# Next-generation salmonid alphavirus vaccine development

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# Next-generation salmonid alphavirus vaccine development

Mia C. Hikke

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## ABSTRACT

Aquaculture is essential to meet the current and future demands for seafood to feed the world population. Atlantic salmon and rainbow trout are two of the most cultured aquaculture species. A pathogen that threatens these species is salmonid alphavirus (SAV). A current inactivated virus vaccine against SAV provides cross-protection against all SAV subtypes in salmonids and reduces mortality amongst infected fish. However, protection is not 100% and due to virus growth at low temperature, the vaccine production process is time consuming. In addition, the vaccine needs to be injected into the fish, which is a cumbersome process. The work described in this thesis aimed to increase the general knowledge of SAV and to assess current vaccine technologies, and to use this knowledge in designing next-generation vaccines for salmonid aquaculture.

An alternative cell line to support SAV proliferation was identified, however, the virus production time could not yet outcompete the current SAV production system. Making use of the baculovirus insect cell expression system, multiple enveloped virus-like particle (eVLP), and core-like particle (CLP) prototype vaccines were produced in insect cells at high temperature. An in vivo vaccination study showed, however, that these vaccines could not readily protect Atlantic salmon against SAV. The low temperature-dependent replication of SAV was attributed to the glycoprotein E2, and it was found that E2 only correctly travelled to the cell surface at low temperature, and in the presence of glycoprotein E1. The biological impact of this finding was confirmed in the development and in vivo testing of a DNA-launched replicon vaccine. The effective DNA-launched replicon vaccine was extended by delivery of the capsid protein in trans. It was hypothesized that viral replicon particles (VRP) were formed in vivo, which would cause an additional single round of infection and might further elevate the immune response in comparison to the replicon vaccine. A second animal trial indicated that the inclusion of capsid did not yet improve vaccine efficacy. This trial however did show that a DNA vaccine transiently expressing the SAV structural proteins provided superior protection over both replicon vaccines (with and without capsid).

In this thesis, some virus characteristics, such as the cause of temperature-dependency of SAV replication, of an unique aquatic virus were further explored. The production and in vivo testing of multiple next-generation vaccines defined the prerequisites for induction of a potent immune response in Atlantic salmon. A prototype DNA-launched replicon vaccine has shown potential for further development. The research described in this thesis contributes to the development of next-generation vaccines in the challenging area of fish vaccinology.

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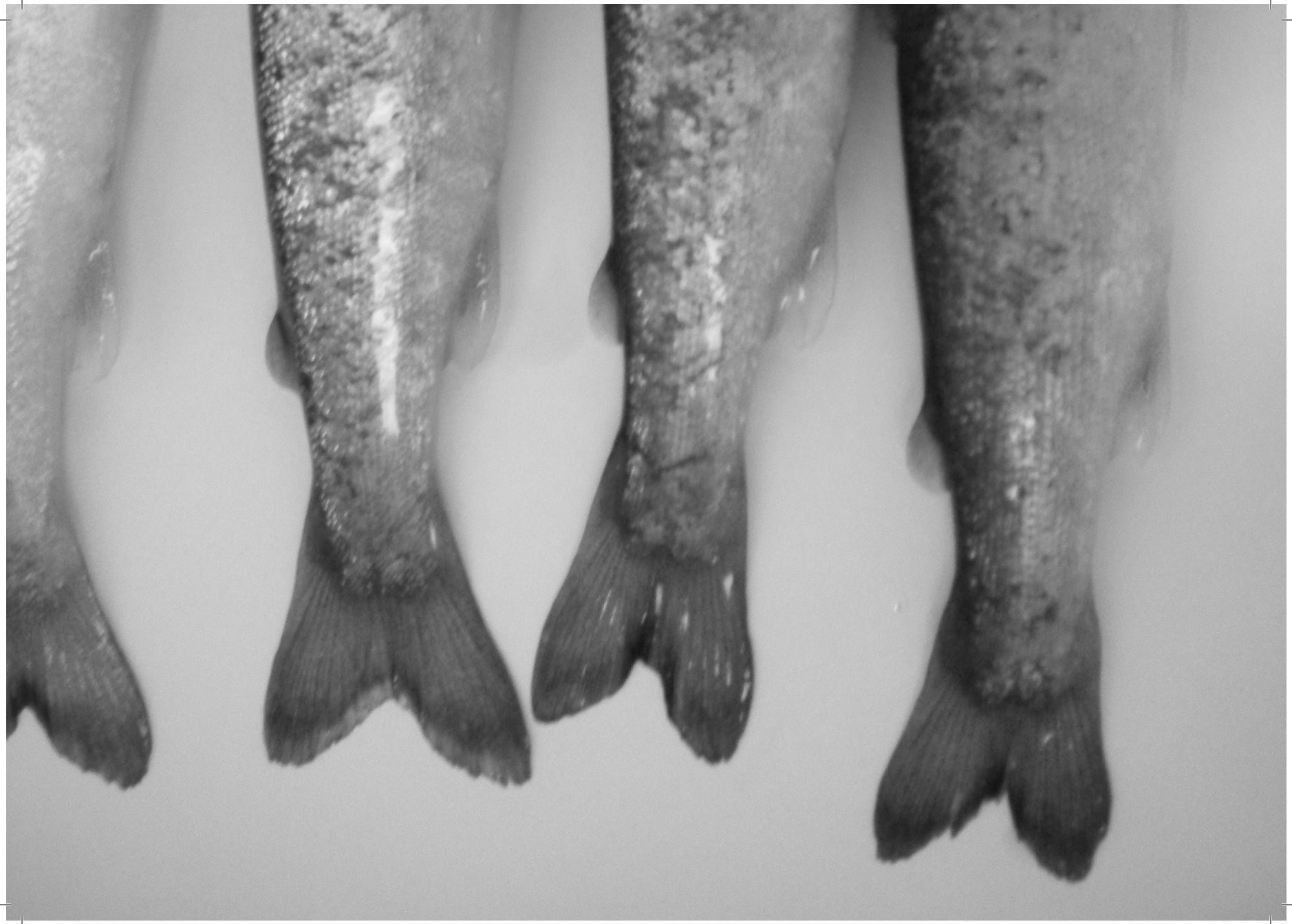
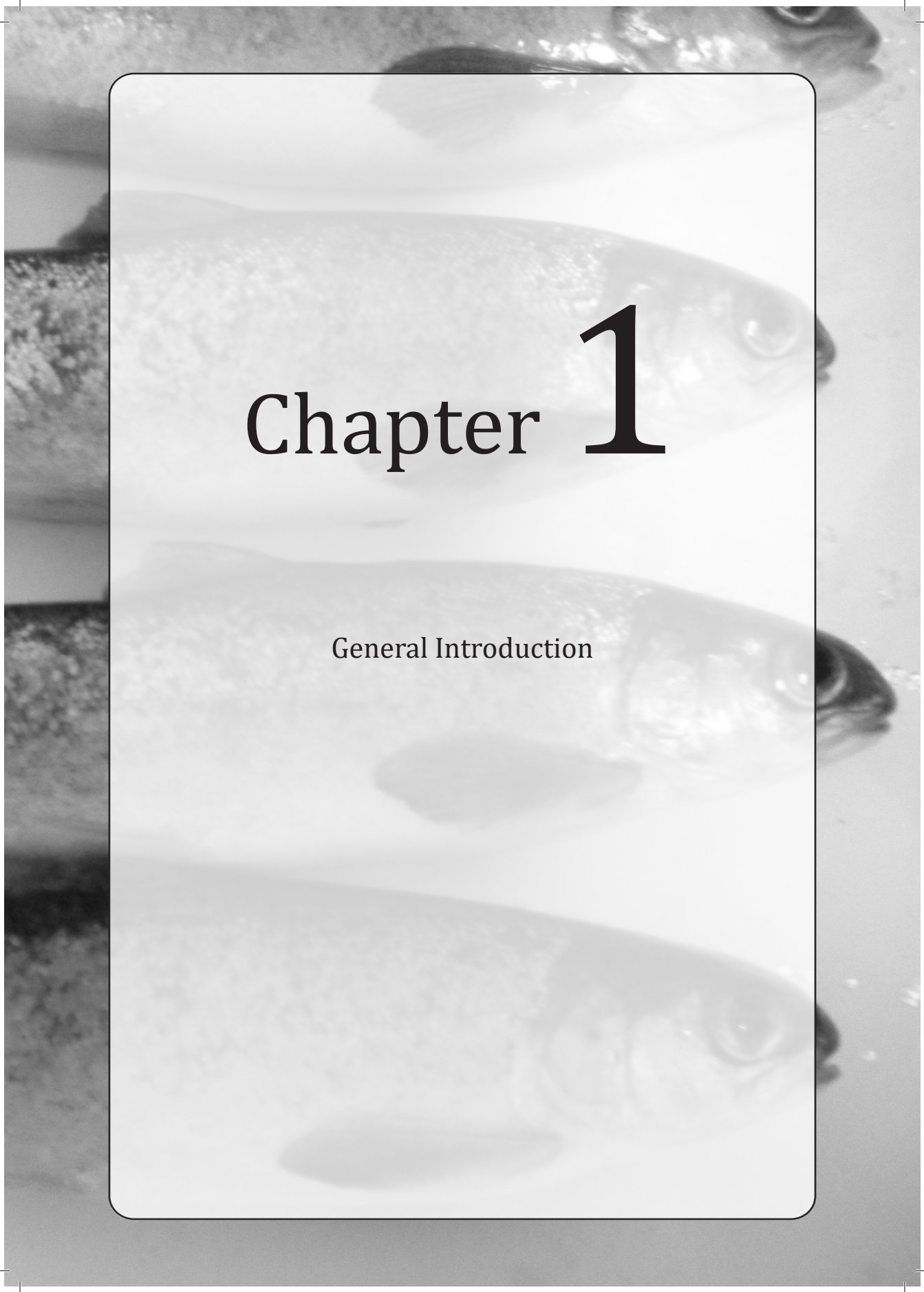




