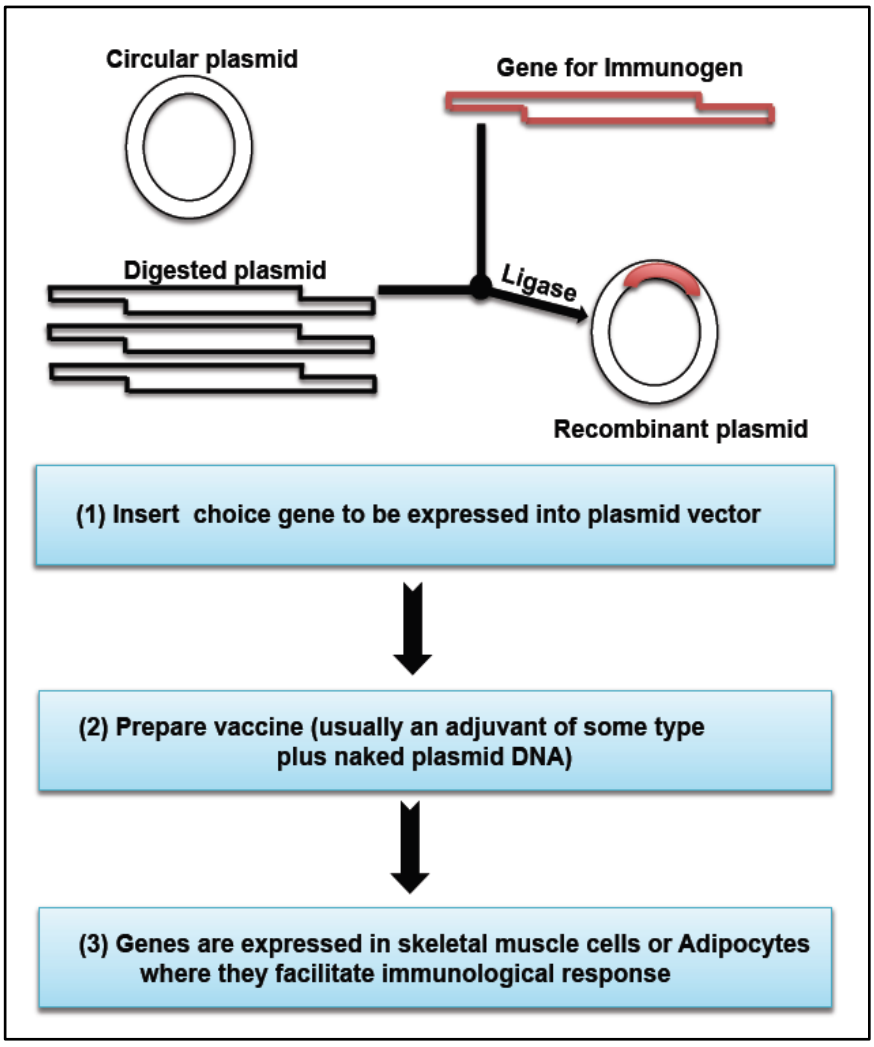

Figure 1.1. Schematic illustration of strategies for the basis of DNA vaccines preparation.

1.2.6. Recombinant vector vaccines

Recombinant vector vaccines are experimental and similar to vaccines based on plasmid DNA, but they use an attenuated virus or bacterium to deliver microbial DNA and immunogen. Vector refers to the virus or bacterium used as the carrier. In live-vectored vaccines, the desired gene coding for target antigens of the virulent pathogen is introduced into a vector and then this vector is used to infect the host. The vector replicates in the inoculated host and serves as the source of the antigen, delivering a large amount of the immunogenic antigen into the system and eliciting a strong immune response. The potential advantage of using live-vectored vaccines is that they are able to activate both humoral and cell-mediated immune responses. Moreover, live vector vaccines can protect vaccinated animals from heterologous virus challenge and provide the possibility that the induced immune response can be differentiated from that resulting from natural infection. Moreover, the vaccine can be produced in cell culture, which makes mass production possible and superior to the traditional vaccine. In the original proof-of-concept studies, the human hepatitis B surface antigen (HBsAg) gene, flanked by the nonessential vaccinia gene for thymidine kinase and an early gene promoter, was inserted into a plasmid vector (Fig. 1.2). Mammalian cells were transfected with the chimeric plasmid, which, after infection with the vector, produced vaccinia virus carrying the HBsAg by homologous recombination (Smith et al., 1983). The recombinant virus was then used as a hepatitis B vaccine. Thereafter, several recombinant viral vectors have been used to deliver immunogen of pathogenic microbes, for example, modified attenuated canarypox virus expressing immunogen of WNV (Boone et al., 2007; Minke et al., 2004) or canine distemper virus (Welter et al., 2000), Fowl poxvirus carrying antigens of avian influenza virus (Qian et al., 2012; Qiao et al., 2009) or Newcastle disease virus (Boursnell et al., 1990), and Modified turkey herpesvirus (HVT) expressing immune stimulating gene of avian influenza virus (Goa et al., 2012). Furthermore previous studies used EHV-1 as a modified live vectored vaccine (Ma et al., 2012; Rosas et al., 2008a; Rosas et al., 2006; Rosas et al., 2007a; Rosas et al., 2008b; Rosas et al., 2007b; Rosas, 2007; Said et al., 2011; Said et al., 2013). Therefore in chapter 2 and 3 of this thesis, we developed an modified live vaccine by using an equine herpesvirus 1 as a vector for expressing the H1 hemagglutinin of pandemic influenza A(H1N1)09 isolate.

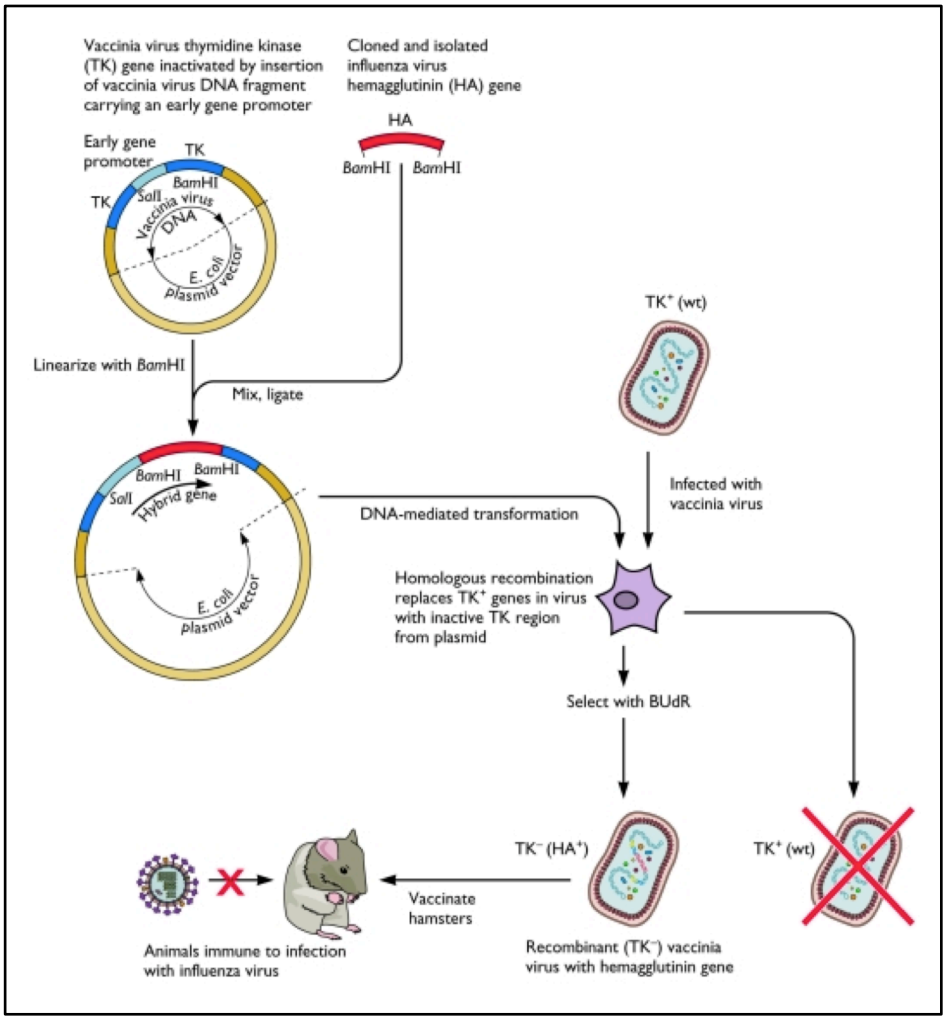

Figure 1.2. Method of constructing a vaccinia virus vector carrying a selected gene from another virus. Adapted with permission from (Dimmock and Primrose, 1994).

1.3. General characteristics of equine herpesvirus type 1 (EHV-1)

1.3.1. Classification and structure of EHV-1

Equine herpesvirus type 1 (EHV-1) is a member of the genus *Varicellovirus* genus within the *Alphaherpesvirinae* subfamily (Davison, 2009). The majority of EHV-1 proteins share extensive homology with human herpes simplex virus (HSV), which is the prototype virus of the *Alphaherpesvirinae* subfamily. The complete genome sequences of EHV-1 was reported (Telford, 1992). It is a double-stranded DNA genome of 150-Kbp in length (Davison, 2009; Telford, 1992). It is divided into a unique long (U_L) and a unique short (U_S) region, the latter being flanked by an inverted internal and terminal repeat sequence (IR_S and TR_S or IR_L and TR_L; respectively). The genome contains 80 open reading frames (ORFs), which encode 76

unique genes; four ORFs are duplicated in the TR_S (Telford, 1992) (Fig. 1.3). The virus genome is contained in the nucleocapsid composed of six proteins (Perdue et al., 1974). All herpesviruses have a similar capsid structure composed by 162 capsomers. Twelve portal proteins form a ring in the nucleocapsid, which is used by viral DNA to enter into the capsid (Baker et al., 1990; Newcomb et al., 1989). Nucleocapsid and tegument are surrounded by an envelope with a total of 11 viral glycoproteins on its surface. The glycoproteins of EHV-1 are conserved in other *alphaherpesviruses* and therefore named according to the nomenclature established for HSV-1. Compared to HSV-1 and the majority of other herpesviruses, EHV-1 encodes an additional glycoprotein, gp2 with a homologue only present in EHV-4.

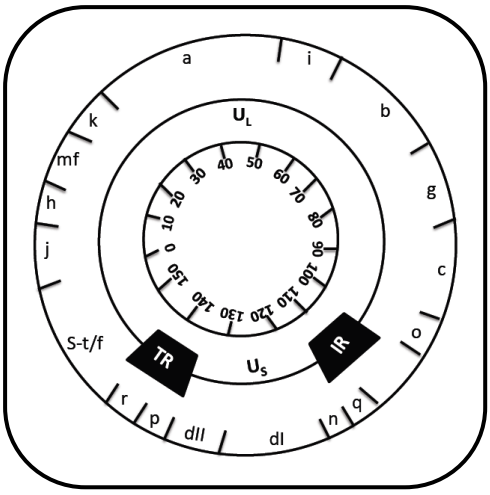

Figure 1.3. Schematic diagram of sequence arrangement of the EHV-1 genome. The unique-long (UL) and unique-short (US) sequences are presented, the US flanked by inverted repeat sequences termed internal and terminal repeats (IR and TR, respectively).

1.3.2. Pathogenesis, replication and latency of EHV-1

EHV-1 is the most important cause of virus respiratory manifestations in horses worldwide and can also lead to severe abortion and nervous system disorders with frequent fatal outcome (Allen and Bryans, 1986; Patel and Heldens, 2005). Infection occurs through the respiratory route and after local replication in tissues of the upper respiratory tract, the virus spreads through a cell-associated viremia to target internal organs such as the uterus or the nervous system (Allen and Bryans, 1986). The pathogenesis of EHV-1 is attributed to the capacity of the virus to rapidly enter lymphatic tissues associated with the upper respiratory tract and to infect mononuclear cells that ultimately reach the bloodstream and lead to cell-associated viremia (Kydd et al., 1994; Wilson, 1997). EHV-1 can be carried by the infected peripheral blood mononuclear cells (PBMC), reach the vasculature of other tissues such as the pregnant uterus or the central nervous system, where the virus can attach to, enter and replicate in endothelial cells (Wellington et al., 1996). Replication of EHV-1 in endothelial cells of uterus

or central nervous systems can result in late term abortion or nervous system disorders (Tearle et al., 1996). EHV-1 can establishes latency in neurons, especially in cells of the trigeminal ganglion, but more frequently in lymphoid cells, primarily monocytes and CD8+ and CD4+-T-lymphocytes (Fig. 1.4) (Allen and Bryans, 1986; Slater et al., 1994; Welch et al., 1992).

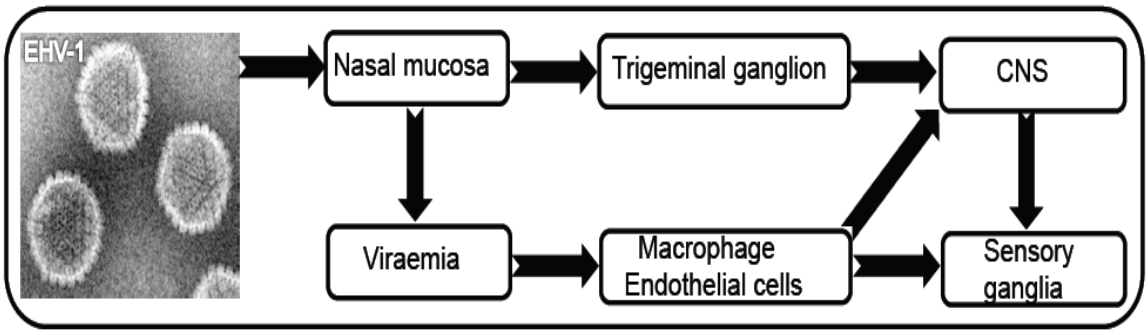

Figure 1.4. EHV-1 can establish latency in trigeminal ganglia via nasal mucosa, modified with permission from (Borchers et al., 2006).

1.3.3. Cloning of herpesviruses as bacterial artificial chromosome (BAC)

1.3.3.A. BACs in Virology

A bacterial artificial chromosome (BAC) is a DNA construct based on a functional fertility plasmid (F-plasmid) that is used for cloning of large DNA in bacteria, usually E. coli (O'Connor et al., 1989; Shizuya et al., 1992). F-plasmids have a critical role in construction of BACs because they contain genes, which promote the distribution of plasmids after bacterial cell division. BAC cloning is not simply issue of ligating the viral genome into the BAC plasmid vector, since large herpesvirus genomes are not typically adjustable to such manipulation. The BAC plasmid cassette is introduced into the herpesvirus genome by ordinary homologous recombination in infected cells. The linear double-stranded DNA genome of herpesviruses circularizes during replication. It is enough to isolate the circular replication intermediate of the BAC mutant and to shuttle it by DNA transformation into competent *E. coli*. The herpesvirus BAC is then propagated and mutated in *E. coli*. Such a viral BAC would be infectious, as transfection of BAC-cloned viral DNA into permissive cells should lead to virus production (She, 2003) (Fig. 1.5). Several EHV-1 strains were cloned as BACs, in most of them the mini-F plasmid sequences were inserted in place of gene 71 (gp2) (Rudolph et al., 2002). Various of these BACs harbor the sequences of chloramphenicol resistance gene that allows selection in bacteria culture as well as the introduction of enhanced green fluorescent protein (EGFP), which allows selection for green

plaques after transfection of BAC DNA into cells (Rudolph et al., 2002). Growth of EHV-1 virus impaired in cell culture after transfection by deletion of gp2 gene, compared to infection with wild-type virus. Co-transfection of the mutated BAC DNA and a plasmid maintaining the gp2 sequence could reconstitute fully infectious virus (Rudolph et al., 2002; von Einem et al., 2007).

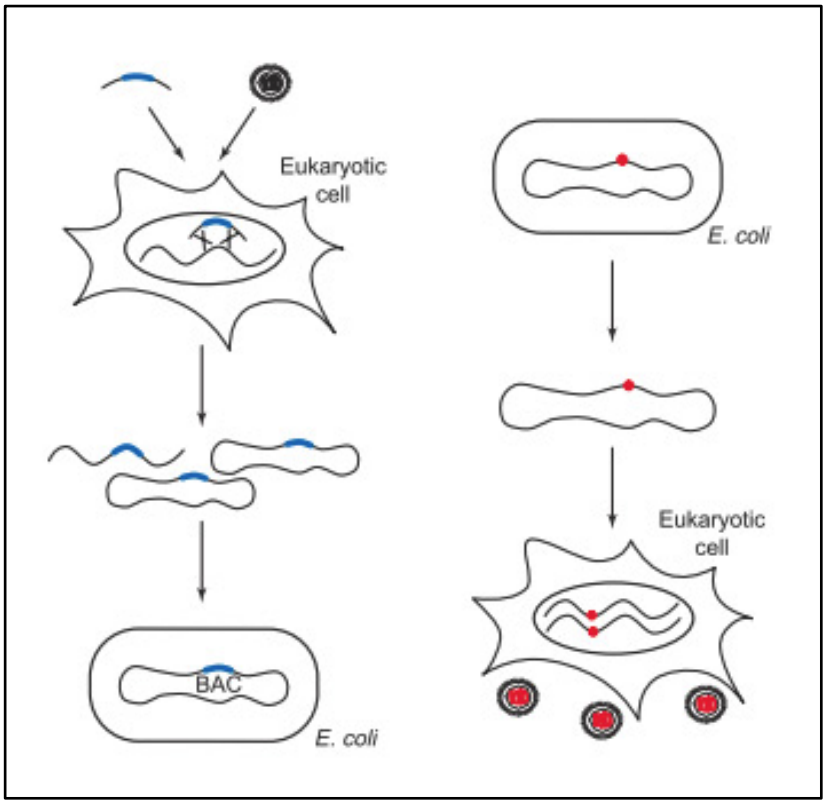

Figure 1.5. Cloning a viral genome as a bacterial artificial chromosome. (a) A mutant virus containing the BAC cassette (blue) is produced by cells by homologous recombination. Circular replication intermediates of the viral genome are isolated and transformed into *E. coli* to obtain a virus BAC. (b) The BAC-cloned viral genome can be engineered in *E. coli* to introduce a mutation (red). Transfection of a BAC containing the mutated viral genome leads directly to mutant viral progeny. Modified with permission from (Brune et al., 2000).

1.3.3.B. Application of BAC technology to herpesviruses

Herpesviruses are important pathogens for animals and humans, and have one of the largest DNA genomes of mammalian viruses (Roizman, 1996). However, the large size of the herpesviruses results in experimental manipulation difficulties (Zhou and Roizman, 2005). Therefore, developments of several human and animals herpesviruses BAC have been created, for example, murine cytomegalovirus, human simplex virus, pseudorabies virus,

equine herpesvirus and bovine herpesvirus. The application of BAC technology to herpesviruses has represented a significant advance in the study of the molecular biology and pathogenesis of these pathogens. The availability of cloned herpesvirus genomes as BACs could allow for rapid sequencing of the unsequenced herpesvirus genomes, and modification of viral genomes by insertion or deletion of sequences or alteration of sequences as discrete as a single-point mutation that makes the BAC system a powerful tool for addressing both mechanistic and functional questions. Furthermore, BACs technology opens the way to additional applications such as expressing therapeutic genes instead of the nonessential genes, which should make these viruses useful as vehicles for gene therapy and vaccine development.

1.3.3.C. Potential of Equine Herpesvirus type 1 as a Vector for Immunization

The EHV-1 strain RacH was established as a BAC (Rudolph et al., 2002) and developed as a universal live vector. Its most important features include its ability to enter a wide group of cell types of different origins (Trapp et al., 2005). RacH has a proven safety record in horses and a number of other species and its attenuation could be returned to a deletion of both copies of gene 67 and other genomic modifications (Hubert et al., 1996; Neubauer et al., 1999; Osterrieder et al., 1996). Previous studies (Ma et al., 2012; Rosas et al., 2008a; Rosas et al., 2006; Rosas et al., 2007a; Rosas et al., 2008b; Rosas et al., 2007b; Rosas, 2007; Said et al., 2011; Said et al., 2013) reported that the RacH strain of EHV-1 can stably and efficiently express immunogenic proteins, induce both humoral and cell-mediated immune responses and protect vaccinated animals from heterologous virus challenge. Moreover, it was shown to be very efficient in non-equine animals, mainly due to its capacity to deliver foreign genes in cells of various species including mice, dogs and cattle (Ma et al., 2012; Rosas et al., 2008a; Rosas et al., 2006; Rosas et al., 2007a; Rosas et al., 2008b; Rosas et al., 2007b; Rosas, 2007) and the lack of pre-existing anti-EHV-1 immunity. EHV-1 has been used to deliver bovine viral diarrhea virus structural proteins in cattle and was shown to induce neutralizing antibodies that were correlated with reduced viremia and virus shedding (Rosas et al., 2007a). While immunization of cattle with the recombinant EHV-1 induced neutralizing antibodies against recombinant EHV-1 and no cross-reactivity with bovine herpesvirus type 1 was observed (Rosas et al., 2007a). On the other hand, the lack of pre-existing anti-EHV-1 immunity in non-equine ruminants will avoid interference with the vector itself. Therefore, development of a vector EHV-1 vaccine against pandemic influenza A H1N1/09 was the focus of this thesis. The strategies for construction of recombinant EHV-1 expressing foreign

genes have included employed two-step en passant mutagenesis (Tischer et al., 2006). During the first recombination event, the insertion of the amplified foreign gene into the previously generated pRacH BAC clone under the control of the HCMV IE promoter resulted in kanamycin-resistant intermediates that exhibited the expected changes in the wild type DNA restriction pattern. The second recombination step resulted in the removal of the aphA1 gene and the final arrangement of recombinant EHV-1 expressing foreign gene is given (Fig. 1.6).

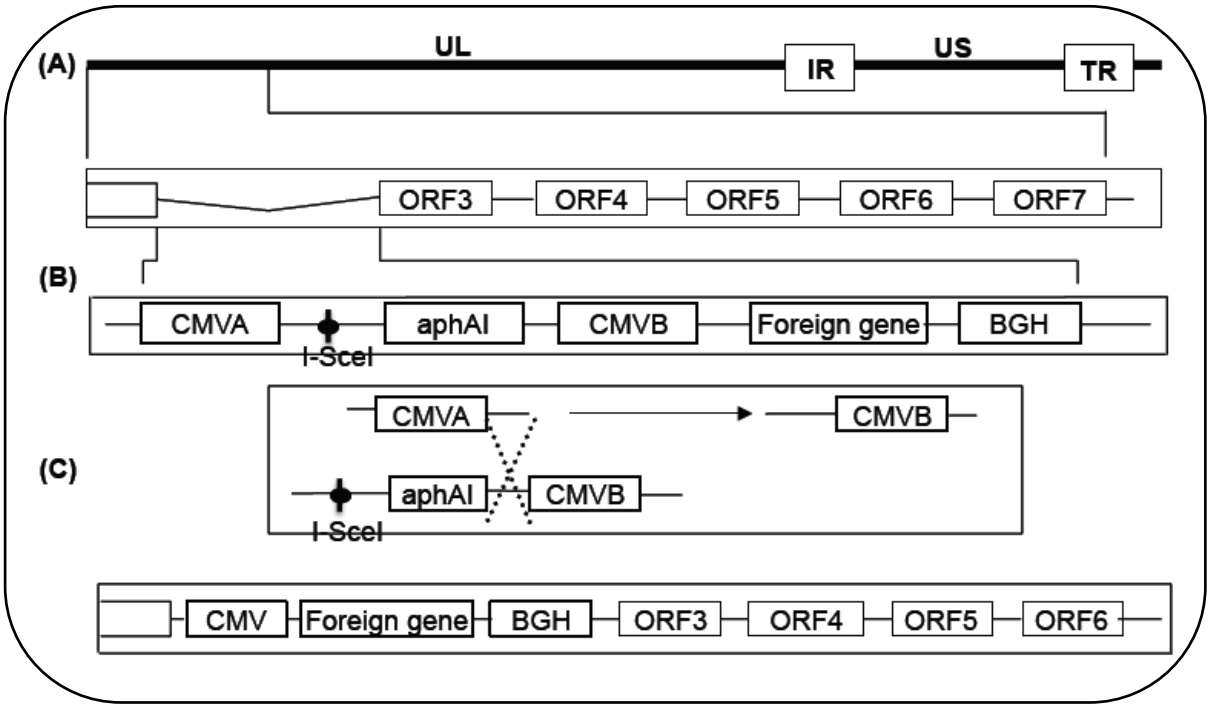

Figure 1.6. Schematic illustration of two-step red mutagenesis applied in the construction of recombinant RacH strain of EHV-1 expressing foreign gene. (A) Depiction of the left terminus of the unique-long segment of EHV-1 strain RacH infectious BAC genome. (B) The first recombination event resulted in the incorporation of foreign gene, HCMV promoter and kanamycin resistance gene in the ORF1/ORF2 locus of the pRacH genome. (C) The second recombination event resulted in resistance gene was removed and the final arrangement of recombinant RacH expressing foreign gene is given.

1.4. Pandemic Influenza A virus

1.4.1. Background and history

Influenza virus is a highly contagious viral disease of the respiratory tract that causes an acute illness including fever, cough, nasal congestion, body aches, redness of eyes, headache and sometimes gastrointestinal symptoms such as diarrhea and abdominal pain. These symptoms

contribute to a significant loss of workdays, human suffering, morbidity and mortality (Barker and Mullooly, 1982; Glezen, 1982; Simonsen et al., 1997). Since the pandemic influenza A (H1N1) 1918 virus "Spanish flu", influenza virus gene reassortment has been documented and observed to occur among human, animals and avian influenza viruses with different subtypes. Such reassortant viruses led to other influenza pandemics in 1947, 1957 and 1968, which were caused by influenza virus subtypes H1N1, H2N2 and H3N2, respectively (Isaacs et al., 1962; Rasmussen et al., 1948; Viboud et al., 2005) The evolutionary interaction between influenza virus strains and antigenic drift of the viral HA resulted in the appearance of new pandemic influenza viruses in 1977 and 1987, which were caused by influenza H1N1 and H3N2 variants, respectively (Scalera and Mossad, 2009; Smith et al., 2004) (Table 1.1). In early 2009, a new influenza A(H1N1) virus emerged among humans in Mexico and California that was able to spread quickly through transmission between animals and humans, and caused the first influenza pandemic of the 21st century (Scalera and Mossad, 2009; WHO, 2009). Then, the virus was transmitted to pigs in May of 2009 (Howden et al., 2009). The virus was antigenically unrelated to human seasonal influenza viruses but genetically related to viruses circulating in pigs. It is referred to as swine-origin influenza virus (S-OIV) A/H1N1, or pandemic influenza A(H1N1)09 virus. Influenze pandemics caused by influenza A viruses from 1918 until the last pandemic influenza virus 2009 are summarized in Table 1.1.

Pandemic influenza caused by influenza A **Major antigenic shifts associated with influenza A pandemics**		
Year	**Sub type**	**Prototype strain**
1918 (Spanish flu)	H1N1	A/Spanish/18
1947	H1N1	A/FM1/47
1957 (Asian flu)	H2N2	A/Singapore/57
1968 (Hong Kong flu)	H3N2	A/Hong Kong/68
1977	H1N1	A/USSR/77
1987	H3N2	No pandemic various strains circulated worldwide
2009	H1N1	Pandemic various strains circulated worldwide

Table 1.1. Pandemic influenza caused by influenza A Virus. Modified from (Ryan and Ray, 2004).

1.4.2. Structure of influenza A virus

Influenza viruses are enveloped viruses with a segmented negative-sense single-stranded RNA genome, which are classified in *Orthomyxoviridae* family (Lamb and Krug, 2001). Influenza viruses are classified as three main types, namely influenza A, B, and C, according to antigenic differences between their matrix and nucleoproteins (M and NP). These viruses further differ with respect to host range, variability of the surface glycoproteins, and genome organization (Lamb and Krug, 2001). Influenza virus type A and B can cause epidemic human disease. Influenza A is composed of eight helices of RNA that encode at least 11 proteins of the virus. The virions are pleomorphic with a diameter of approximately 80-120 nm (Fujiyoshi et al., 1994; Lamb and Choppin, 1983). The viral envelope is composed of a lipid bilayer containing the viral HA, neuraminidase (NA), and ion-channel (M2) proteins, and a layer of matrix protein (M1) can be found. The remaining proteins including nucleoprotein (NP) and polymerase proteins (PB1, PB2 and PA) that are bound to the viral RNA and constitute the viral ribonucleoprotein (vRNP), are located inside the virion (Lamb and Choppin, 1983; Nayak et al., 2009).

1.4.3. History of pandemic influenza A(H1N1)09 reassortment

Genetic reassortment is important for influenza virus evolution and the generation of novel viruses with pandemic potential (Rambaut et al., 2008; Taubenberger et al., 2005). In May 2009, the pandemic influenza virus was first detected in pigs in Canada (Howden et al., 2009), and then the virus spread to and infected pigs in different countries (WHO, 2010). Influenza pandemics are caused by the introduction of a virus with an HA subtype that is new to human populations, which can result from reassortment (Wright et al., 2007). The last three pandemic viruses, the Asian influenza/1957, Hong Kong influenza/1968, and the pandemic influenza A(H1N1)09 viruses, have one or more polymerase genes from a nonhuman source, besides the HA and NA genes (Kawaoka et al., 1989; Smith et al., 2009), which suggests the essential role of these genes in the generation of pandemic influenza viruses through genetic reassortment. The pandemic influenza A(H1N1)09 virus appeared to be a product of reassortment of classic swine H1N1, human H3N2, Avian, and Eurasian swine influenza viruses over the years (Trifonov et al., 2009). The polymerases, HA, NPs and NS proteins of pandemic influenza A(H1N1)09 virus show high similarities with swine influenza A/H1N2/North American, which was isolated in the late 1990's. The segments coding for NA and matrix proteins are related to swine influenza A/H1N1, which was isolated in Europe in 1992 (Trifonov et al., 2009) (Fig. 1.7).

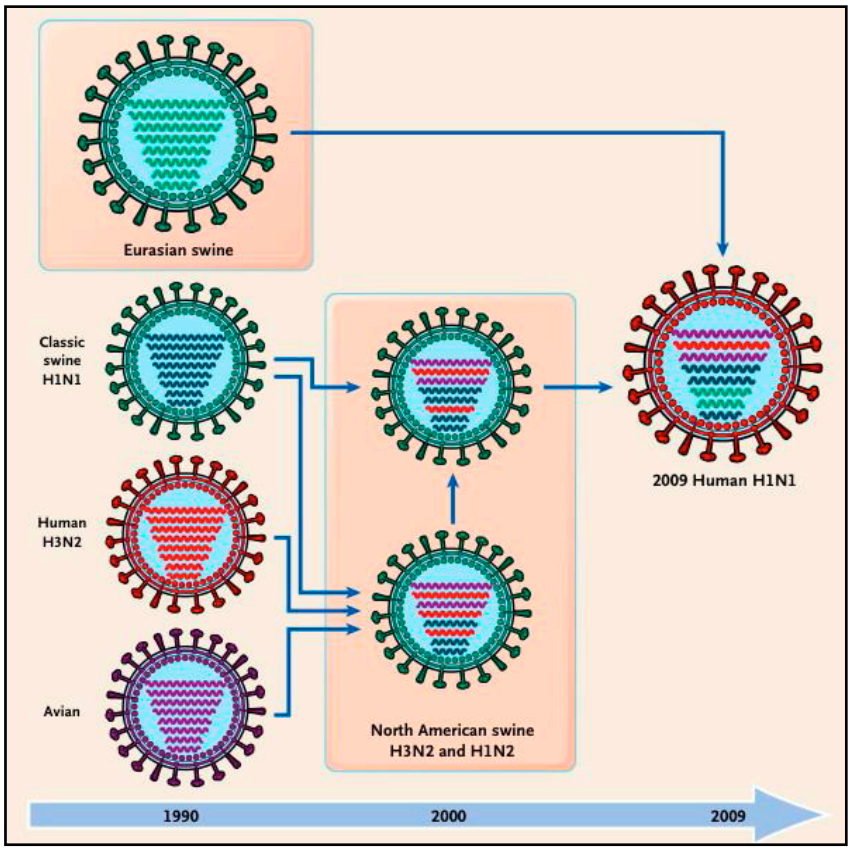

Figure 1.7. History of Reassortment Events in the Evolution of the 2009 Influenza A(H1N1) Virus. Adapted with permission from (Trifonov et al., 2009).