# Chapter 1

General Introduction

## 1. AQUACULTURE

Seafood is an essential part of our diet. It provides us with proteins, essential fatty oils, and vitamins, and fits in a healthy lifestyle. Therefore, the Dutch government currently recommends to eat fish twice per week (The 'Schijf van Vijf', The Netherlands Nutrition Centre Foundation). On average, a person in Europe consumes 23.1 kg seafood per year, 24 percent of which comes from aquaculture (European Commission, 2015). Aquaculture is indispensable, as wild-catch of seafood cannot meet the growing human consumption demands. In addition, aquaculture may allow the recovery of those wild fish stocks that have been overexploited in the past. Over seventy different fish species are grown in aquaculture within Europe, and for many years, 90% of the production is dominated by five species, including Atlantic salmon and rainbow trout (Fig. 1) (FEAP, 2014).

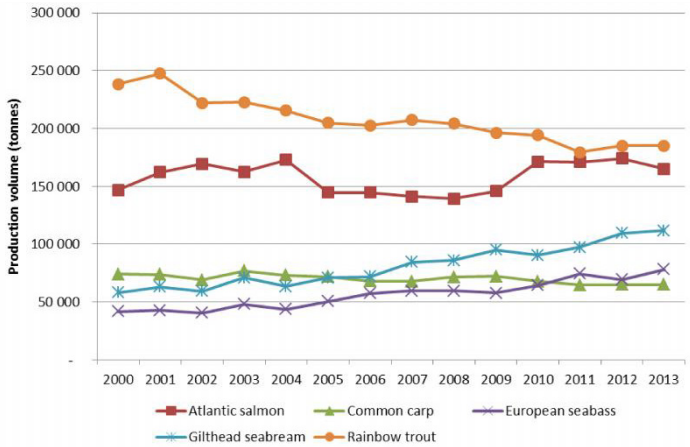



**Figure 1. Production of the top 5 EU farmed fish (2000-2013).** Source: FEAP (2014)

In Norway, aquaculture is one of the major industries along the northern and western coastline. In 2002, the total turnover (production, processing and marketing) was estimated to be around US$ 2800 million (FAO 2015). Considering high density production systems, often off-shore in open water, it is important that the fish species are protected against pathogens, as an infection might spread rapidly throughout the farm site (Jansen et al., 2010). Together with correct culture tank maintenance (e.g. good hygiene, avoid escaped fish) and control of parasites (Jansen et al., 2012), many vaccines have been developed against viral and bacterial diseases (recently reviewed by Dhar et al., 2014). Fish species are vaccinated against a range of different pathogens based on their susceptibility to the pathogen, the region they are cultivated in and in accordance with local regulations. Vaccination will result in herd immunity, thereby increasing protection in the full tank or net pen and reduction of pathogen spread between aquafarms. It is an accepted form of disease prevention in aquaculture industry and has substantially aided in the decrease of antibiotic use (Gudding and Van Muiswinkel, 2013; Midtlyng et al., 2011).

## 2. SALMONID ALPHAVIRUS

### 2.1 Viral occurrence

Within cold-water marine and freshwater fish cultivation, Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* W.) are the prime production species, respectively (FEAP, 2014). However, salmon aquaculture farms are often located at open sea, where control of environmental factors like water temperature is more difficult, and where the fish are threatened by many pathogens. One major burden to aquaculture of salmonid species is salmonid alphavirus (SAV), which is the aetiological agent of pancreas disease (PD) and sleeping disease (SD) in Atlantic salmon and rainbow trout, respectively. Salmonid alphavirus, also known as salmon pancreas disease virus (SPDV, or SAV1), was first isolated in 1995 from an Atlantic salmon in Ireland (Nelson et al., 1995). This was followed by isolation of a similar virus from trout, also better known as sleeping disease virus (SDV, or SAV2) in France (Castric et al., 1997). These two viruses are closely related subtypes of the same virus species, and the collective name salmonid alphavirus was proposed (Weston et al., 2002). Another virus isolate was found in Norway (Christie et al., 1998), which is now recognized as Norwegian salmonid alphavirus (NSAV) or SAV3 (Hodneland et al., 2005). So far, at least 6 subtypes have been identified, although subtypes 4, 5 and 6 result from few, or only one isolate (Table 1).

**Table 1. The SAV subtypes specified and their endemic regions.**

| Subtype | Disease | Species | Region | Ref |
|---|---|---|---|---|
| SAV1 | PD | Atlantic salmon | Ireland, Scotland | (Nelson et al., 1995; Rowley et al., 1998) |
| SAV2 | SD | Fresh water trout | France, UK, Germany, Italy, Spain, Poland, Croatia | (Bergmann et al., 2008; Borzym et al., 2014; Branson, 2002; Castric et al., 1997; Graham et al., 2007; Graham et al., 2003; Smrzlic et al., 2013) |
| | PD | Atlantic salmon | Scotland, Norway | (Fringuelli et al., 2008; Hjortaas et al., 2013) |
| SAV3 | PD | Atlantic salmon<br>Marine water trout | Norway | (Christie et al., 1998; Hodneland et al., 2005) |
| SAV4 | PD | Atlantic salmon | Ireland, Scotland | (Fringuelli et al., 2008) |
| SAV5 | PD | Atlantic salmon | Scotland | (Fringuelli et al., 2008) |
| SAV6* | PD | Atlantic salmon | Ireland | (Fringuelli et al., 2008) |

*Only a single virus isolate was found

PD = pancreas disease; SD = sleeping disease

In Norway, the predominant subtype is SAV3, but since 2010, also SAV2 has spread through the northern sea areas. A PD outbreak causes substantial losses to the farmer. For an average Norwegian Atlantic salmon farm stocked with 500 000 smolts, costs of disease are around NOK 15.6 million (EURO 1.7 million) (Aunsmo et al., 2010; Pettersen et al., 2015). To prevent the salmon from disease development, they are vaccinated during the freshwater rearing phase with an inactivated SAV virus vaccine, grown on a salmonid cell line at low temperature (10-15°C). This vaccine is administered by injection into the intraperitoneal cavity of the fish (Box 1), where an immune response is mounted that will protect from viral infection.

**Box 1 Maintenance and vaccination of salmon in the fresh water phase**

Rearing of Atlantic salmon comprises both a fresh water and sea water phase. The initial egg hatching and rearing occurs in fresh water and vaccination of salmon is performed during this fresh water phase. Fresh water aquaculture farms raise salmon until they undergo seawater adaptation and become smolts. This 'smoltification' is a process triggered by changing photoperiod. If a farmer misses the transfer from fresh- to sea water during this smoltification period, the fish are no longer able to be transferred, and have to remain in fresh water until a new smoltification phase. Farmers may choose to simulate the correct light/dark conditions for smoltification multiple times a year, resulting in a higher turnover for the farmer. Salmon are vaccinated when they are approximately 20 cm long and the time to vaccination depends on the temperature at which they are grown (measured in degree-days). Current vaccination is performed by intraperitoneal injection, which can be done manually or by an injection machine (pictures). In both cases, a lot of human labour is involved. Though, following vaccination, the fish are protected from disease and hopefully, they will not suffer any SAV infections during their life-time until harvest for consumption around the age of 2.

**A vaccine injection machine.** Visible are the tubes that contain the infection needle and the slide exit from the machine.

**Intraperitoneal injection.** Visible is the 'immune response' to the vaccine in the intraperitoneal cavity (white substance).

## 2.2 SAV aetiology

SAV is the causative agent of pancreas disease (PD) and sleeping disease (SD) in Atlantic salmon and rainbow trout (Mérour and Brémont, 2014). Depending on subtype and environmental conditions, PD or SD is caused in either salmon and trout (Table 1). Infection might lead to up to 48% mortality (McLoughlin and Graham, 2007) and decreased fillet quality (Lerfall et al., 2012). PD causes loss of appetite, lethargy, an increase in faecal casts and increased mortality. The most prominent histopathological signs are severe skeletal muscle lesions, pancreatic necrosis, and heart myopathy. Due to these lesions, the fish are unable to maintain a normal posture in the water and they become very sensitive to handling. Sudden deaths may appear amongst infected fish. In addition, late stage PD infections might result in lesions in the kidneys and brain (McLoughlin and Graham, 2007).

SD's most prominent clinical sign is the inability of the fish to maintain their position in the water. They lie sideways on the bottom of the tank, hence it looks as if they are 'sleeping'. This clinical sign is caused by extensive necrosis of red skeletal muscle. In addition, similar lesions in heart and pancreas occur as with PD (McLoughlin and Graham, 2007).

## 2.3 SAV transmission

Most alphaviruses are transmitted by invertebrate vectors, typically mosquitoes (Strauss and Strauss, 1994). Well known alphaviruses are amongst others Semliki Forest virus (SFV), Sindbis virus (SINV), and chikungunya virus (CHIKV), which are known to cause disease in humans. However, there are some exceptions. Eilat virus replication was shown to be restricted to replication in mosquito cells and thus represents the first insect-only alphavirus (Nasar et al., 2014; Nasar et al., 2012). In contrast, SAV3 replication is limited to a small range of vertebrate fish cell lines and it was never shown to be transmitted by an invertebrate vector (Weston et al., 1999). The common ancestor of the alphaviruses is still unknown, but they are proposed to have an invertebrate (Ventoso, 2012) or marine origin (Forrester et al., 2012).

SAV was shown to be present in the sea louse *Lepeophtheirus salmonis*, caught when feeding on an infected salmon (Petterson et al., 2009). However, active virus replication within sea lice has never been demonstrated. Since its first discovery, possible reservoirs have been searched for, and SAV was detected in several species of flatfish (McCleary et al., 2014; Simons et al., 2015; Snow et al., 2010). This may suggest that SAV is not a virus specifically infecting salmonids. Cohabitation trials with infected shedders have shown that SAV is certainly horizontally transmitted from fish to fish, so high density aquaculture tanks are ideal for horizontal SAV transmission (Graham et al., 2011). So far, there is no proof of vertical virus transmission (Kongtorp et al., 2010) and the role of a vector remains enigmatic (Petterson et al., 2009).

## 2.4 SAV virion structure and replication cycle

SAV belongs to the genus *Alphavirus*, within the *Togaviridae* family. Like all other alphaviruses, SAV is an enveloped virus of approximately 70 nm (Fig. 2), and contains a positive, single-stranded RNA genome of around 12 kb, encoding two polyproteins (Villoing et al., 2000). The viral genomic RNA is 5'-capped and contains a 3'-polyadenylation signal. The virion contains the structural proteins capsid (C), envelope (E) protein 3, glycoprotein E2, 6K and glycoprotein E1. Together, the structural proteins make up the virion (Kuhn, 2007). The order of SAV structural proteins encoded on the viral genome is similar to that of other alphaviruses, however, on average the structural proteins are somewhat larger (e.g. the SAV structural polyprotein is ~6% larger than that of CHIKV) (Villoing et al., 2000).

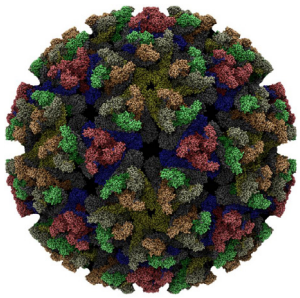


**Figure 2. Alphavirus virion.** Visible are the trimeric spikes on the virion surface. Picture courtesy of Jean-Yves Sgro.