1.4.4. Control and prevention of pandemic influenza

Governments and health authorities worldwide are planning how to best prepare for and deal with future influenza pandemics. Pandemic influenza happens when new variants of influenza viruses emerge and little immunity against the newly emerged virus exists in the general population. Pandemic influenza strains are transmitted more rapidly than seasonal strains across several countries, and make more people ill. Therefore, improvement of influenza drugs and vaccines will help us to reduce the effects of the future influenza outbreaks. Oseltamivir (Tamiflu) is the one of the antiviral drugs that able to prevent viral particles from being released by infected mammalian cell. However, influenza viruses can develop resistance to these drugs and new resistant strains have appeared. (Bonhoeffer et al., 1997; Lipsitch, 2001). Therefore, influenza vaccines have been developed to limit or prevent appearance of the new pandemic influenza viruses. These vaccines either contain inactivated influenza virus, or live attenuated virus that cannot cause influenza. Viruses can loose their pathogenicity when inactivated or killed after growth in embryonated chicken eggs

(Hoffmann et al., 2002). Whole virus vaccine inactivated with formaldehyde or β-propiolactone to destroy the viral envelope of the virus. Several live attenuated vaccines were developed against influenza virus (Block et al., 2011; Heikkinen et al., 2012; Phonrat et al., 2013). However, the use of either inactivated or MLV vaccines are not DIVA vaccines. In contrast to the traditional inactivated or MLV vaccines, a DIVA strategy can be achieved using live-vectored vaccines. Moreover, the using of recombinant vaccine against influenza A virus not only eliminates the industry's dependence on chicken embryos but also addresses the general safety concerns associated with vaccines based on the whole pathogen. Therefore, modified live vector vaccines are generated based on expression of immunogenic protein of influenza virus by using several modified live attenuated virus as vehicles, for example, a modified Vaccinia virus (Goodman et al., 2011; Kreijtz et al., 2010; Rimmelzwaan and Sutter, 2009), a modified canarypox virus (Minke et al., 2007), a recombinant fowl pox virus (Qian et al., 2012; Qiao et al., 2009; Qiao et al., 2003), and a modified EHV-1 (Rosas et al., 2008a; Said et al., 2011; Said et al., 2013; Van de Walle et al., 2010). Therefore, development of a vaccine against pandemic influenza A virus 2009 by using a modified equine herpesvirus was the focus of this thesis.

1.5. Outline of the thesis

The overall goals of this thesis were to develop pandemic influenza vaccines based on the established EHV-1 vector expressing the H1 protein (rH_H1) of H1N1. Although vaccines against pandemic influenza A(H1N1) are available, their efficacy is limited and immunity established after either infection or vaccination is short-lived and incomplete. Therefore, the main objective of this study was to design new vaccines capable of inducing long and efficient protection against heterologous infection with H1N1 pandemic influenza. Such immunity should be based on both humoral and cellular immune responses. Here, we developed vaccine based on EHV-1 virus vector against pandemic influenza A(H1N1)09, and this vaccine may provide the opportunity for a differentiation of infected and vaccinated pigs.

The specific aims of this study are:

1- To construct recombinant pandemic influenza virus expressing H1.
2- To evaluate whether insertion of H1 in EHV-1 BAC has an effect on virus growth *in vitro*.
3- To detect expression of H1 protein in rH_H1 *in vitro*.
4- To test the efficacy and safety of the recombinant vaccine *in vivo*.

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Chapter 2: An equine herpesvirus 1 (EHV-1) vectored H1 vaccine protects against challenge with swine-origin influenza virus H1N1

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2.1. Summary

In 2009, a novel swine-origin H1N1 influenza A virus (S-OIV), antigenically and genetically divergent from seasonal H1N1, caused a flu pandemic in humans. Development of an effective vaccine to limit transmission of S-OIV in animal reservoir hosts and from reservoir hosts to humans and animals is necessary. In the present study, we constructed and evaluated a vectored vaccine expressing the H1 hemagglutinin of a recent S-OIV isolate using equine herpesvirus 1 (EHV-1) as the delivery vehicle. Expression of the recombinant protein was demonstrated by immunofluorescence and western blotting and the *in vitro* growth properties of the modified live vector were found to be comparable to those of the parental virus. The EHV-1-H1 vaccine induced an influenza virus-specific antibody response when inoculated into mice by both the intranasal and subcutaneous routes. Upon challenge infection, protection of vaccinated mice could be demonstrated by reduction of clinical signs and faster virus clearance. Our study shows that an EHV-1-based influenza H1N1 vaccine may be a promising alternative for protection against S-OIV infection.

2.2. Introduction

Swine influenza is an acute and highly contagious respiratory disease caused by type A influenza viruses. Influenza A viruses are enveloped viruses with a segmented negative-sense single-stranded RNA genome, which are members of the *Orthomyxoviridae* family (Palese and Shaw, 2007). The haemagglutinin (HA) and neuraminidase (NA) proteins present in the viral envelope are the key antigens against which humoral immune responses are directed. Pigs have been hypothesized to act as the reservoir host for mammalian influenza viruses and played an important role in the evolution of swine-origin (S-OIV) H1N1 influenza A virus

that emerged in 2009 (Vijaykrishna et al., 2010). This virus is antigenically and genetically divergent from seasonal H1N1 strains and was responsible for the 2009/2010 flu pandemic in humans (Garten et al., 2009).

HA is essential for influenza virus replication and mediates virus binding to sialic acid receptors on the cell surface and fusion of viral and endosomal membranes, ultimately leading to viral entry into the host cell (Palese and Shaw, 2007; Skehel and Wiley, 2000). HA-specific antibodies can block Influenza A virus infection by preventing receptor binding and/or fusion. Immunization with plasmid DNA encoding HA is capable of inducing cell mediated and humoral immunity (Bot et al., 1999; Deck et al., 1997; Trapp et al., 2005; Webster et al., 1994). An HA-based DNA vaccine was able to induce higher HA-specific antibody levels than similar vaccines containing other S-OIV genes (Wang et al., 2006). Plasmid vaccination of newborn mice and swine with HA is capable of inducing protective immune responses (Larsen et al., 2001; Robinson et al., 1993). Taken together, these studies showed the potential of HA-based DNA vaccines as a potential strategy for S-OIV control (Steitz et al., 2010).

Equine herpesvirus type 1 (EHV-1) is a member of the *Alphaherpesvirinae* and its genome is a double-stranded DNA of 150-kbp in length. EHV-1 can cause serious clinical disease in horses including respiratory disease, abortion and neurological disorders (Allen and Bryans, 1986; Slater, 2007). Recombinant EHV-1 was developed as an immunization vector and prominent features include its ability to enter a wide array of cell types of different origins and the induction of protective immune responses in various species and laboratory animals (Trapp et al., 2005). The large genome of EHV-1 allows insertions of foreign DNA, and EHV-1 can easily be manipulated using infectious genomes cloned in Escherichia coli (Trapp et al., 2005). The RacH modified live vaccine strain of EHV-1 has been cloned as an infectious bacterial artificial chromosome (BAC) (Rudolph et al., 2002). RacH has a proven safety record in horses and a number of other animal species and its attenuation could be attributed to a deletion of both copies of gene 67 (Hubert et al., 1996a; Neubauer et al., 1999b; Osterrieder et al., 1996a). We reported previously that RacH-based vaccine vectors can stably and efficiently express immunogenic proteins, induce humoral immune responses and protect vaccinated animals from heterologous virus challenge (Rosas et al., 2008a; Rosas et al., 2006; Rosas et al., 2007a, b).

In the present study, we developed an EHV-1 based vaccine expressing the H1 protein

derived from S-OIV California/4/2009. The recombinant vaccine was able to induce an immune response in a mouse model of influenza virus infection. Vaccinated mice were shown to be protected against clinical disease and were able to clear challenge virus from the respiratory tract of infected animals. We found that intranasal vaccination was superior to parenteral administration of the vaccine, which opens the possibility for local administration of the vaccine and control of virus replication at the portal of entry.

2.3 Materials and Methods

2.3.1 Viruses and cells

Rabbit kidney (RK13) cells were maintained in Earle's minimum essential medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). EHV-1 strain rRacH_EF1 (rH) (Ma and Osterrieder unpublished) and recombinant EHV-1 expressing H1 of S_OIV (rH_H1) were propagated in RK13 cells. For challenge infection, the second passage of strain A/Bayern/74/2009 H1N1 (kindly provided by B. Schweiger, Robert Koch-Institute, Berlin, Germany) originating from a human patient was used (Kalthoff et al., 2010). The virus was propagated on Madin–Darby canine kidney (MDCK) cells (Collection of cell lines in veterinary medicine, FLI Insel Riems, RIE1061). All experiments using S-OIV H1N1 virus were conducted using biosafety level 3 agriculture containment procedures.

2.3.2. BAC cloning and mutagenesis

The pRacH-EF1 (pRacH) BAC clone was generated previously by replacing the CMV promoter located inside the mini-F vector sequences (Rudolph et al., 2002) by a human elongation factor-1 alpha promoter (Ma and Osterrieder, unpublished). E. coli GS1783 cells harboring pRacH were maintained in Luria-Bertani (LB) broth or on LB agar containing 30 µg/ml of chloramphenicol. E. coli strain DH10B harboring plasmid pEP_H1 (Fig. 2.2.1). was grown in LB broth or LB agar containing 50 µg/ml of kanamycin and 50 µg/ml of ampicillin. The H1 gene of S-OIV A/California/4/2009 was commercially synthesized after codon optimization (Geneart, Germany). H1 was PCR-amplified using oligonucleotides SIV-FW and SIV-RV (Table 2.1) containing flanking restriction enzymes sites (*KpnI* and *XbaI*) for cloning into the multiple cloning site of pEP-CMVin (Rosas et al., 2007a and Tischer et al., 2006) and resulted in transfer plasmid pEP-CMVin. Recombinant pRacH1 harboring the

S-OIV H1 gene (pH_H1) was constructed using pH and pEP_H1 and two-step en-passant Red recombination (Tischer et al., 2006). GS1783 cells were grown at 32 °C and made electrocompetent exactly as described earlier (Tischer et al., 2006). The transfer cassette

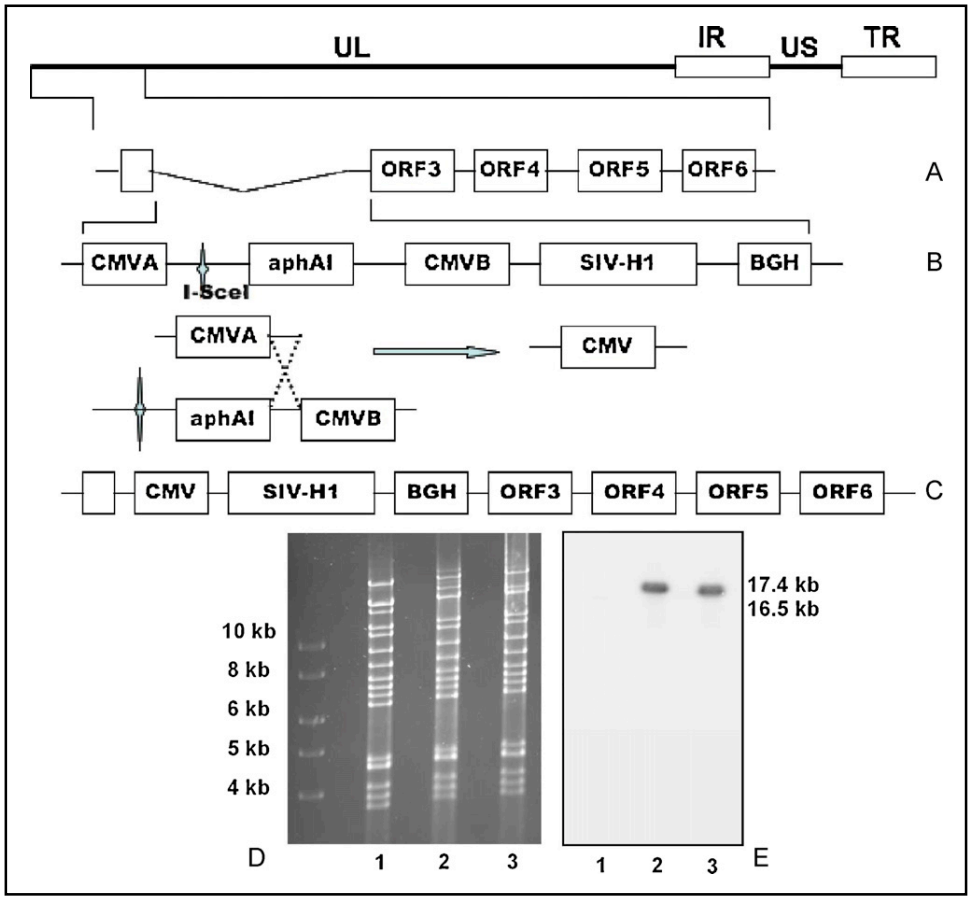

Figure 2.1. Schematic illustration of two-step (en-passant) red mutagenesis applied in the construction of recombinant rH_H1 virus. (A) Depiction of the left terminus of the unique-long segment of EHV-1 strain RacH infectious BAC genome termed pRacH. (B) The first recombination event resulted in the incorporation of S-OIV HA gene, HCMV promoter and kanamycin resistance gene in the ORF1/ORF2 locus of the RacH genome. (C) Second step of the en passant protocol in which resistance gene was removed and the final arrangement of rH_H1 genome including the SIV structural sequences is given. (D and E) Restriction fragment length polymorphisms (RFLPs) and Southern blot of pRacH, the cloning intermediate, and the final pH_H1 construct. An ethidium bromide-stained agarose gel is shown in the left panel with the *BamHI* restriction patterns of pRacH (lanes 1), the kanamycin-resistant intermediate (lanes 2), and pH_H1 (lanes 3). In the right panel, a Southern blot of the same gel is shown after hybridization with a digoxigenin-labeled S-OIV H1 probe.

containing H1 under control of the CMV IE promoter (Fig.2.1) was PCR-amplified from pEP_H1 with EP1-FW and EP2-RV primers (Table 2.1) and used for electroporation as described. After the first Red recombination, BAC DNA was isolated from chloramphenicol- and kanamycin-resistant colonies by using alkaline lysis, digested with *BamHI* and analyzed by 0.8% agarose gel electrophoresis. Positive clones were subjected to the second round of Red recombination to obtain the final construct after excision of the aphAI gene (Tischer et al., 2006). DNA from colonies that were kanamycin-sensitive but chloramphenicol-resistant was extracted and again checked by RFLP analysis after digestion with *BamHI*. Southern blotting was used to further verify the structure of the recombinant BAC clones. DNA fragments were transferred onto a positively charged nylon membrane and hybridized using a digoxigenin-labeled H1 probe (PCR DIG Probe Synthesis Kit, Roche). Hybridization was detected with an anti-digoxigenin AP antibody (Roche) and the bound antibody conjugate visualized by chemoluminescence (CDP-Star, Roche).