On the virion surface, 80 trimeric spikes, consisting of three heterodimers of the glycoproteins E2 and E1, are present (Fig. 2). Upon binding of glycoprotein E2 to viral receptor on the surface of the host cell, the virion undergoes initial conformational changes, that cause the particle to be endocytosed (Flynn et al., 1990) (Fig. 3). Acidification of the endosome causes the E1/E2 heterodimers to destabilize and this exposes the fusion loop of the E1 glycoprotein, which is incorporated into the endosomal membrane, thereby initiating fusion (Li et al., 2010). Fusion releases the nucleocapsid in the cytoplasm, where it dissociates and releases the viral RNA (Jose et al., 2009). Translation of the viral RNA generates a non-structural polyprotein, which consists of non-structural protein (nsP) 1, 2, 3 and 4, necessary for replication. NsP2 contains protease activity and cleaves the non-structural polyprotein initially into nsP1-3 and nsP4. These proteins form a complex that results in the transcription of negative-strand RNA, which remains bound to the incoming plus strand in a double-stranded RNA (dsRNA) form. Later during infection, also nsP1-3 is processed by nsP2 into the separate nsPs. This leads to formation of a replication complex that primarily synthesizes positive-strand RNA (Shirako and Strauss, 1994). Most alphaviruses contain an opal, leaky stop codon between nsP3 and nsP4 (Strauss et al., 1983), and translational read-through occurs in 5-20% of the transcripts (Myles et al., 2006). This opal stop codon is expected to regulate the production levels of nsP4 during infection, however, salmonid alphavirus was shown to lack this leaky stop codon (Hodneland et al., 2005). A viral subgenomic RNA (26S) is translated into the structural proteins. Upon translation, C is autocatalytically released from the forming polyprotein into the cytoplasm, while the remaining envelope cassette is translocated into the ER. (Fig. 4). Host signalases cleave at both ends of 6K, resulting in precursor E2 (PE2), 6K and E1. In the ER, PE2 and E1 heterodimerize, form trimeric spikes, and travel via the Golgi apparatus to the

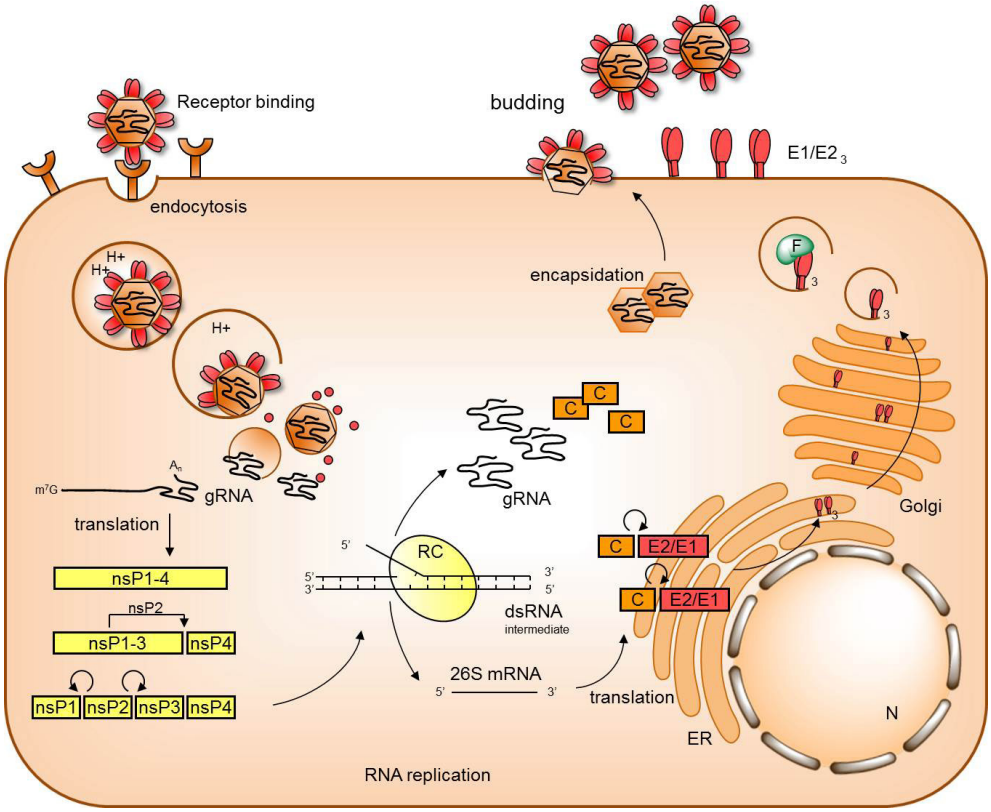



**Figure 3. Replication cycle of salmonid alphavirus.** The virus enters the cell where it fuses with the cell membrane, releasing the nucleocapsid and subsequently the genomic RNA (gRNA). The non-structural polyprotein (nsP) is directly translated from the genomic strand. The nsPs together form replication complexes (RC), that produce new gRNAs and a subgenomic messenger RNA (26S mRNA). The 26S mRNA encodes for the structural polyprotein. Capsid protein (C) encapsidates new genomic RNA in the cytoplasm, while the glycoproteins mature in the host cell its secretory pathway (see Fig. 4). Trimeric spikes of heterodimers of the E2 and E1 protein are located at the cell surface. Together with the nucleocapsid, new viruses bud from the cell, thereby taking along a host-cell derived lipid bilayer.

cell surface (Mulvey and Brown, 1996). In the secretory pathway, the glycoproteins are glycosylated. SAV glycoproteins E2 and E1 are known to be N-glycosylated once on amino acid (aa) $N_{319}$ and aa $N_{35}$, respectively, however, a second glycosylation site is predicted on E2 aa $N_{90}$ (NetNGlyc prediction server). Before the proteins appear at the cell surface, host furin processes PE2 into mature E2, thereby cleaving off protein E3, which remains closely associated with the spike (Uchime et al., 2013). Meanwhile, in the cytoplasm, 240 copies of the viral capsid protein have encapsidated the new viral genomic RNA. The cytoplasmic domain of E2 docks into the hydrophobic pocket of the C protein and new particles bud from the surface of the cell, thereby acquiring the viral envelope (Lee et al., 1996; Skoging et al., 1996) (Fig. 3).

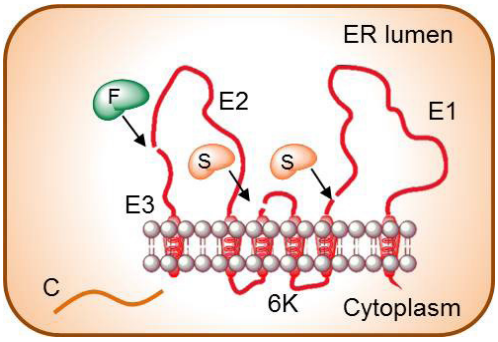



**Figure 4. Processing of the alphavirus structural polyprotein.** Capsid protein (C) is autocatalytically cleaved from the forming polypeptide, which is embedded in the ER membrane. Host signalases (S) cleave at the N- and C-terminal end of protein 6K. Before arrival at the plasma membrane, host furin (F) cleaves PE2 into E3 and E2.

## 2.5 Immunogenic epitopes

The alphavirus virion contains 80 trimeric spikes, each comprising three heterodimers of E2 and E1. As shown in Fig. 5A, E2 covers the most outer D-II domain of glycoprotein E1, thereby preventing premature fusion in the acidic environment of the secretory pathway. The distal tip of the E2 protein (the B-domain, Fig. 5B) is the most external part of the virion and is essential in provoking an effective immune response. Neutralizing antibodies against both E2 and E1 were identified (Hernandez et al., 2008; Hunt et al., 2010), although, antibodies binding to the E2 protein were found to be more potently neutralizing (Fox et al., 2015; Hunt et al., 2010; Mérour et al., 2014; Moriette et al., 2005; Weger-Lucarelli et al., 2015). For instance, a study using chimeric alphaviruses of CHIKV and SFV showed that the E2 B-domain was the primary determinant of neutralization in

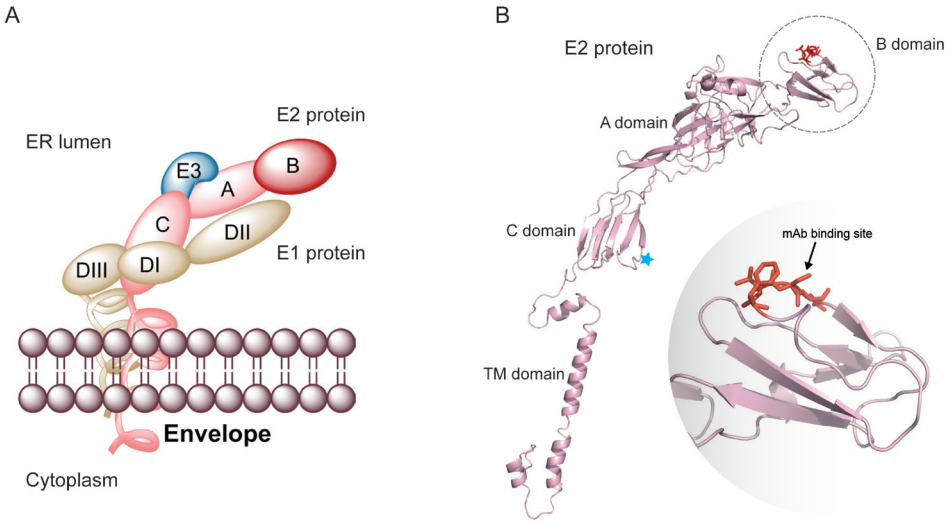



**Figure 5. Schematic presentation of the SAV glycoproteins.** (A) Depicted is a rough estimate of the glycoprotein E2-E1 heterodimer, embedded in the ER membrane. Indicated are the protein domains. (B) Model of the E2 glycoprotein. The blue star indicates the glycosylated amino acid $N_{319}$. Inset: binding peptide of the neutralizing, monoclonal antibody used in this thesis, 17H23. Model by Stephane Villoing.

both human and mice (Weger-Lucarelli et al., 2015), and epitopes on the E2 B-domain that cross-neutralize between alphaviruses, were found to block both viral entry and egression steps (Fox et al., 2015). In addition, binding of the antigen-binding fragment (Fab) of the mAb CHK-265, directed against an epitope centred on the CHIKV E2 B-domain, caused the E2 A-domain to shift, thereby allowing cross-linkage between the E2 B- and A-domain of multiple spikes. This cross-linkage made it less likely that the E1 fusion peptide was exposed, and may therefore have inhibited viral entry (Fox et al., 2015). A SAV neutralizing monoclonal antibody (mAb 17H23), binds the glycoprotein E2 B domain. By epitope mapping using 17H23 binding to linear peptides, the minimum required amino acid (aa) sequence for correct folding of the E2 B domain and related antibody binding was determined (E2 aa 158-252) (Villoing et al., 2009). However, later, by an alanine scanning mutagenesis approach, the antibody-binding region was further narrowed down to the short, linear peptide FTSD in the B-domain, explaining why the 17H23 mAb recognizes both native and denatured E2 protein (Mérour et al., 2014; Moriette et al., 2005).

## 3. SAV LOW-TEMPERATURE DEPENDENT VIRION PRODUCTION

SAV replication is most efficient at temperatures between 10 and 15°C (McLoughlin and Graham, 2007). This lower temperature may explain why SAV infections of salmonids are currently restricted to the colder waters of Europe. In contrast, other alphaviruses replicate well at 27°C, i.e. in their mosquito host, or 37°C, in their mammalian host. Previous research on alphavirus vaccines had led to the development of a chikungunya virus (CHIKV) eVLP vaccine by expression of the structural polyprotein using baculoviral vectors in insect cells (Metz et al., 2013), and a similar strategy was subsequently proposed for the production of SAV eVLPS. However, the baculovirus optimum replication temperature in insect cells is 27°C (King and Possee, 1992), and at this temperature no SAV eVLPs were secreted in the medium (Metz et al., 2011) (Fig. 6). Further analysis showed that at 27°C no E2 appeared at the cell surface of the insect cells and that PE2 was not processed by furin into mature E2. A temperature decrease until below 18°C resulted in surface translocation of the glycoproteins and rescued eVLP secretion (Metz et al., 2011). Thus, low temperature was shown to be a requirement for virion production as well as for SAV eVLP generation. The underlying mechanism for the inability of SAV to secrete new particles at higher temperatures is not well understood.

## 4. SCOPE OF THE THESIS

Current vaccination of young salmon presmolts with a commercial inactivated whole virus vaccine results in effective protection against SAV infection. It provides long-lasting immunity against multiple SAV subtypes, and reduces mortality, viral replication

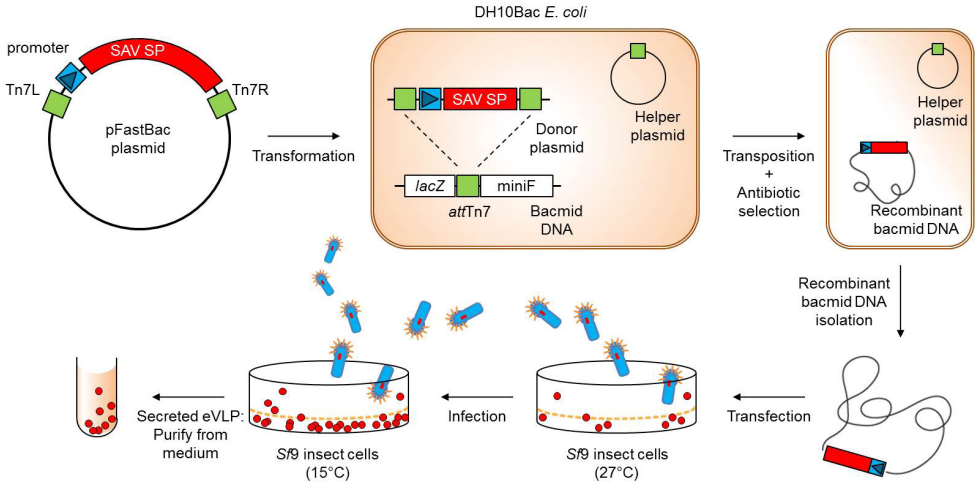



**Figure 6. SAV eVLP production by recombinant baculoviruses using Bac-to-Bac cloning technology (Invitrogen).** Initial recombination of the donor plasmid, coding for the SAV structural proteins (SP), with the bacmid DNA occurs in E. coli in the presence of a helper plasmid. Following recombination, antibiotic selection allows for selection of recombinant clones. Isolated recombinant bacmid DNA is used to transfect a susceptible insect cell line, where recombinant baculovirus virions are formed. Concurrently with viral replication, the SAV structural proteins are produced and eVLP are secreted in the culture medium.

and pathology (Mérour and Brémont, 2014). However, the protection obtained is not always 100%, and virus propagation on a fish cell line at low temperature makes vaccine production a time-consuming process. Furthermore, the inactivated virus vaccine needs to be administered to the juvenile fish by injection, which is labour intensive and stressful to the fish. Finally, injection of salmon can only be done once in their life span, and only after the fish have reached a specific size, which results in a very short time frame for farmers to vaccinate, and leaves the fish unprotected until they reach the desired size for vaccination. An oral or immersion vaccine would therefore be much easier in this respect as these vaccine modalities are non-invasive and can be applied to small fish. Also, oral or immersion vaccines may provide opportunities for the development of e.g. booster vaccination regimes, with an oral or immersion vaccine as a first immunization, followed by a second immunization via injection.