Name	**Sequence 5'-3'**
SIV-FW	ATTGGTACCGCCACCATGAAGGCCATCC
SIV-RV	TTGTCTAGATCAGATGCAGATCCGGCAT
pEP1-FW	TCGCCGCCATGAGACCCGAGGGAGTTTCGCGG GGCCGCGCCTCCTCTGTCTCCATCTCCAACTAG TTATTAATAGTAATC
pEP2-RV	CCCCGTACCCAGTGGGCAATAAGCGCCTCGGG CGTGAAGACGCAGCTTTTTAGCCCCGGGGCTA TTGTCTTCCCAATCCT
HA-FW1	ATCCTCGTCGTGCTGCTGTA
HA-FW2	CCTCACGCTGGCGCCAAGAG
HA-FW3	TAAGTACGTGAAGTCCACCA
HA-FW4	TGCGACAACACCTGTATGGA
HA-RV1	CAGTGCCGGATCTGCATCTTG
HA-RV2	ACCGCCGTGGGCAAAGAGTT
HA-RV3	GACCAGGAAGGCCGGATGAA
HA-RV4	AGTGCAATATCGCCGGCTGG

Finally, **primers listed in Table 2.1** (HA-FW1‑4 and HA-RV1‑4) were used for DNA sequencing to verify the integrity of the H1 insert in the recombinant pH_H1 BAC.

Sequencing reactions were performed with ABI PRISM Big Dye Terminator Cycle Sequencing V3.1 Ready Reaction kit (Applied Biosystems) and analyzed by using an ABI PRISM 3730 automated sequencer (Applied Biosystems). Assembly of the nucleotide sequences and translation into amino acid sequences were performed with Vector NTI version 9 software (Invitrogen).

2.3.3. Reconstitution of infectious virus from BAC DNA and virus growth assays

H1-expressing RacH was reconstituted after transfection of DNA into RK13 cells using the calcium phosphate transfection method (Rudolph and Osterrieder, 2002). Briefly, 5 μg of pH_H1 DNA was cotransfected into RK13 cells with 2 μg of plasmid DNA p71H expressing the full-length glycoprotein gp2 (von Einem et al., 2004). At day 5 after cotransfection, nonfluorescing virus plaques were picked and transferred to fresh RK13 cells. After two rounds of plaque purification, a homogenous virus stock was prepared and stored at −80 °C.

For plaque size measurements, confluent monolayers of RK13 cells in 12-well plates were infected with approximately 30 PFU/well of rH_H1. After 2 h the inoculum was replaced with EMEM containing 1.5% methylcellulose (400 cp, Sigma), 5% FBS and antibiotics. At 3 days post-infection (p.i.), plaques were stained by indirect immunofluorescence (see Section 2.4) using an anti-EHV-1 glycoprotein M (gM) monoclonal antibody (MAb) (Rosas et al., 2008c). Fifty plaques were photographed, and average plaque areas were determined using the ImageJ software (http://rsb.info.nih.gov/ij/). Values were calculated and compared to plaque areas induced by parental rH virus, which were set to 100%. Average percentages of plaque areas and standard deviations were determined from three independent experiments. The Shapiro–Wilks test was used to assess for normality and the Student's t-test was employed to compare the mean areas of the plaques of the examined viruses.

For single-step growth kinetics, RK13 cells were infected at a multiplicity of infection (MOI) of 3. Virus was allowed to attach for 1 h at 4 °C, then a penetration period of 1.5 h at 37 °C followed after which a treatment with ice cold citrate-buffered saline for 3 min was done to remove and inactivate unbound virus. At 0, 4, 8, 12, 24, 28 and 36 h after infection, culture supernatants and cells were harvested separately, and cell-associated and extracellular viral titers were determined by plating onto RK13 cells. At day 4 p.i., cells were stained with 0.3% crystal violet, and plaques were counted. Single-step growth curves were determined in three

independent experiments and means and standard deviations were computed and plotted. Student's t-test was used to test the differences of viral growth kinetics of examined viruses.

2.3.4. Indirect immunofluorescence assays (IFA) and western blotting

IFA was used to detect EHV-1 gM and gp2 or S-OIV HA expression by the recombinant viruses. Briefly, RK13 cells were seeded in 6-well plates and infected with rH_H1 or parental rH virus. At 2 h p.i., medium was removed and infected cells were overlaid with EMEM-1.5% methylcellulose. At 48 h p.i., cells were fixed with 3.7% formaldehyde in PBS for 20 min at RT and subsequently permeabilized with 0.2% Triton X-100 in PBS for 3 min. After washing with PBS, free binding sites were blocked using PBS containing 0.5% bovine serum albumin (BSA) for 30 min at RT. Cells were incubated with MAbs 3B12 (anti-gp2) (Rudolph et al., 2002) or A8 (anti-gM) (Rudolph et al., 2002) at a 1:10 dilution in PBS–0.5% BSA or with S-OIV H1N1 specific porcine serum after experimental infection (1:100 dilution) for 1 h at RT. After extensive washing with PBS, the secondary antibody, anti-mouse IgG or goat anti-swine IgG conjugated with Alexa 488 (Molecular Probes), was added at a 1:500 dilution for 30 min at RT. After thorough washing, plaques were inspected by using an inverted fluorescence microscope Zeiss Axiovert 100 and recorded with the Axiocam (Zeiss).

H1 expression by rH_H1 virus was also assessed by western blot analysis. Monolayers of RK13 cells in 12-well plates were either mock-infected or infected with recombinant viruses at an MOI of 1. At 24 h p.i., cells were resuspended in detergent buffer (50 mM Tris, pH 7.4; 1% Triton X-100; 0.25% Na-deoxycholate and 150 mM sodium chloride). Samples were mixed with sample loading buffer (1 M Tris/HCl, pH 6.8; 0.8% SDS; 0.4% glycerol; 0.15% β-mercaptoethanol; 0.004% bromophenol blue), heated at 95 °C for 5 min, and subjected to sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-10%-PAGE). Separated proteins were transferred to a polyvinyl flouride membrane (Roth) by the semi-dry method (Sambrook et al., 1989). Free binding sites on the membranes were blocked by incubation with PBS containing 2.5% skim milk, 2.5% BSA and 0.3% Tween 20 (PBS-T) overnight before the membrane was incubated with a 1:500 dilution of polyclonal antibodies raised in sheep against influenza A/California/7/2009 H1N1 (National Institute for Biological Standards and Control, code 09/152) in PBST. Bound antibodies were detected with

peroxidase-conjugated anti-sheep IgG at a 1:6000 dilution in PBS-T (Rockland). Reactive bands were visualized by enhanced chemoluminescence (ECL plus, Amersham).

2.3.5. Animal experiments

In a first animal experiment, 3-week-old female Balb/c mice (Charles River) were allocated randomly to two groups of 5 mice each and immunized with rH_H1 virus for a total of 3 times in 3-week intervals. Mice were anesthetized with 100 mg/kg ketamin and 10 mg/kg xylazine and inoculated intranasally (IN) with 1×10^5 PFU/animal in 40 µl EMEM. The second group was inoculated subcutaneously (SQ) with 1×10^5 PFU/animal in 100 µl EMEM. All mice were bled at days 14, 28, 35, 49 and 56. Serum titers to S-OIV H1 were determined by virus neutralization test (VNT) as described below as well as hemagglutination inhibition (HI) using influenza A/Bayern/74/2009/H1N1 and human erythrocytes according to the (WHO, 2010b).

In the second animal experiment, a vaccination-challenge infection was performed. Eight female Balb/c mice were vaccinated twice in a 3-week interval with rH_H1 by either the IN or SQ route. Mice from the IN group were immunized after a short anesthesia with isoflurane with 1×10^5 PFU/mouse in 40 µl of EMEM. SQ immunization was also achieved under isoflurane anesthesia using 1×10^5 PFU in 100 µl EMEM. Negative (non-inoculated) and positive (challenge infection only) control groups consisted of 3 mice per group. Challenge infection was performed by IN inoculation 10 days after the booster immunization with $10^{6.5}$ TCID50 of influenza A/Bayern/74/2009 under isoflurane anesthesia. All mice were monitored daily for clinical signs and euthanized on day 10 after challenge infection. All animal experiments were reviewed and approved by the state ethics committee (LALLF M-V/TSD/7221.3–1.1.-052/09).

Tissues from mice were collected at the time of necropsy and RNA was extracted with the nucleospin 96 RNA tissue kit (Macherey&Nagel) on an open robotic platform (Freedom EVO, Tecan). Individual samples were tested with real-time RT-PCR (rRT-PCR) specific for H1 (Hoffmann et al., 2010) and genomic loads were subjected to a semi-quantitative evaluation exactly as described previously (Hoffmann et al., 2009).

Serum samples obtained during the vaccination-challenge study were heat-inactivated at 56 °C for 30 min and tested using a nucleoprotein antibody enzyme-linked immunosorbent assay (ELISA, ID-Vet), which was performed exactly following the manufacturers' instructions for testing chicken sera. Serum samples were tested using a VNT, which was

modified according to a previously described procedure (Rowe et al., 1999). Briefly, 2-fold serial dilutions of sera were prepared in 50 µl EMEM in 96-well plates. The diluted serum samples were mixed with an equal volume of media containing A/Bayern/74/2009 adjusted at a concentration of 10 tissue culture infectious doses 50% (TCID50) per well. After 1 h incubation at 37 °C in a 5% CO2 humidified atmosphere, 1.5×10^4 MDCK cells were added to each well. The plates were incubated for 3 days as described and viral replication was assessed by visually scoring the cytopathic effect without staining. Each assay was validated by comparison with positive and negative control sera from chickens and cattle as well as back titration of the virus dilutions. Titers were expressed at log2 of the reciprocal of dilutions that caused 50% neutralization (ND_{50}).

2.4. Results

2.4.1. Generation of a vectored RacH vaccine expressing S-OIV HA (rH_H1)

To obtain recombinant rH_H1, pRacH was manipulated such that a codon-optimized version of the S-OIV H1 gene derived from influenza A/California/4/2009 was inserted into the infectious clone under the control of the HCMV IE promoter. To this end, the optimized H1 gene was first cloned into transfer plasmid pEp_CMVin and two-step en passant mutagenesis was employed for manipulation (Tischer et al., 2006). During the first recombination event, the insertion of the amplified cassette into the previously generated pH BAC clone resulted in kanamycin-resistant intermediates that exhibited the expected changes in the DNA restriction pattern. As predicted in silico, the insertion of the cassette resulted in a *BamHI* fragment of 17.4 kbp in size compared to the 13.8 kbp fragment present in the parental pRacH (Fig. 2.1.D). The second recombination event resulted in the removal of the aphA1 gene, which led to the reduction in size of the 17.4 kbp *BamHI* fragment to 16.5 kbp (Fig. 2.1.D). The results of the RFLP analysis were confirmed by Southern blotting, which revealed that only the 17.4- and 16.5-kbp *BamHI* bands containing the introduced S-OIV H1 sequences were reactive with the HA1-specific probe (Fig. 2.1.E). Nucleotide sequencing further confirmed the correct insertion of the gene at the left genomic terminus of the pRacH clone that otherwise appeared unaltered (data not shown). From the above results, we concluded that the generated recombinant pH_H1 BAC harbored the S-OIV H1 gene in the targeted locus. After confirmation of the genetic integrity of pH_H1, recombinant rH_H1 virus was reconstituted by co-transfection of p71 (Rudolph and Osterrieder, 2002) and pH_H1 DNA. rH_H1 expressing gp2 was obtained after two rounds of purification of plaques that did not exhibit

autofluorescence following the absence of EGFP in rH_H1 after replacement with gp2-encoding sequences (data not shown).

2.4.2. *In vitro* characterization of rH_H1

To evaluate expression of the H1 gene by the recombinant virus, RK13 cells were infected with rH_H1 and the expression of the S-OIV H1 gene was demonstrated by IFA. In RK13 cells infected with rH_H1, virus plaques were reactive with S-OIV H1-specific antibodies, whereas those induced by parental rH virus were not (Fig. 2.2.A). High-level expression of S-OIV H1 protein by rH_H1 was also confirmed by western blot analysis. A protein of approximately 80-kDa in size was specifically reactive with a commercial anti-H1 antibody in lysates of cells infected with the H1 expressing rH_H1, but was absent in cells that were mock-infected or infected with parental virus (Fig. 2.2.B). We concluded from these results that the recombinant rH_H1 efficiently expressed the S-OIV H1 protein *in vitro*.

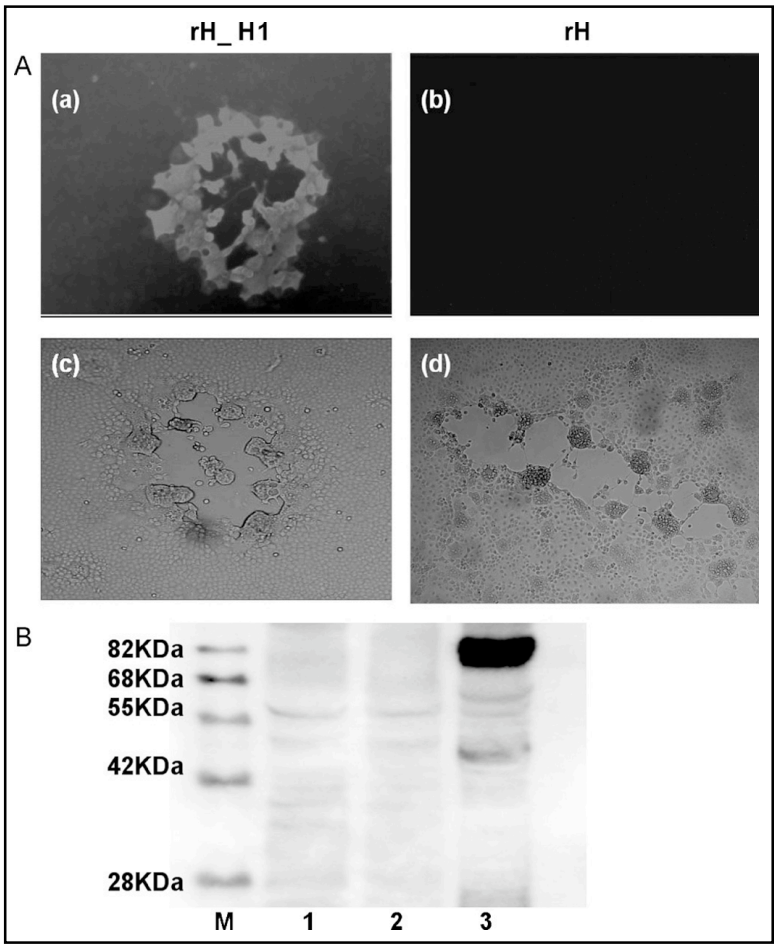

Figure 2.2. Visualisation of H1 expression from the rH_H1 virus (A) Immunofluorescence staining of RK13 cells infected with rH_H1 or rH virus. Plaques were stained with an anti-S-OIV H1 polyclonal antiserum. Bound antibodies were detected with conjugated goat anti-swine IgG antibody and visualized by fluorescence (a and b) or phase contrast (c and d) microscopy. (B) Western blot analysis of mock, rH and rH_H1 infected cells (lanes 1, 2 and 3, respectively). A specific band of approximately 80 kDa was visualized by enhanced chemoluminescence.