Indeed, a recombinant, live attenuated SAV2 virus was developed a decade ago, which could be administered via bath immersion and provided complete and long-lasting protection upon challenge with a pathogenic wildtype virus (Moriette et al., 2006). However, there are considerable safety concerns associated with the use of live-attenuated virus (LAV) vaccines, as reversion to virulence might occur, and therefore LAV vaccines are currently not used in the field (Shoemaker and Klesius, 2014). It can be concluded that there is room for improvement when it comes to vaccination against SAV infections. Therefore, the overall aim of this thesis is to develop and evaluate next-

generation vaccines against SAV infection.



SAV is currently propagated in vitro on Chinook salmon embryo (CHSE-214) cells at low temperature. A faster growing cell line might shorten the production time of the initial cell mass prior to virus infection. In **Chapter 2**, we set out to find an alternative cell line for SAV propagation. Since all other alphaviruses can replicate in invertebrate cells, the replication efficiency of SAV3 in a selection of insect cells is compared with replication in CHSE-214 cells.

The current inactivated virus vaccine against SAV, and therefore also a similar vaccine produced on a different cell line, is a classical, first generation vaccine. A nanoparticle vaccine may be a good alternative for the inactivated vaccine as it resembles the structure of the native virion. As was mentioned in section 3, before the onset of this study, an enveloped virus-like particle (eVLP) prototype vaccine was developed by expressing SAV structural proteins using recombinant baculoviral vectors in insect cells. SAV eVLPs could only be obtained when the incubation temperature was lowered to below 18°C. However, total protein expression levels at low temperatures are lower than at 27°C. In **Chapter 3**, another prototype vaccine is developed in the shape of core-like particles (CLP), which consist of self-assembled SAV capsid proteins. These CLPs can be produced at high levels at 27°C and are used as protein carriers for the most immunogenic epitope of SAV. In addition, a simple purification method is presented to purify the CLPs, free from baculovirus budded viruses

To better understand the SAV replication cycle and circumvent SAV eVLP production at low temperature, in **Chapter 4**, the molecular basis of low-temperature dependent SAV virion production is studied. A synthetic SAV infectious clone is designed that can be easily adapted for molecular studies and vaccine purposes. Using recombinant baculoviral vectors, the conditions for correct SAV glycoprotein folding and processing are examined. Furthermore, the specific region within the structural polyprotein that is responsible for low-temperature dependent virion production is identified.

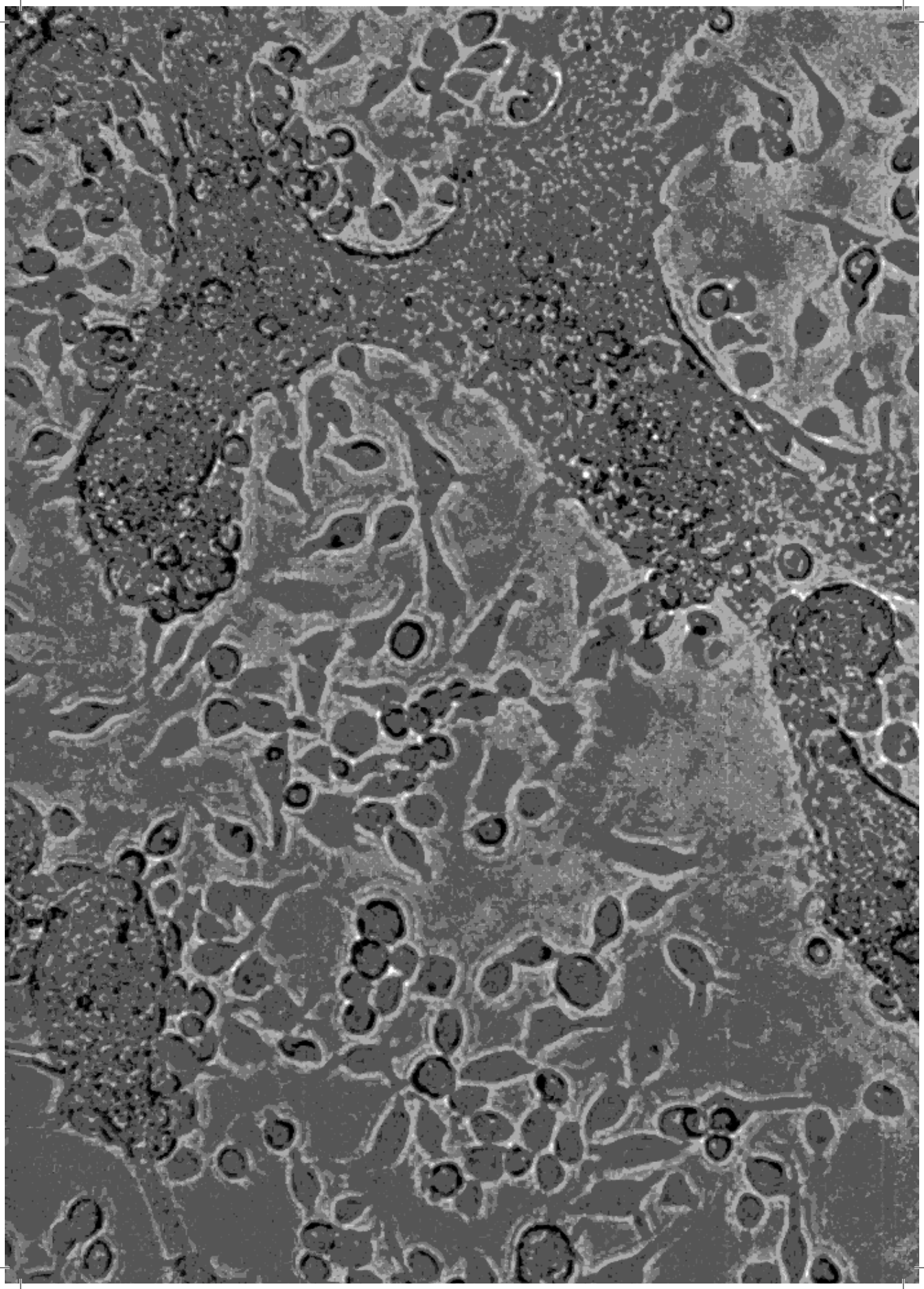
In **Chapter 5**, using different combinations of chimeric viral polyproteins, the region responsible for low-temperature dependency of SAV virion production is mapped in more detail. The obtained knowledge is subsequently used to generate a chimeric eVLP at 27°C. The chimeric eVLP combines the best characteristics of the SAV eVLP (overall particle structure) and chimeric CLP (production at 27°C) vaccines. The efficacy of all CLP and eVLP prototype vaccines is evaluated in a cohabitation challenge-trial of Atlantic salmon.

A more recent vaccination approach is the use of nucleic acid (i.e. DNA or RNA) vaccines. DNA vaccination encompasses transient antigen expression from a suitable promoter in the host. However, the efficacy of DNA vaccines is not always optimal. Another class of vaccines, based upon self-replicating RNAs (replicons), has shown great potential

in vaccine development. These replicons, which can be delivered as naked RNA, as viral replicon particles (VRPs), or in a DNA-launched form, often trigger a more potent immune response than classical DNA vaccines and may work at a lower dose due to their self-replicating ability. In **Chapter 6**, the design and mode of action of self-replicating, viral replicon vaccines are introduced, and a compiled overview of the veterinary applications for the different types of self-replicating replicon vaccines is given.