To determine whether the insertion of H1 had an effect on viral growth *in vitro*, plaque sizes and growth kinetics of rH_H1 were determined and compared to those of parental rH virus. The average size of rH_H1 plaques formed at 3 days p.i. were reduced in size by approximately 15% compared to parental virus (Fig. 2.3.A), however, this reduction did not reach statistical significance ($p > 0.05$). When viral replication was evaluated by single-step growth kinetics, both viruses exhibited comparable virus titers during the 36 h observation period, with respect to both extracellular and intracellular titers (Fig. 2.3.B). Virus titers at the end of the observation period were virtually identical between the analyzed viruses. Based on these results, we concluded that the insertion of H1 had negligable effects on viral growth *in vitro.*

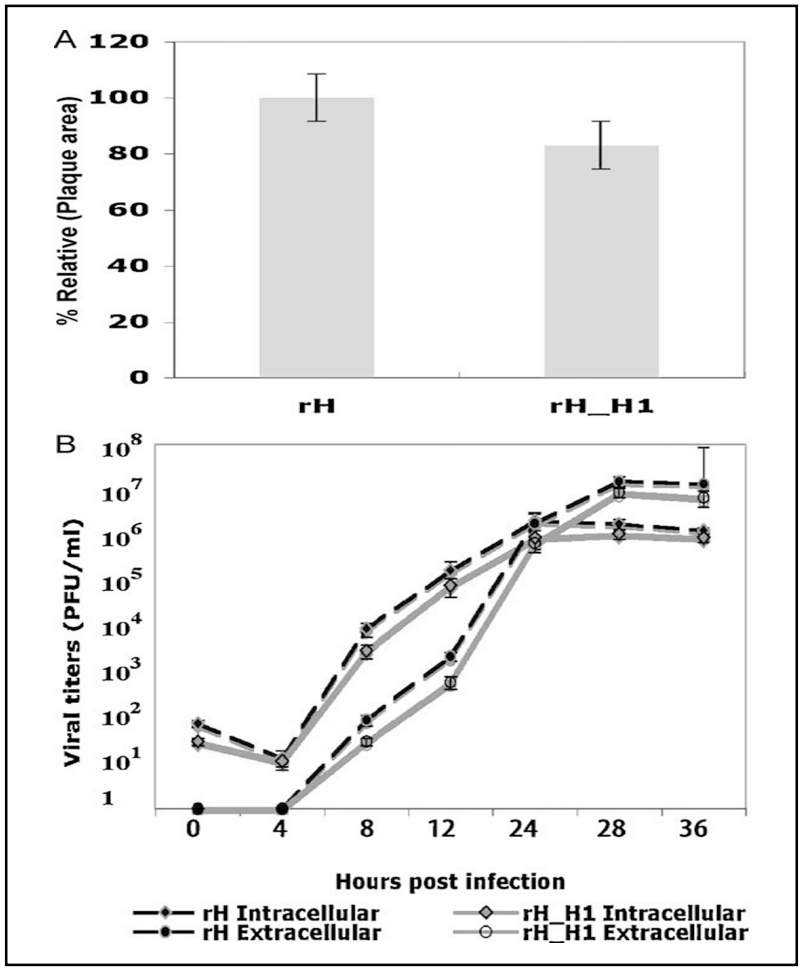

Figure 2.3. Comparison of *in vitro* growth properties of rH_H1 with those of parental virus. (A) Fifty plaques per virus were measured. *In vitro* growth properties of rH_H1 were comparable to those of the parental virus. (B) Analyses of single-step growth kinetics revealed no major differences in growth properties of parental and recombinant virus. Error bars represent standard deviations. These results are representative of three independent experiments.

2.4.3. Serological responses induced in mice immunized with rH_H1

Pilot vaccination studies were done in Balb/c mice to determine whether rH_H1 was capable of inducing humoral immune responses against H1N1 influenza virus. Antibody responses following vaccination with rH_H1 were detected as early as day 14 after the first dose of vaccine administered in the IN (1:64) group (Fig. 2.4.). A high HI antibody titer of 1:1024 was detected one week after the first booster vaccination in the IN group. The antibody levels remained stable throughout the observation period until the end of the experiment (day 56). Animals inoculated by the SQ route exhibited a weaker antibody response with a peak HI titer of 1:64 determined at the end of the observation period. These results indicated that the EHV-1 recombinant virus is capable of expressing the H1 protein *in vivo* in the mouse and can induce the production of HI-specific reactive serum, especially after IN application.

Virus neutralizing antibodies to H1N1 virus after SQ or IN vaccination were also evaluated by VNT. Consistently high levels of neutralizing antibodies were detected in the IN group, while animals in the SQ group exhibited lower titers. The first neutralizing response in the IN group was detected already by day 14 after the 1st immunization. Titers on day 49 reached values of more than 210.6 ND_{50} in the IN group, while the SQ group had lower titers of 23.6 (Table 2.2). The data of the VNT corroborated those obtained by HA and ELISA and suggested a robust immune response after immunization with the generated EHV-1-vectored H1N1 vaccine, especially after IN administration.

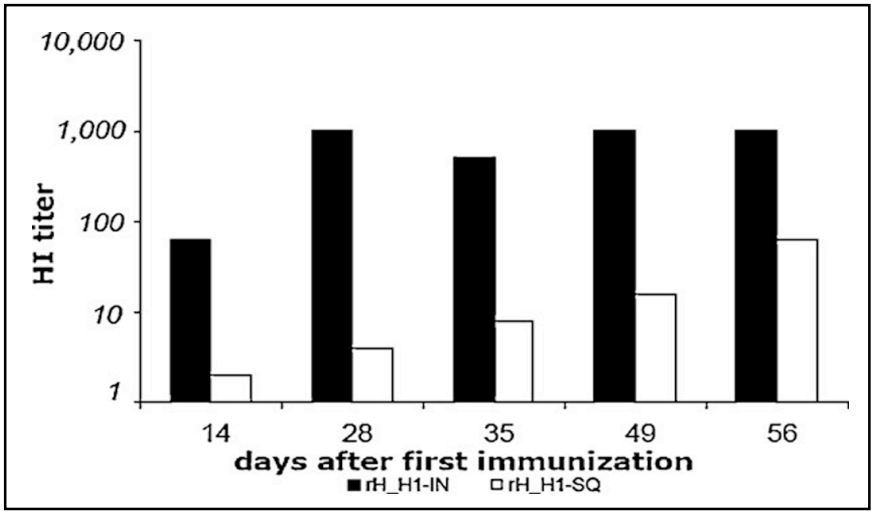

Figure 2.4. Immunization of mice with rH_H1 induces anti-H1-specific antibodies. Mice were immunized IN or SQ at days 0, 21 and 42. Serum from immunized mice (5 mice per group) was pooled and titrated. HI titers are given at different time points after first inoculation.

Table 2.2. Virus neutralizing antibody titers in vaccinated mice.

	Vaccinated mice[a]	
	Days post immunization	Neutralization titer ND_{50} (log2)
IN	14	3.3
	49	10.6
SQ	14	<3
	28	<3
	49	3.6
	56	9

[a]Serum not available for days 28 and 56 in the IN group.

2.4.4. Protective efficacy of EHV1-expressed H1 in mice

To determine the protective efficacy of the rH_H1 vaccine against challenge infection, groups of mice were immunized twice in a 3-week interval by either the IN or the SQ route and challenged 10 days after the second vaccination with Influenza A/Bayern/74/2009 (H1N1), a strain that is capable of reliably inducing morbidity in naive mice. None of the mice showed any signs of illness or adverse effects after vaccination. Daily clinical evaluation of the mice revealed ruffled fur observed in mock-immunized animals from 3 days post challenge (p.c.) until day 10 p.c. Additionally, the mice in this group showed reduced mobility and apathy starting from day 4 p.c., when one mock-immunized mouse died. In contrast, ruffled fur was observed only on day 4 p.c. in individuals of both groups immunized with rH_H1 and mice in these groups were clinically inconspicuous otherwise. Sera of immunized and challenged mice were investigated for the presence of influenza A virus nucleoprotein-specific antibodies as measured by a commercial competitive ELISA. All but one serum sample were clearly positive following challenge, indicating the development of antibodies against replicating challenge virus (Fig. 2.5). The one negative serum originated from a mouse of the IN group. Similarly, VNT titers at day 10 after challenge reached titers of more than 211 in all cases and regardless of the route of immunization, thus corroborating a robust anamnestic response of vaccinated animals following challenge infection.

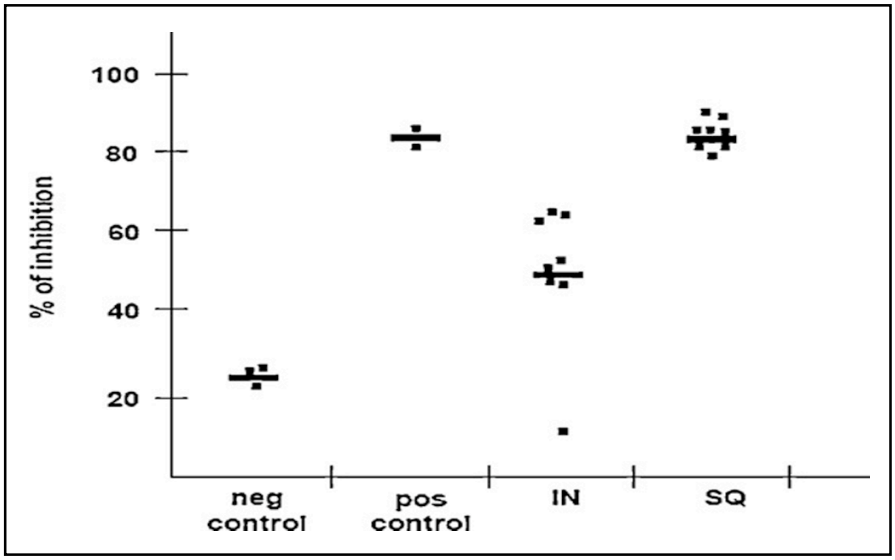

Figure 2.5. Induction of humoral immune responses in vaccinated and unvaccinated mice after challenge infection. A competitive NP ELISA (ID-VET) was used. Results are given as % of inhibition. Samples with over 35% of inhibition were considered being positive.

To further evaluate whether vaccination provides control of challenge virus replication, viral RNA present in samples from different organs was measured by qRT-PCR (Table 2.3). All 3 mice in the mock-immunized group exhibited medium to high viral loads in respiratory organ samples and the brain. Additional organs (brain, myocardium, spleen) of the deceased individual also presented detectable viral loads. In contrast, viral RNA could not be detected from the organs in any of the mice immunized by the IN route with the recombinant rH_H1 virus. In the SQ-immunized group, only low levels of viral genome loads were detected in respiratory organ samples. As expected, all samples from the negative control group were negative for H1N1 genomes. From the results of these experiments we concluded that rH_H1 vaccination can protect mice against challenge infection and can confer effective control of viral replication, especially after IN application.

Table 2.3. Distribution of viral genomic load in tissues of inoculated mice.

Groups		Viral RNA load in tissues[a]				
		Lung	Brain	Myocardium	Trachea	Nose
unvaccinated and uninfected control	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
unvaccinated and infected control	1	+++	-	-	++	++
	2	++	-	-	+++	+
	3[b]	+++	+	++	+++	+++
IN-vaccinated and infected	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
	4	-	-	-	-	-
	5	-	-	-	-	-
	6	-	-	-	-	-
	7	-	-	-	-	-
	8	-	-	-	-	-
SQ-vaccinated and infected	1	+	-	-	+	-
	2	-	-	-	-	-
	3	-	-	-	-	+
	4	-	-	-	+	+
	5	-	-	-	+	+
	6	+	-	-	-	+
	7	-	-	-	+	+
	8	++	-	-	+	+

Real time RT-PCR results are given as cycle of threshold (Ct) values: − (no viral RNA, Ct of ≥40), + (low viral RNA load, Ct of 35–39), ++ (moderate viral RNA load, Ct of 30–34) and +++ (high viral RNA load, Ct of ≤29).

[a]Spleen and liver samples were negative in all groups.

[b]This mouse died at day 4 after challenge.

2.4. Discussion

Influenza viruses, and the 2009 S-OIV is no exemption, can cause severe respiratory disease characterized by predominantly respiratory and gastrointestinal symptoms in a number of species. While infection of livestock can result in a significant negative economic impact for the industry, infections of companion animals, livestock and wild birds are of importance with respect to public health. Swine are commonly referred to as a "mixing vessel" for new influenza reassortants because of their susceptibility to both avian and human influenza A viruses and the possibility to generate new viruses by dual infections. Some of these viruses

may have the potential to transmit to humans (Karasin et al., 2000). The new 2009 S-OIV H1N1 virus likely was the result of such a recombination event and contains a combination of gene segments that had not been previously identified in swine or human influenza isolates (Chang et al., 2009; Garten et al., 2009). The virus rapidly spread throughout the world and caused a human pandemic (Michaelis et al., 2009). However, companion animals may also be important for the introduction of influenza viruses into the human population. (Sponseller et al., 2010) Reported the presence of S-OIV in domestic cats, most likely transmitted to the feline by infected owners. Thus, the circulation of S-OIV between human and household animals including dogs and cats would be a potential source of cross-species adaptation and spread of influenza virus (Bao et al., 2010). In addition, other influenza viruses including avian influenza virus H5N1 and H3N2 are capable of infecting dogs and cats (Giese et al., 2008; Song et al., 2008).

It is clearly important to develop effective strategies to control influenza replication in both companion animals and livestock and to prevent circulation of viruses between the human and animal populations. Vaccines may be able to prevent cross-species adaptation and thereby decrease the possibility of creating potentially pandemic human influenza virus reassortants (Haque et al., 2007). Currently, modified live vector vaccines (MLV) expressing codon-optimized antigens, which are copies of the naturally encoded genes, are being intensively studied. Such vaccines have been evaluated in several species for their potential to stimulate both humoral and cell-mediated responses against influenza viruses (Bublot et al., 2006a; Karaca et al., 2007; Minke et al., 2007a; Minke et al., 2007b). In addition, recombinant EHV-1-based vectored vaccines can stably and efficiently express immunogenic proteins of other viral pathogens (Robinson et al., 1993; Rosas et al., 2008a; Rosas et al., 2006; Rosas et al., 2007a; Rosas et al., 2008c; Rosas et al., 2007b; Rosas et al., 2007b).

In this study, we constructed and evaluated an EHV-1 recombinant vaccine, based on the well-characterized RacH MLV, expressing a codon-optimized version of S-OIV H1. Recombinant rH_H1 virus was generated through manipulation of the RacH genome as described earlier (Tischer et al., 2006). In line with previous studies (Rosas et al., 2008a; Rosas et al., 2006; Rosas et al., 2007a; Rosas et al., 2008c; Rosas et al., 2007b; Rosas et al., 2007b), insertion of the S-OIV H1 gene into the EHV-1 genome did not affect *in vitro* growth characteristics and the recombinant virus was able to propagate in cell culture as efficiently as the parental virus.

When the recombinant virus was inoculated into mice, humoral responses against H1N1 influenza virus were induced following either IN or SQ administration and antibody titers were maintained at high levels up to day 56 after immunization, the time point when the experiment was terminated. Interestingly, the magnitude of the immune response was greater after IN administration, which may be caused by the fact that EHV-1 can replicate in the epithelium of the respiratory tract that contains dendritic cells and macrophages, potent antigen-presenting cells, which may result in a more specific presentation of the vectored antigen (Siedek et al., 1999).

As humoral antibody responses against HA are considered good indicators for evaluating potentially protective immunity upon natural infection or vaccination (Couch and Kasel, 1983; Schild et al., 1975), we surmised a protective ability of the vectored EHV-1-H1 vaccine. In addition, as antigen is produced in the immunized animal after application of the vectored vaccine, induction of a cell-mediated immunity is likely (Bot et al., 1997; Deck et al., 1997; Ulmer et al., 1993; Webster et al., 1994) and probably also the case for EHV-vectored vaccines. We therefore tested the ability of rH_H1 to induce protection against challenge infection with a local S-OIV strain, A/Bayern/74/2009 (H1N1). This strain possesses a high 98.7% homology on the HA amino acid level compared to that of the recombinant virus rH_H1 (Fig. 2.6). Two routes of vaccination (IN and SQ) were used to study whether immunization routes had an effect on vaccine efficacy against challenge infection similar to what was observed with respect to the induction of a humoral immune response. Importantly, all mice vaccinated with rH_H1 and challenged with S-OIV survived and none showed any clinical signs of illness or adverse effects after vaccination. Daily clinical observation of mice after challenge infection revealed that individuals from both immunized groups showed ruffled fur for one day only (day 4 p.c.). In contrast, mock-immunized mice were depressed and presented with ruffled fur for a total of 5 days and one mouse died on day 4 p.c. Faster virus clearance was also observed in both vaccinated groups, with the IN route showing more efficient virus clearance. Although not measured here, the IN route may have induced secretory IgG or IgA with neutralizing activity, which might have not been achieved by the SQ route. Because the respiratory tract is the natural site of influenza virus entry and replication, the use of EHV-1 and its protective potential make this virus an attractive alternative for the construction of recombinant vaccines against influenza. Overall, robust serum anti-NP antibodies as measured by ELISA correlated with high levels of

neutralizing antibodies. Although the number of tested animals was low, one out of eight mice vaccinated IN was negative in an ELISA test for NP-specific antibodies. One explanation is that this individual inhibited viral replication so efficiently that immunity against NP did not develop.