In **Chapter 7**, the knowledge obtained in the previous chapters is used to design and produce two DNA-launched replicon vaccines. Following vaccination with these DNA constructs, DNA-launched viral replicon RNA and viral replicon particles (VRP) are generated *in vivo*. The efficacy of both the DNA-launched viral replicon vaccine and the VRP vaccine are evaluated in individual cohabitation-challenge trials of Atlantic salmon, and compared to a classical inactivated virus and a classical DNA vaccine.

Finally, the data obtained in this thesis is discussed in the context of the current knowledge in **Chapter 8**.



# Chapter 2

Salmonid alphavirus replication in mosquito cells: towards a novel vaccine production system

## ABSTRACT

Salmonid alphavirus (SAV) causes pancreas disease and sleeping disease in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) and confers a major burden to the aquaculture industry. A commercial inactivated whole virus vaccine propagated in a salmon cell line at low temperature provides effective protection against SAV infections. Alphaviruses (family *Togaviridae*) are generally transmitted between vertebrate hosts via blood-sucking arthropod vectors, typically mosquitoes. SAV is unique in this respect since it can be transmitted directly from fish to fish and has no known invertebrate vector. Here, we show for the first time that SAV is able to complete a full infectious cycle within arthropod cells derived from the Asian tiger mosquito *Aedes albopictus*. Progeny virus is produced in C6/36 and U4.4. cells in a temperature-dependent manner (at 15°C but not at 18°C), can be serially passaged and remains infectious to salmonid CHSE-214 cells, without amino acid adaptations. This suggests that SAV is not a vertebrate-restricted alphavirus after all and may have the potential to replicate in invertebrates. The current study shows the ability of SAV to be propagated to high titers in mosquito cells, thereby possibly providing an alternative SAV production system for vaccine applications.

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## 1. INTRODUCTION



Salmonid alphavirus (SAV) is a major burden in aquaculture of Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* W.) and is the causative agent of pancreas disease (PD) and sleeping disease (SD). At present, at least six different SAV subtypes have been distinguished based on genotype and geographic distribution (Fringuelli et al., 2008; Graham et al., 2012; Graham et al., 2011). Infections may lead up to 48% mortality (McLoughlin and Graham, 2007) and deteriorated fillet quality (Lerfall et al., 2012). Salmonid alphavirus 3 (SAV3) is originally found in Norway and infection of salmon results in loss of appetite, impaired swimming behaviour and myopathy of heart and skeletal muscles (Hodneland et al., 2005). A commercial inactivated 'whole virus' vaccine propagated in a salmon cell line at low temperature (10-15°C) (Norvax® Compact PD) provides effective protection against SAV infections. A prototype vaccine, based upon virus-like particles produced in *Spodoptera frugiperda Sf*9 insect cells by the baculovirus expression system, was recently developed. In this system, SAV3 envelope glycoproteins E1 and E2 were correctly folded when expressed at low temperatures and E1 retained fusogenic activity as observed by syncytia formation (Metz et al., 2011).

SAV3 is a unique member of the genus *Alphavirus* (family of *Togaviridae*), the genome consists of a single, positive-stranded RNA of approximately 12kb, which is 5'-capped and 3'-polyadenylated allowing direct translation by the host cell machinery. Non-structural proteins (nsP1-4) are directly translated into one polyprotein which is processed into separate nsPs by the viral protease activity present in nsP2. Together the nsPs form a replication complex allowing the synthesis of negative-stranded RNA and subsequent positive-stranded progeny viral RNA (Kuhn, 2007). In addition to the directly translated 'non-structural' polyprotein, a 'structural' polyprotein is translated from a subgenomic RNA strand, encoding the virion proteins capsid (C), envelope (E) 3, E2, 6K and E1. Upon translation, C is autocatalytically cleaved off while proteolytic processing between E2, 6K and E1 occurs by host signalases in the endoplasmic reticulum (ER). After translocation to the trans-Golgi system, host furin cleaves between E3 and E2, rendering the viral particle sensitive for low pH-induced activation (Kuhn, 2007).

Alphaviruses are generally characterised by the ability to transfer between hosts via blood-sucking arthropod vectors, typically mosquitoes. Well known examples are Sindbis virus, Semliki Forest virus and chikungunya virus, the latter causing mainly fever, rash and arthralgia. Via their arthropod vector they infect a wide range of vertebrate hosts including human and non-human primates (Jose et al., 2009). However, there are a few exceptions as well. Recently, Eilat virus replication was shown to be restricted to replication in mosquito cells and thus represents the first insect-only alphavirus (Nasar et al., 2012). In contrast, replication of SAV3 is limited to a small range of vertebrate fish cell lines and it does not have a known invertebrate vector (Weston, 1999). Furthermore,

no evidence of vertical transmission of SAV is available (Kongtorp et al., 2010). SAV was also shown to be effectively transmitted horizontally in cohabitation experiments and this is assumed to be the main route of transmission in high-density aquaculture settings (Graham et al., 2011). Nonetheless, the haematophagous salmon louse *Lepeophtheirus salmonis* was found to contain the virus when caught feeding on the skin of an infected salmon, though no direct evidence of active viral replication within these arthropods was provided (Karlsen et al., 2006; Petterson et al., 2009). As outbreaks of SAV often coincide with a high sea lice burden in aquaculture tanks (Rodger and Mitchell, 2007), it is possible that salmon lice could serve as a transmission vector. If this is the case, the question is whether SAV is productively replicating within the sea lice and thus can be classified as an arthropod-borne (arbo)virus or not.

In this paper it is shown for the first time that SAV is able to replicate within arthropod cells derived from the Asian tiger mosquito *Aedes albopictus*. This is the first indication that SAV is not a vertebrate-restricted alphavirus only and may have the potential to replicate in invertebrate hosts. The ability of SAV to replicate within mosquito cells provides a potential alternative system for SAV vaccine production.

## 2. RESULTS AND DISCUSSION

At the moment vaccination of young salmon parr/fingerlings occurs using adjuvanted, inactivated SAV virus from Chinook salmon embryo cells (CHSE-214) grown at low temperature. This low temperature-dependency of the production process makes upstream processing cumbersome and quite expensive. To examine alternative SAV

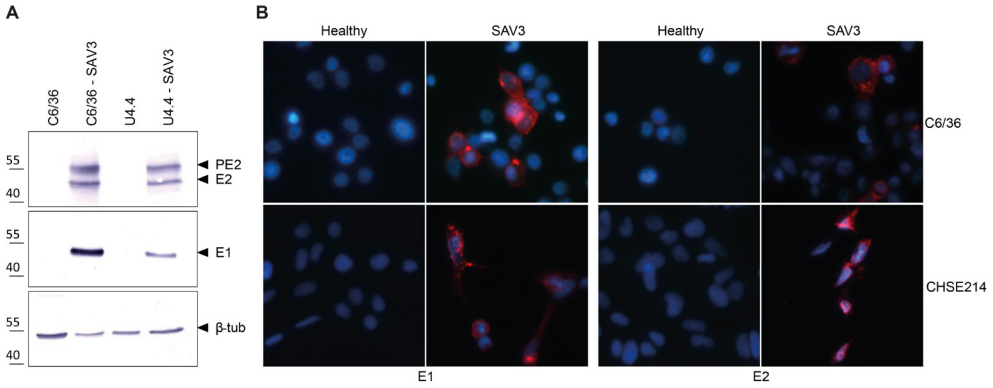



**Figure 1. SAV3 WT infection of mosquito cell lines.** (A) Western blot analysis of proteins from invertebrate cell lines nine weeks after primary infection (15°C) with SAV3. Detection of SAV3 proteins presence was performed using mAb against SAV E1 (1:1000) and E2 (1:2000) glycoprotein. As loading control a polyclonal Ab against β-tubulin (1:2000, Abcam) was used. Protein sizes in kDa are indicated at the left. (B) Immunofluorescence detection of surface expressed SAV glycoproteins on C6/36 (upper panel) and CHSE-214 cells (lower panel). C6/36 and CHSE-214 cells were infected with SAV3 at a MOI 0.005 $TCID_{50}$ units/cell and incubated at 15°C for 10 days. E1 (left) and E2 (right) proteins are shown in red (Alexa-546). Hoechst 33258 nuclear staining (blue) was used to indicate cells.