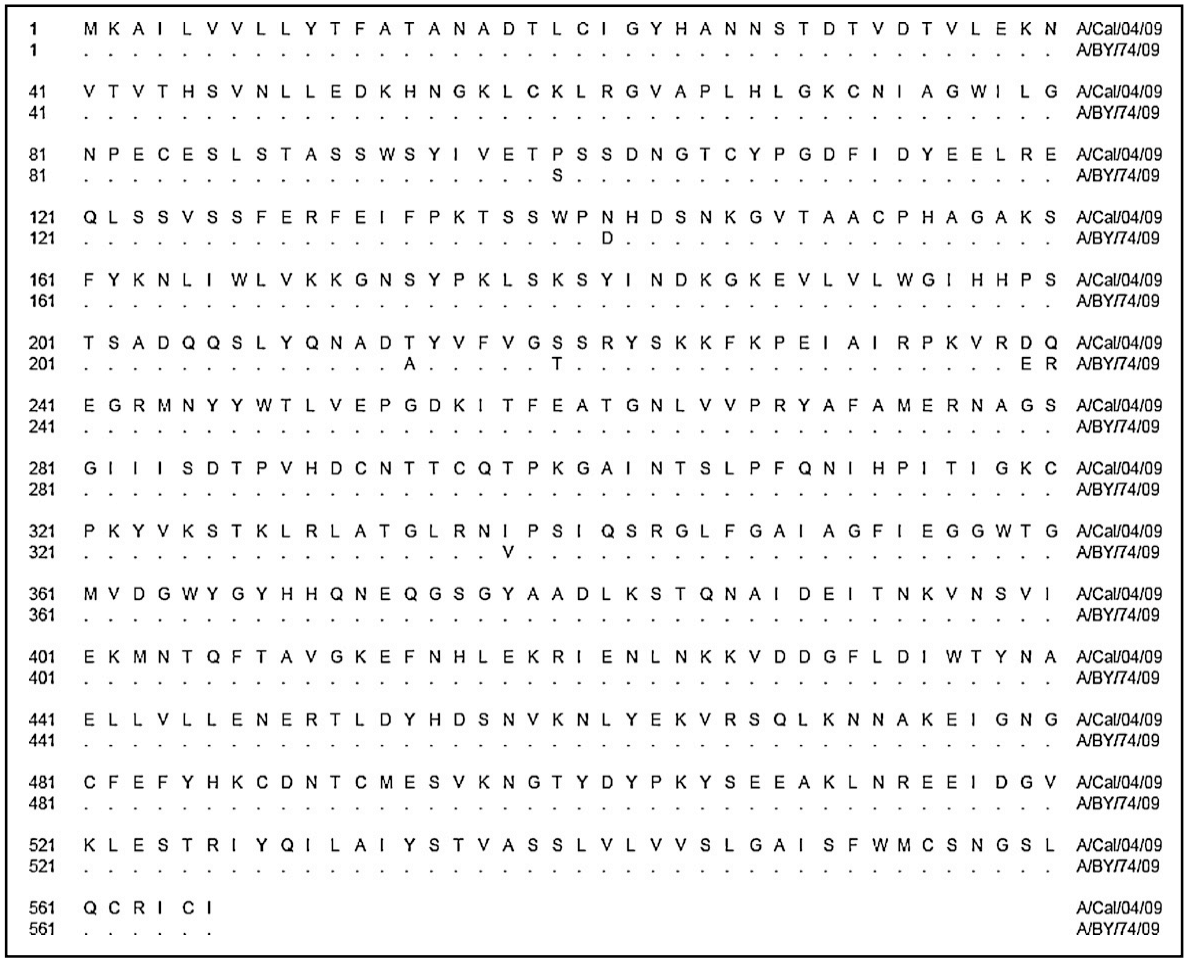

Figure 2.6. Amino acid sequence alignment of influenza A/California/4/2009 (A/Cal/04/09) with that of A/Bayern/74/2009 (A/BY/74/09). High sequence similarity (98.7% homology) between the two HAs is evident.

In summary, we developed a recombinant EHV-1 vaccine encoding the HA gene from S-OIV H1N1 (Influenza-A/California/4/2009 H1N1) and evaluated its efficacy as a vaccine. Our study demonstrated that the EHV-1 recombinant generated, rH_1H1, is capable of inducing high levels of H1 antibodies in the mouse model of influenza virus infection. Moreover, Balb/c mice were protected against clinical disease induced by S-OIV H1N1 and virus clearance was greatly enhanced. In future studies, we plan to determine the efficacy of our

vaccine in the protection of livestock (pigs) and companion animals (dogs) from infection with S-OIV H1N1.

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Chapter 3: Recombinant equine herpesvirus 1 (EHV-1) vaccine protects pigs against challenge with influenza A(H1N1)pmd09

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3.1. Summary

Swine influenza virus (SIV) is not only an important respiratory pathogen in pigs but also a threat to human health. The pandemic influenza A(H1N1)pdm09 virus likely originated in swine through reassortment between a North American triple reassortant and Eurasian avian-like SIV. The North American triple reassortant virus has genes from avian, human and swine influenza viruses. An effective vaccine may protect the pork industry from economic losses and curb the development of new virus variants that may threaten public health. In the present study, we evaluated the efficacy of a recombinant equine herpesvirus type 1 (EHV-1) vaccine (rH_H1) expressing the hemagglutinin H1 of A(H1N1)pdm09 in the natural host. Our data shows that the engineered rH_H1 vaccine induces influenza virus-specific antibody responses in pigs and is able to protect at least partially against challenge infection: no clinical signs of disease were detected and virus replication was reduced as evidenced by decreased nasal virus shedding and faster virus clearance. Taken together, our results indicate that recombinant EHV-1 encoding H1 of A(H1N1)pdm09 may be a promising alternative for protection of pigs against infection with A(H1N1)pdm09 or other influenza viruses.

3.2. Introduction

Swine influenza (SI) is a highly contagious viral infection in pigs and is characterized by coughing, nasal discharge, elevated temperatures, breathing difficulties and reduced appetite (Amorij et al., 2010; Tang et al., 2002; Wang and Palese, 2009b). The disease is caused by SIV, which belongs to the influenza A virus genus in the *Orthomyxoviridae* family. The influenza A viruses are characterized by a segmented genome of eight single-stranded RNA molecules of negative polarity (Castrucci et al., 1994; Palese and Shaw, 2007; Webster and Bean, 1978). Influenza A viruses undergo antigenic shift (or reassortment) and drift processes

leading to the continued generation of new virus variants. Pigs have both major types of viral *receptors in the respiratory tract that are* characterized by sialic acids with α2,3 and α2,6 linkages, respectively (Ito et al., 1998, 2000). Therefore, pigs have been considered an important reservoir host or even a mixing vessel for the generation of new reassorted strains with pandemic capacity (Castrucci et al., 1994; Garten et al., 2009; Kida et al., 1994; Schultz et al., 1991).

In April 2009, a novel swine-origin H1N1 influenza A virus, later by convention referred to as A(H1N1)pdm09 (WHO, 2011), was identified. The virus has a distinct combination of gene segments from both North American and Eurasian swine influenza lineages as well as avian and human lineages (Furuse et al., 2010; Garten et al., 2009; Girard et al., 2010; Smith et al., 2009). The susceptibility of pigs to this particular virus strain was confirmed in several experimental studies (Lange et al., 2009; Vincent et al., 2010b). The clinical signs associated with A(H1N1)pdm09 infection in pigs were similar to those caused by endemic swine influenza strains. As a consequence of the appearance of A(H1N1)pdm09, the porcine industry was severely impacted as pork consumption dropped primarily because of the misconception that the disease can be transmitted through meat (Pappaioanou and Gramer, 2010).

Infected pigs may, however, become a source of infection for humans, even if the virus does not succeed in becoming endemic in pig populations (Irvine and Brown, 2009; van Reeth et al., 2007). Therefore, vaccination of pig populations by highly effective vaccines that are able to reduce virus excretion may help reduce the risk for human infection. The viral hemagglutinin (HA), one of the two envelope glycoproteins, is the main viral antigen (Hessel et al., 2011; Hghihghi et al., 2011). A number of studies have demonstrated that immunization with recombinant HA is capable of inducing both cell-mediated and humoral immune responses (Hessel et al., 2011). Currently available commercial swine influenza vaccines are traditional, inactivated whole virus preparations containing the H3N2 and H1N1 subtypes. The inactivated virus vaccines are produced either in eggs or in cell culture and usually efficacious at reducing clinical signs, although it was shown that they may enhance disease in some cases (Kobinger et al., 2010; Vincent et al., 2008). Previous studies suggested that cell-mediated and/or mucosal responses, which are not stimulated by inactivated virus vaccines, are essential for induction of heterologous immunity (Ma and Richt, 2010; Van Reeth et al., 2004). Moreover, the use of whole virus vaccines does not allow for differentiation between infected and vaccinated animals. Pigs are often asymptomatic after SIV infection but can still

spread the virus. For these reasons, efforts have concentrated on the development of alternative vaccine production methods by engineering recombinant virus vaccines against SIV (Sipo et al., 2011; Tang et al., 2002; Tian et al., 2006; Wesley et al., 2004). In our study, we used equine herpesvirus type 1 (EHV-1) as a delivery vector for influenza virus HA to protect pigs against challenge infection with the A(H1N1)pdm09. EHV-1 is a member of the genus *Varicellovirus*, which belongs to the subfamily *Alphaherpesvirinae* in the *Herpesviridae* family. The EHV-1 genome is a double-stranded DNA of 150-Kbp in length (Davison et al., 2009). The virus is highly prevalent in the horse population and causes mild to severe clinical disease that includes respiratory distress, abortion storms and neurological disorders in equines (Allen and Bryans, 1986; Telford et al., 1992). The potential of the EHV-1 RacH modified live virus (MLV) vaccine strain as an immunization vector has been highlighted by its ability to stably and efficiently deliver immunogenic proteins and induce both humoral and cellular immune responses (Osterrieder et al., 1996; Rosas et al., 2008; Rosas et al., 2007a; Rosas et al., 2007b). The RacH strain has a proven safety record in horses and in a number of other animal species including mice, dogs and cattle (Rosas et al., 2008; Rosas et al., 2007a; Rosas et al., 2007b; Said et al., 2011). Its attenuation could be attributed to a deletion of both copies of gene 67. Other genomic alterations, such as the truncation of the glycoprotein B, also contribute to its complete a pathogenicity for various of species (Hubert et al., 1996; Neubauer et al., 1999; Osterrieder et al., 1996).

We previously reported on the construction of an EHV-1-based vaccine expressing the H1 derived from A(H1N1)pdm09 A/California/4/2009 that was termed rH_H1 (Said et al., 2011). We demonstrated that the rH_H1 vaccine was able to induce an immune response in mice in which a reduction of clinical signs and faster virus clearance from the respiratory tract was documented. Here, we evaluate the extent to which the vaccine is able to protect pigs against challenge infection with the A(H1N1)pdm09 virus. We show that vaccinated pigs mounted robust immune responses and had significantly reduced virus loads in the respiratory tract after challenge infection.

3.3. Materials and Methods

3.3.1. Viruses and cells

Rabbit kidney (RK13) cells were maintained in Earle´s minimal essential medium (EMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 U/ml

penicillin and 0.1mg/ml streptomycin). Modified live EHV-1 expressing H1 (rH_H1) of A(H1N1)pdm09 (A/California/4/2009) was propagated in RK13 cells (Said et al., 2011). For challenge infection, A/Bayern/74/2009/H1N1 (kindly provided by B. Schweiger, Robert Koch-Institut, Berlin, Germany) originating from a human patient was used (Kalthoff et al., 2010). The virus was propagated on Madin–Darby Canine kidney (MDCK) cells (collection of cell lines in Veterinary Medicine, FLI Insel Riems, RIE1061).

3.3.2. Animals

Nine-week-old piglets were obtained from a high health status-breeding herd in which no seroconversion against any pathogenic diseases including influenza had been observed. Moreover, all animals were screened with an enzyme linked immunosorbent assay to test for the presence of antibodies against influenza A virus nucleoprotein before vaccination using a commercial kit (ELISA NP, ID Screen®Influenza A Antibody Competition, ID Vet, France). All piglets were housed in isolation rooms in biosafety level 3 facilities at the Friedrich-Loffler-Institut. Animal care procedures were in accordance with state animal welfare guidelines under the supervision of an ethics committee.

3.3.3. Experimental design

Ten piglets were allocated randomly to two groups. In group 1 (n = 5), piglets were vaccinated intramuscularly (IM) with the modified live EHV-1 vector vaccine expressing H1 of H1N1 (pdm09). Per pig, $1x10^5$ PFU/ml rH_H1 were applied twice in a 3-week interval (rH_H1-immunized group). Group 2 (n = 5) piglets were left unvaccinated and served as controls (mock-immunized group). Three weeks after the second vaccination, the animals in both groups were challenged intranasally (IN) with a suspension containing $1x10^6$ tissue culture infectious doses 50% ($TCID_{50}$) per ml of A/Bayern/74/2009/H1N1. The challenge dose was estimated to be within the range of natural exposure to the virus during an outbreak.

3.3.4. Clinical records and sampling

Clinical symptoms and body temperatures were recorded daily from the first day after challenge infection until the end of the experiment. Serum samples were collected during various time points after vaccination (0, 7 and 21 days post vaccination, dpv) and challenge infection (0, 7, 10, 14 and 21 days post infection, dpi). Serum samples were heat-inactivated

at 56°C for 30 min and tested using an NP ELISA. In addition, serum samples were subjected to hemagglutination inhibition (HI) assays to detect antibodies against H1. Nasal swabs were collected from 0 until 10 dpi, and suspended in 2 ml EMEM containing 5% FBS and 10% antibiotics. The samples were stored at -70°C until analysis by a quantitative real-time RT-PCR (qRT-PCR) to determine viral genome copies in swabs as described previously (Lange et al., 2009).

3.3.5. Hemagglutination inhibition (HI) assay

The immunogenicity of the recombinant vaccine was evaluated by HI. Sera were treated with receptor-destroying enzymes followed by heat inactivated at 56 °C for 30 min. Sera were then treated to remove non specific hemagglutinin inhibitors and natural serum agglutinins, and finally pre-adsorbed with 1% chicken red blood cells according to procedures described previously (Vincent et al., 2010a). The HI assays were then performed with A/Bayern/74/2009/H1N1, A/Regensburg/D6/2009/H1N1 and A/swine/Germany-MV/ Wessin8/2011/H1N1 viruses as antigens. Titers were determined using 2-fold serial dilutions to detect end points of agglutination.

3.3.6. Quantitative reverse transcriptase real time PCR (qRT-PCR)

Viral RNA was extracted from swab samples using the QIAamp viral RNA Kit (Qiagen) according to the manufacturer's instruction. One-step real-time RT-PCR was performed using the AgPath-ID^{TM} One-Step RT-PCR Kit (Applied Biosystems, USA) with primers and probes (Table 3.1) targeting the influenza A virus M gene (Lange et al., 2009). The RT-PCR assay was optimized using a total volume of 25 µl. Briefly, 4.5 µl RNase-free water, 12.5 µl 2x RT-PCR buffer, 1.0 µl 25x RT-PCR Enzyme Mix and 2 µl primer-probe-mix (final concentration of 0.8 µM for the forward and 0.6 µM for the reverse primers) and 0.1 µM for the probe were pooled as a master mix. Finally, 5 µl RNA was added. The amplification conditions were as follows: reverse transcription at 45°C for 10 min; initial denaturation reaction at 95°C for 10 min; 42 PCR-cycles of 15 sec at 95°C (denaturation), 20 sec at 55°C (annealing) and 30 sec at 72°C (elongation). Fluorescence values were collected during the annealing step.

Table 3.1: Oligonucleotide primers and probe used in this study

Primers	Sequence 5'-3'	Position
SWH1FW	GTGCTATAAACAGAATYC CA	934-956
SWH1RV	CGGGATATTCCTTAATCCTGTRGC	1017-994
SWH1Probe-FAM	FAM-TCAGGCCCCCTCAAAGCCGA-BHQ1	968-944

Statistical analysis

Student's *t*-test was used to compare ELISA NP and HI titers in serum samples as well as viral loads in nasal swabs in rH_H1- and mock-immunized groups. The significance level was set at 0.05.

3.4. Results

3.4.1. Serological responses are induced in rH_H1-immunized pigs prior to experimental challenge

Serological studies were done in piglets to determine whether rH_H1 was capable of inducing humoral immune responses against A(H1N1)pmd09 and heterologous H1 strains. Serum samples were examined by HI after the second vaccination, which showed that all animals vaccinated with rH_H1 mounted high antibody titers against A(H1N1)pmd09 (Fig.3.1).

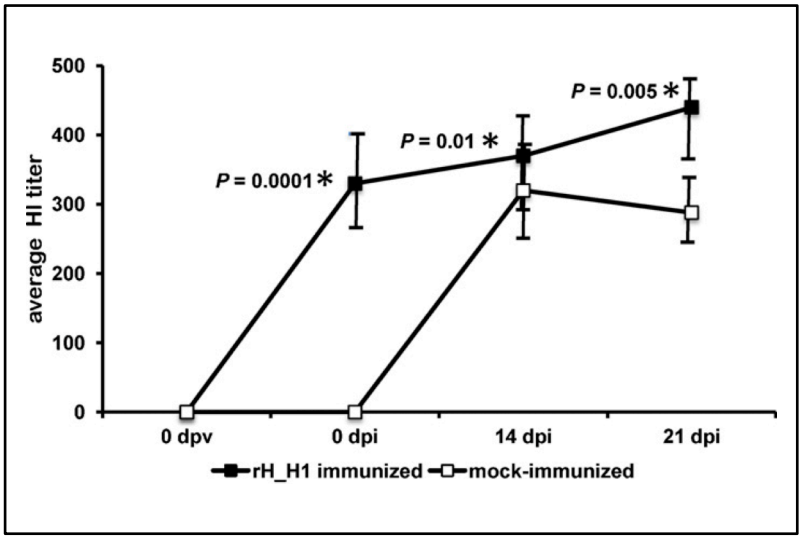

Figure 3.1. HI antibody titers induced by vaccination and challenge. Serum samples were tested at the indicated days post-vaccination (dpv) and post-challenge (dpi). Each data point represents the mean ± SD of HI antibody titers. The asterisk (*) denotes statistically significant differences between groups ($p<$ 0.05).

As expected, all animals in the mock-immunized group did not show any H1 specific antibody Moreover, anti-influenza virus NP antibody levels were determined in serum samples from all animals at 0, 7 and 21 dpv using ELISA NP. All animals, including those in

the vaccination group, remained seronegative for influenza A virus during the vaccination period (data not shown). It must be noted that one of the piglets from the immunized group died between the first and second vaccination for causes unrelated to vaccination. We concluded from our results that the engineered generated EHV-1-vectored H1 vaccine is capable to induce robust immune responses in vaccinated animals.

3.4.2. Immunization of pigs with rH_H1 results in protection against experimental challenge infection

To determine the protective efficacy of the rH_H1 vaccine against challenge infection, pigs were challenged by intranasal inoculation 3 weeks after the second vaccination. The mock-immunized control group was challenged with the same virus and route. Clinical signs and induction of specific humoral immune responses upon challenge infection were recorded. Moreover, the amount and duration of virus shedding in nasal swabs was investigated in both groups. No clinical symptoms were observed in the vaccinated animals, while two piglets in the mock-immunized group showed coughing and conjunctivitis at 7 dpi. In addition, body temperatures of immunized animals remained within the normal range during the entire experiment. In contrast, four animals in the mock-immunized group exhibited fever at 1 and 4 dpi (temperatures > 40 °C) (Fig. 3.2) with no other associated clinical signs. The results indicate that rH_H1 vaccine is able to protect pigs against challenge infection as concluded from the absence of clinical signs.

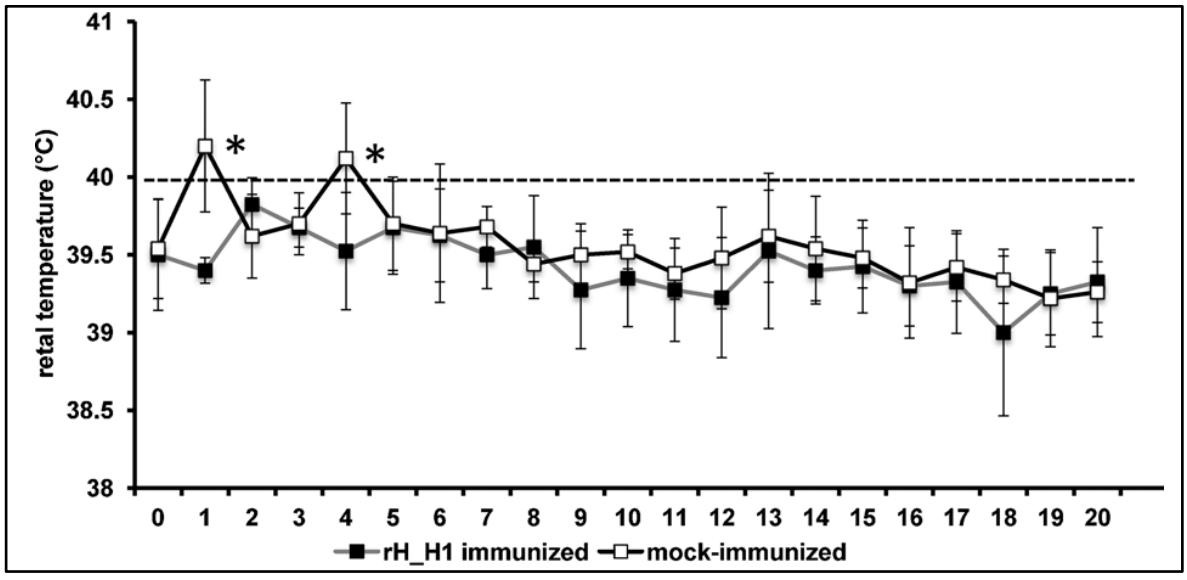

Figure 3.2. Graphical representation of febrile responses upon challenge infection. A body temperature above 40°C (dotted line) indicates fever. Data are represented as means ± SD of daily

rectal temperatures for each group. The asterisk (*) denotes statistically significant differences between groups ($p< 0.05$).

3.4.3. Immunized animals developed a specific humoral anamnestic response that interfere with replication of challenge virus

To test the ability of rH_H1 in induction of an anamnestic humoral immune response against challenge infection with A/Bayern/74/2009, serum samples were tested by HI assay. In animals vaccinated with rH_H1, the H1-specific antibody responses detected at the day of challenge exhibited only a slight increase following challenge infection (Fig.3.1). On the other hand, mock-immunized animals also produced an H1-specific antibody response, which, however, remained lower than those in the vaccinated group and were on the decline at 21 dpi (Fig.3.1). To further evaluate whether vaccination of animals with the rH_H1 vaccine provides cross reactivity, we tested the HI-specific antibody response after the second vaccination to A/Bayern/74/2009, A/Regensburg/D6/2009 and A/swine/Germany-MV/Wessin8/2011. The viruses share 96.6%, 97.8% and 97.3% amino acid identity, respectively, with the H1 of California/4/2009/H1N1 used for vaccination. The mean HI antibody response against A/swine/Germany-MV/Wessin8/2011 was 1:320 and 1:20 against A/Regensburg/D6/2009.

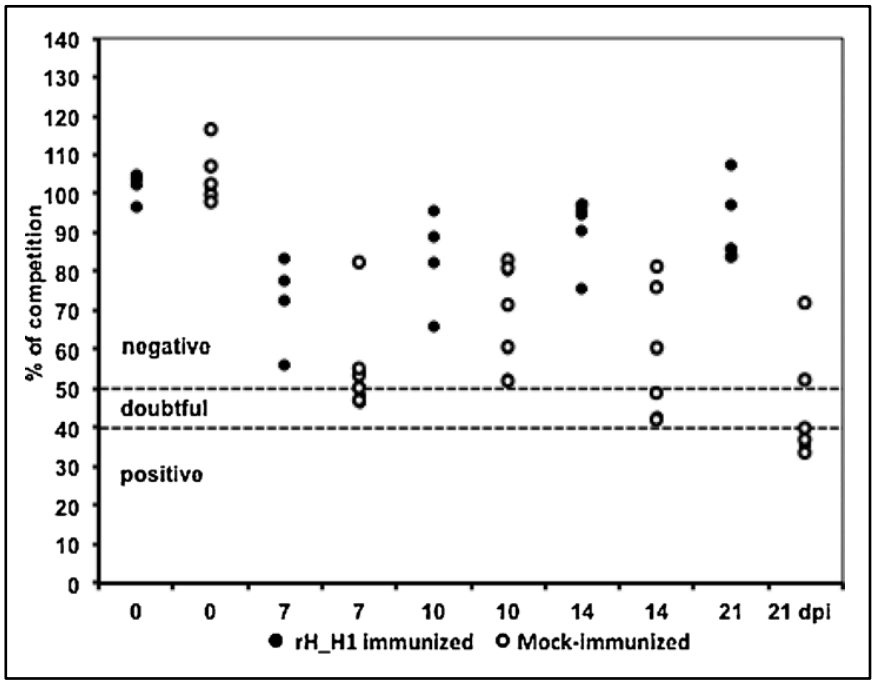

Figure 3.3. Detection of anti-NP antibodies after challenge infection. Serum samples were collected at different time points (0, 7, 10, 14 and 21 dpi) and a competitive NP ELISA was used to

measured antibody levels to influenza NP. The percentage of competition was calculated with the formula OD sample/OD negative control X 100. Samples up to 40% of the negative control are considered NP antibody positive. Samples between 40%-50% are considered doubtful and those over 50% negative.

Similarly, the presence of influenza A virus specific anti-NP antibodies in immunized and mock-immunized pigs was investigated. Our results revealed that all animals in the mock-immunized group developed a positive or at least doubtful serological response to NP after challenge infection within the 21-day observation period, which was in contrast to pigs in the immunized group. The latter did not seroconvert to NP during the observation period after challenge infection at any time (Fig.3.3). We concluded from the negative ELISA results that the rH_H1 vaccine was able to induce an (H1N1)pmd09-specific immune response in pigs that interfered with replication of challenge virus as evidenced by the absence of an NP-specific immune response after challenge infection.

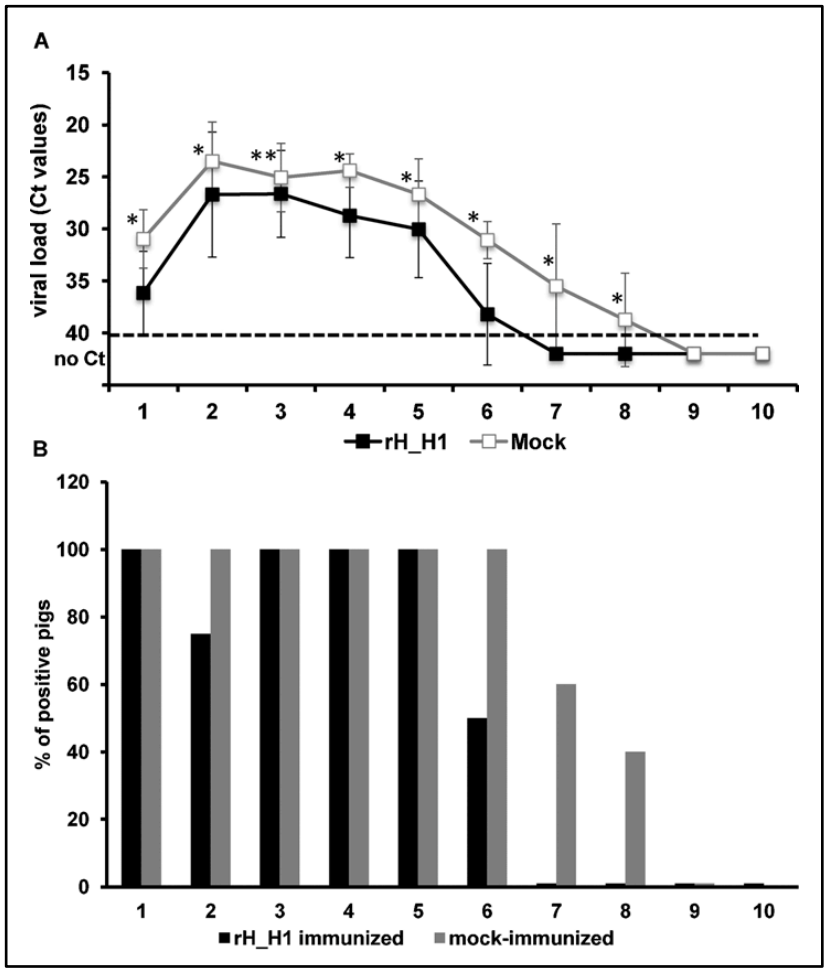

Figure 3.4. Viral RNA detection in nasal swabs after challenge infection. Viral loads were examined by RT-qPCR. (a) Cycle threshold (Ct) values in nasal swabs collected from immunized and

mock-immunized animals at the indicated time points (1 to 10 dpi) after challenge. The mean Ct values are given. *P* values for statistical significance are given; the asterisk (*) reflects significant differences ($p<0.05$). Data are shown as means ± SD of two independent measurements. (b) Percentage of RT-qPCR positive infected animals.

3.4.4. Vaccination reduced viral shedding of challenge virus

To further evaluate whether vaccination has an influence on virus replication, the magnitude and duration of viral shedding in nasal swab samples of both groups were analyzed by qRT-PCR after challenge infection. Our results demonstrated that virus shedding was 10 times higher in the mock-immunized group when compared to the immunized group (Fig.3.4a) according to the differences determined by qRT-PCR (Ct values with 3.3 cycles). In addition, viral RNA loads in nasal swabs of animals in the rH_H1-immunized group were significantly lower when compared to the mock-immunized group between 1 and 6 dpi except on day 3 pi. Moreover, viral RNA copies could still be detected in 100% of the mock-immunized animals at 6 dpi, whereas only 50% of immunized-animals shed virus at this time point. By 7 dpi, viral RNA was absent in the immunized animals but not in the non-immunized control animals (Fig.3.4b). These results suggest that vaccination of piglets with the rH_H1 vaccine had an effect in reducing virus replication and shedding after challenge infection with A(H1N1)pmd09.

3.5. Discussion

Pandemic influenza A(H1N1)pdm09, antigenically and genetically divergent from seasonal H1N1, caused a flu pandemic in humans starting in 2009. The development of an effective vaccine to limit transmission of A(H1N1)pdm09 in animal reservoir hosts and from reservoir hosts to humans and other animals is necessary. Preparation of inactivated influenza vaccines is labor-intensive, may not protect for long periods, and usually requires repeated immunizations to induce protective immunity in swine. On the other hand, for live attenuated vaccines, the risks of adverse reactions and potential re-assortment with circulating wild-type viruses are of concern. In addition, the use of conventional inactivated or live attenuated vaccines will not allow differentiation between infected and vaccinated animals. To improve influenza vaccines, new strategies focus on the development of vaccines that closely mimic natural infection. Vaccines using viral vectors for heterologous gene delivery have been

shown to induce immune responses similar to those observed during natural infection. Upon entry of the viral vector into cells, antigen will be produced and induce humoral and cellular immune responses (Brave et al., 2007). Moreover, the use of viral vectors has been studied intensively and evaluated in several species with respect to stimulation of humoral and cell-mediated immune responses against influenza viruses (Bublot et al., 2006; Minke et al., 2007; Minke, 2007; Rosas et al., 2008; Said et al., 2011).

In a previous study (Said et al., 2011), we constructed and evaluated a recombinant EHV-1 vaccine expressing the H1 protein derived from the California/4/2009/H1N1 strain. We demonstrated that insertion of the H1 gene into the EHV-1 genome did not affect *in vitro* growth characteristics, and the recombinant virus was able to propagate in cell culture as efficiently as parental virus. In addition, the rH_H1 vaccine induced an influenza virus-specific antibody response when inoculated into mice. Upon challenge infection, protection of mice was demonstrated by reduction of clinical signs and faster virus clearance. Here, we further characterized the rH_H1 vaccine and evaluated its ability to protect the natural host, pigs, from challenge infection with a A(H1N1)pmd09 A/Bayern/74/2009/H1N1. This strain has a high (98.7%) identity at the H1 amino acid level compared to California/4/2009, the template sequence used for H1 synthesis in the construction of rH_H1 (Said et al., 2011). Pigs vaccinated with the rH_H1 vaccine did not show clinical signs of illness or adverse effects after vaccination and challenge. In contrast, fever, coughing and conjunctivitis were recorded in mock-immunized pigs after challenge infection. Our data are in line with previous studies showing that A(H1N1)pmd09 causes clinical symptoms in unprotected pigs (Lange et al., 2009; Vincent et al., 2010a). Humoral antibody responses against HA are considered good indicators for evaluating protective immune responses after natural infection or vaccination (Couch and Kasel, 1983). Here, we tested the ability of rH_H1 vaccine to elicit humoral immune responses and protect pigs against challenge with A(H1N1)pmd09 virus.

Development of a serological response to NP in non-immunized animals, but not in previously immunized animals, was detected by competitive ELISA following challenge infection. We concluded from the results that the rH1_H1 vaccine was able to induce a robust immune response against A(H1N1)pmd09. This immune response interfered with replication of challenge virus, which in turn resulted in the absence of an NP-specific response immediately following challenge infection. Moreover, an H-specific antibody response was detected by HI in all immunized pigs after booster vaccination. Limited serological cross-

reactivity with A(H1N1)pmd09 was demonstrated with sera from pigs infected or vaccinated with contemporary H1 SIV (Vincent et al., 2010b). In our study, we assessed by HI the serological cross-reactivity with three influenza-A H1N1 isolates using sera from pigs vaccinated with rH_H1. Cross-reactivity was high between H1 of California/4/2009/H1N1 used for vaccination and H1 of A/Bayern/74/2009 and A/swine/Germany-MV/Wessin8/2011. Surprisingly, cross-reactivity was low between H1 of the vaccine strain and the A/Regensburg/D6/2009 isolate, although the amino acid sequence similarity between the two viruses is very high and reaches nearly 98%. The HA1 of influenza A virus forms a membrane-distal globular domain that contains the receptor-binding site and most antigenic sites recognized by virus-neutralizing antibodies preventing attachment of the virus to the host cell (Wang and Palese, 2009a). Based on the prediction of the H1 amino acid sequences of A/Regensburg/D6/2009, we identified 4 amino acid differences compared to H1 of California/4/2009/H1N1. Therefore, H1N1 with different amino acid sequences such as the A/Regensburg/D6/2009 isolate may avoid complete neutralization in response to vaccination with the rH_H1 vaccine, which, nonetheless, was able to induce a cross-reactive immune response. Our results suggest, therefore, that the vaccine may provide some level of protection against other A/H1N1 that may circulate in swine or man. In a previous vaccination-challenge study in pigs, a DNA vaccine exhibited only partial protection against a classical swine influenza virus strain with significant viral loads still demonstrable at 5 dpi or later (Larsen et al., 2001; Larsen and Olsen, 2002; Macklin et al., 1998). Moreover, commercial inactivated vaccine preparations also provided only partial protection against A(H1N1)pmd09, with viral load reductions starting at 5 dpi (Vincent et al., 2010b). In our study, viral RNA loads in nasal swabs were detected rapidly at 1 dpi in mock- and rH_H1 immunized animals. Importantly, however, animals immunized with rH_H1 vaccine showed reduction in viral load in nasal swabs already at 1 dpi when compared to the unvaccinated controls. In addition, complete virus clearance from nasal swab of vaccinated animals was evident at 6 dpi.

Taken together, the rH_H1 vaccine was capable of reducing viral shedding, enabling earlier viral clearance and abrogating viral replication in the lung. In addition, clinical signs were virtually absent in vaccinated animals after challenge infection and the vaccine candidate is able to induce a robust influenza virus–specific antibody response in immunized pigs. Therefore, this recombinant vaccine prototype may be an alternative approach to prevent and control A (H1N1) pdm09 in swine.

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Chapter 4: General Discussion

4.1. Pandemic Influenza Virus

Influenza virus is a member of the *Orthomyxoviridae* and is an enveloped virus containing a negative-sense RNA genome (Flint et al., 2004; Nayak et al., 2009). The influenza viruses are divided into three types A, B, and C that are distinguished on the basis of the differences in NPs and M proteins. The influenza A virus RNA genome comprises 8 segments that encode at least 11 proteins and the virus causes the most serious respiratory illness in humans together with type B influenza. In contrast, influenza C infection is only of sub-clinical importance. Influenza type A occurs more often and leads to more deaths than influenza type B, which is a significant source of morbidity. Swine influenza is one representative of influenza A viruses and characterized by respiratory distress, weight loss, fever and high morbidity but low mortality (Amorij et al., 2010; Tang et al., 2002; Wang and Palese, 2009). Swine are commonly referred to as a mixing vessel for new influenza reassortants because they have both receptors of human and avian influenza A alpha 2,6 and alpha 2,3 receptors, respectively. This physiological peculiarity provides the possibility to generate new viruses by double infections. Some of the viruses generated in pigs may have the potential to transmit to humans or other animals before ultimately being transmitted to humans (Karasin et al., 2000). In 2009, a novel swine-origin H1N1 influenza A virus appeared that was later referred to as pandemic influenza A(H1N1)09 (WHO, 2011). It was identified to be a variant resulting from a combination of gene segments from both North American and Eurasian swine influenza lineages as well as human and avian influenza lineages (Furuse et al., 2010; Garten et al., 2009; Girard et al., 2010; Smith et al., 2009). The virus quickly spread throughout the world and caused a human pandemic (Michaelis et al., 2009). In previous studies, the susceptibility of pigs to this particular virus strain was confirmed (Lange et al., 2009; Vincent et al., 2010). The clinical manifestations associated with pandemic influenza A(H1N1)09 infection in pigs were similar to that caused by endemic swine influenza strains. As a result of the appearance of pandemic influenza A(H1N1)09, pork consumption and the swine industry were affected by the misconception that the disease was transmitted through meat (Pappaioanou and Gramer, 2010). Therefore, development of an effective vaccine to limit transmission of influenza viruses in animal reservoir hosts and from reservoir hosts to humans and other animals is deemed necessary.

4.2. Immune Responses to Influenza Virus

Humoral and cellular immune responses play important roles in influenza virus clearance following infection. Influenza virus infection induces virus-specific antibody responses (Mancini et al., 2011). The specific antibodies directed against the two surface glycoprotein HA and NA is essential, since the presence of antibodies recognizing these proteins correlates with protective immunity (Gerhard, 2001). HA-specific antibodies are able to inhibit virus attachment and entry into the host cell (de Jong et al., 2000). Some of the HA-specific antibodies are able to recognize and bind HA molecules from different subtypes and have broad neutralizing capacity that can protect against heterologous strains of influenza virus (Ekiert et al., 2009; Ekiert et al., 2011; Sui et al., 2009). The enzymatic activity of the NA protein results in cleavage of the cellular receptors, sialic acid residues, on the cell surface that also facilitate release and spread of newly formed virus particles. Therefore, NA-specific antibodies have some protective effect, mainly through slowing the spread of virus release and transmission to new host cells (Wright et al., 2007). Also neutralizing antibodies to the M2 protein have protective immunity through inhibition of membrane fusion (Treanor et al., 1990; Zebedee and Lamb, 1988). Based on infection with influenza viruses, cytotoxic T lymphocytes (CTL) are activated in the lymphoid tissues and recruited to the site of infection. The activities of CTL result in inhibition of virus replication through clearance of virus-infected cells (Wright et al., 2007).

4.3. Influenza Virus Vaccines

Currently available vaccines can be produced at low cost and are relatively effective in reducing the impact of influenza virus infection. Thus, development of a novel influenza vaccine has been relatively limited. The inactivated virus vaccines are produced either in eggs or in cell culture and usually effective at reducing clinical signs, although it was shown that they may enhance disease in some cases (Kobinger et al., 2010; Vincent et al., 2008). Application of these vaccines reduces the severity of the disease but does not provide long-term protection from infection. Moreover, it is labor-intensive and usually requires multiple vaccination doses to induce protective immunity in swine. Previous studies proposed that cell-mediated and humoral immune responses are not stimulated by inactivated virus vaccines, which are essential to induce heterologous protective immune responses (Ma and Richt, 2010; Reeth et al., 2004). Moreover, the use of whole virus vaccines does not allow for differentiation between infected and vaccinated animals. Therefore, in chapter 2 and 3, we focused on the development of new vaccines and strategies that are able to stimulate both

humoral and cellular immune responses, and that allow differentiation between infected and vaccinated animals.

4.4. Development of New Vaccines

Production of an effective and fast vaccine during a pandemic outbreak is essential. However, current vaccines can only be generated when the virulent pandemic strain is identified and propagated. Therefore, it is necessary to develop a novel vaccine strategy, which is time- and cost-effective, and able to induce a broad protective immunity by stimulating both cell-mediated and humoral immune responses. To improve influenza vaccines, our strategy has focused on the development of vaccines that mimic natural infection and are therefore expected to provide the best protection. Modified live vectored vaccines (MLV) expressing immunogenic antigens of influenza virus and stimulate both humoral and cellular immune responses are being studied intensively (Bublot et al., 2006; Karaca et al., 2007; Minke et al., 2007a; Minke et al., 2007b). Viral vectors are natural favorite vehicles for heterologous gene delivery, which can produce similar immune responses to those observed during natural infection. Upon entry of the viral vector into cells, antigens will be presented through classical antigen presenting pathways, thus not only resulting in humoral immune responses stimulation but also cell mediated immune responses stimulation, which will lead to longer, stronger, and more effective immunity (Brave et al., 2007). Therefore, previous studies have concentrated on the development of alternative vaccine production methods by engineering a several recombinant virus vaccines against SIV (Sipo et al., 2011; Tang et al., 2002; Tian et al., 2006; Wesley et al., 2004). Moreover in our study, we used equine herpesvirus type 1 as a modified live vector to express the HA protein of pandemic influenza A(H1N1)09 virus.

4.5. Development of EHV-1 as vector expressing the H1 of pandemic influenza A(H1N1)09

The two major viral transmembrane glycoproteins; HA and NA proteins are used to classify influenza A viruses into different subtypes and HA- and NA-specific neutralizing antibodies are correlated to protection (Hamilton et al., 2012; Nicasio et al., 2012). In our study, we developed a modified live viral vaccine using a heterologous virus, EHV-1, as a vehicle to delivery the complete HA protein of H1N1 of pandemic influenza A virus because the HA proteins are (i) the key antigens for stimulation of humoral immune responses to induce specific antibodies against influenza virus infection; (ii) essential for influenza virus replication and mediates virus binding to sialic acid receptors on the cell surface and fusion of

viral and endosomal membranes, and finally (iii) leading to viral entry into the host cell (Palese and Shaw, 2007; Skehel and Wiley, 2000). In addition, HA-specific antibodies can block Influenza A virus infection by preventing receptor binding and/or fusion. The HA antibodies are detected using a hemagglutinin inhibition test (HI) that is modified for each subtype by use a reference strain capable of detecting a wide range of different strains within the corresponding subtype (Long et al 2004). Therefore, the HA is an ideal protein for use it in influenza vaccination strategies.

Equine herpesvirus type 1 (EHV-1) is an *Alphaherpesvirus* of the genus *Varicellovirus* and is the one of the most important respiratory pathogen in the horse population, causing respiratory, abortion and neurological manifestations (Allen and Bryans, 1986; Davison, 2009; Telford, 1992). The MLV vaccine of RacH strain of EHV-1 is commonly used to vaccinate horse against EHV-1 in Europe. The large genome of EHV-1 allows insertion of foreign DNA and EHV-1 can easily be manipulated using infectious genomes cloned in E.coli (Trapp et al., 2005). The MLV RacH-based vaccine is capable of entering a wide variety of cell types of different origins and induction of protective immune responses in various species and laboratory animals. Attenuation of EHV-1 RacH strain could be attributed to deletion of both copies of gene 67 (IR6), which originated during its 256 passages on primary kidney cells. In addition, other genomic modification such as truncation of the glycoprotein B also contributes to its attenuation in a variety of species (Hubert et al., 1996; Neubauer et al., 1999; Osterrieder et al., 1996). The EHV-1 vaccine strain RacH has been cloned as an infectious bacterial artificial chromosome (BAC) (Rudolph et al., 2002; Rudolph and Osterrieder, 2002) and developed as a modified live virus vector against various pathogens. RacH strain has a proven safety record and were shown to induce both humoral and cellular immune responses and provide protection in vaccinated animals, including mice, dogs, cattle and swine (Ma et al., 2012; Rosas et al., 2008a; Rosas et al., 2007a; Rosas et al., 2008b; Rosas et al., 2007; Rosas et al., 2007b; Said et al., 2011; Said et al., 2013).

Since equine herpesvirus type 1 (EHV-1) has been developed as a vector to express foreign genes, the efficacy of the vector in inducing immune responses should be involved. Firstly, RacH vector vaccines were developed by insertion of foreign genes instead of EGFP gene in the mini-F sequences. The resulting final recombinant virus conserve mini-F sequences that including resistance gene, which might cause problems in immunized animals. In this thesis, we developed a modified live RacH vector vaccine by inserting the H1 hemagglutinin of

pandemic influenza A(H1N1)09 isolate (rH_H1) under the control of earl HCMV promoter, bovine growth hormone (BGH) and poly A sequences, into the ORF1/ORF2 deleted locus. The whole mini-F sequences were removed by reconstituting gp2-encoding ORF71 gene, and final clean recombinant viruses were developed.

Our study in chapter 2, demonstrated that the rH_H1 virus showed plaque sizes and growth kinetics that were comparable to those of the parental virus. Stable expression of the codon-optimized, synthetic H1 sequences of pandemic influenza A(H1N1)09 virus was detected even after several passages in RK13 cells by IFA and western blotting. When the rH_H1 virus was inoculated into mice, humoral responses against H1 of pandemic influenza A virus were induced following either IN or SQ administration. From cell-culture and evaluation of neutralizing antibody detection studies, we concluded that insertion of the Hemagglutinin protein of H1N1 of pandemic influenza A virus in the selected locus of RacH strain of EHV-1 was stable and did not change the replication of EHV-1 *in vitro*. Upon challenge infection, protection of immunized mice with rH_H1 vaccine was demonstrated by reduction of clinical signs and faster virus clearance.

In chapter 3, we further characterized the rH_H1 vaccine and evaluated its ability to protect the natural host (pigs). Our results demonstrated that rH_H1 vaccine was able to induce humoral immune responses and protect pigs against challenge with pandemic influenza A (H1N1)09 virus. Competitive ELISA following challenge infection detected development of a serological response to NP in non-immunized and not in immunized animals. We concluded from the results that the rH1_H1 vaccine was capable of inducing a robust immune response against pandemic influenza A(H1N1)09. This immune response interfered with replication of challenge virus, which in turn resulted in the absence of an NP-specific response immediately following challenge infection. Furthermore, HI detected an H1-specific antibody response in all immunized pigs after booster vaccination. In addition, rH_H1 vaccine achieved a reduction in viral shedding by diminishing clinical sigs in immunized animals in compare to non-immunized animals. Taken together, our results indicate that recombinant EHV-1 encoding H1 of pandemic influenza A(H1N1) may be a promising alternative for protection of pigs against infection with pandemic influenza A(H1N1)09 or other influenza viruses.

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Selbständigkeitserklärung

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

Berlin, den 16.04.2013 **Abdelrahman Said**