propagation cell lines, a number of lepidopteran (*Spodoptera frugiperda Sf*9 and *Sf*21, *Trichoplusia ni* Hi5) and dipteran (*Culicoides* Kc, *Drosophila melanogaster* S2, *Aedes albopictus* U4.4 and C6/36) cell lines were infected with SAV3 and incubated at both 15°C and 18°C. These are the temperatures at which SAV replicates (15°C) or not (18°C) in salmonid CHSE-214 cells (Graham et al., 2008; McLoughlin and Graham, 2007). Replication of SAV3 in C6/36 and U4.4 mosquito cell lines at 15°C was observed when the proteins were analysed by SDS-PAGE (10%) followed by western blot detection of E1, precursor E2 (PE2) and E2 protein using α-E1 (kindly provided by MSD Animal Health, Boxmeer) and α-E2 (17H23 (Moriette et al., 2005)) monoclonal antibodies, respectively, in cell fractions at 4 weeks post infection (wpi) (MOI ~30) or 9 wpi (MOI 10) (Fig. 1A). At 15°C, the other cell lines did not show a positive signal for SAV3 proteins on western blot (data not shown). In contrast, at 18°C, no virus replication was detected in any of the cell lines tested.

After infection of C6/36 cells at 15°C, progeny virus was harvested to infect fresh C6/36 cells. E2 was still detected in infected cell fractions after 4 serial, undiluted passages on the C6/36 mosquito cell line, suggesting that C6/36 cells support the complete SAV3 infectious cycle (entry, replication, assembly and secretion). Additionally, glycoprotein production and posttranslational cellular transport in C6/36 cells was confirmed by surface immunofluorescence detection of the E1 (Fig. 1B, top left, 1:1000) and E2 (Fig. 1B, top right, 1:2000) glycoproteins. As a positive control, CHSE-214 cells were subjected to the same treatments as the C6/36 cells (Fig. 1B, bottom). Because the neutralizing 17H23 α-E2 mAb is expected to be a conformational antibody (Metz et al., 2011), detection of its epitope indicates correct folding of the E2 protein in C6/36 cells.

A syncytia assay was performed to show that infection of C6/36 cells with SAV3 also results in the presence of a functional fusogenic E1 glycoprotein on the cell surface. Since alphavirus infection is cholesterol dependent and mosquitoes are cholesterol auxotrophs (Kielian and Helenius, 1984), incubation (15°C) was performed in

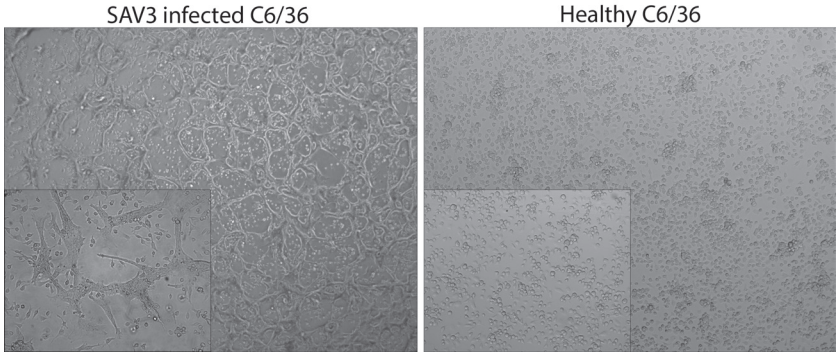



**Figure 2. Syncytia formation of SAV3 infected C6/36 cells.** SAV3 infected C6/36 cells (left) were incubated for two weeks at 15°C in cholesterol-enriched medium (0.1 mg/mL). Infected and healthy cells were subjected to acidified (pH 5.5) medium for 2 min. Infected cells show syncytia formation 4 h post acidification. Insets: magnification of the cell morphology.

cholesterol-supplemented medium (0.1 mg/mL). Two weeks post-infection the medium was removed and the cells were subjected to acidified medium (pH 5.5) for 2 min. Extensive syncytia formation was observed only in those samples infected with SAV3 (Fig. 2, left) confirming that the fusogenic activity of the E1 protein is retained when expressed during SAV3 replication in C6/36 cells. Thus, efficient functional glycoprotein production was observed in C6/36 cells. The virus could be serially passaged at least 4 times.

To further confirm the presence of *de novo* virion production, medium from SAV infected C6/36 cell culture was fractionated via discontinuous sucrose gradient centrifugation (Fig. 3A). One band (B1) was visible on top of the 20% (w/v) sucrose layer, one band (B2) at the 20-50% (w/v) interphase and two bands (B3, B4) were observed around the 50-70% (w/v) sucrose interphase. The harvested bands were diluted 1:10 in Leibovitz (L15) medium and each was used to infect fresh C6/36 cells. Analysis of this infection resulted again in detection of the E2 protein, indicating that the purified B3 and B4 sucrose fractions indeed contained infectious SAV3 particles (Fig. 3A).

An additional infection on C6/36 cells was performed to visualize C6/36-derived SAV particles from the medium by transmission electron microscopy. Analysis of sucrose purified samples showed particles of 60-85 nm in diameter (Fig. 3B), indistinguishable from SAV particles (Metz et al., 2011).

The *Aedes albopictus* cell line C6/36 is quite susceptible to viral infections and often used for in vitro cultivation of arboviruses (Arunrut et al., 2011; Sudhakaran et al., 2007; White, 1987). Most likely this is due to a dysfunctional antiviral RNAi system (Brackney et al., 2010). We have shown that C6/36 cells also support the full replication cycle of SAV3. The fact that also U4.4 cells, another *A. albopictus* cell line but with a functional RNAi system, are susceptible to SAV3 infection raises the question whether SAV replication in mosquito cells is under RNAi control (Sanchez-Vargas et al., 2004).

C6/36 cells are robust and fast growing cells and easy to culture. Because they are lowly adherent by nature and do not need EDTA or trypsin for detachment of monolayers from culture flasks, they are easily adaptable to growth in suspension (Morita and Igarashi, 1989). Additionally, no cooling equipment is needed for the proliferation of these cells (27-28°C) in the absence of virus infection and therefore this may be cheaper in upstream processing. Even though the production of infectious SAV3 particles is still restricted to a low temperature (<15°C), up-scaling of C6/36 cells at higher temperatures (27-28°C) prior to infection may decrease vaccine production times significantly. For this, a prerequisite would be that similar SAV viral titers are obtained when grown on C6/36 cells in comparison to virion production in CHSE-214 cells. To do this, C6/36 cells were infected with SAV WT virus in duplo (MOI~0.1), incubated at 15°C and samples were taken at multiple time point post-infection and the viral titer determined (Reed and Muench, 1938). The growth curve showed that SAV could reach a maximum titre in

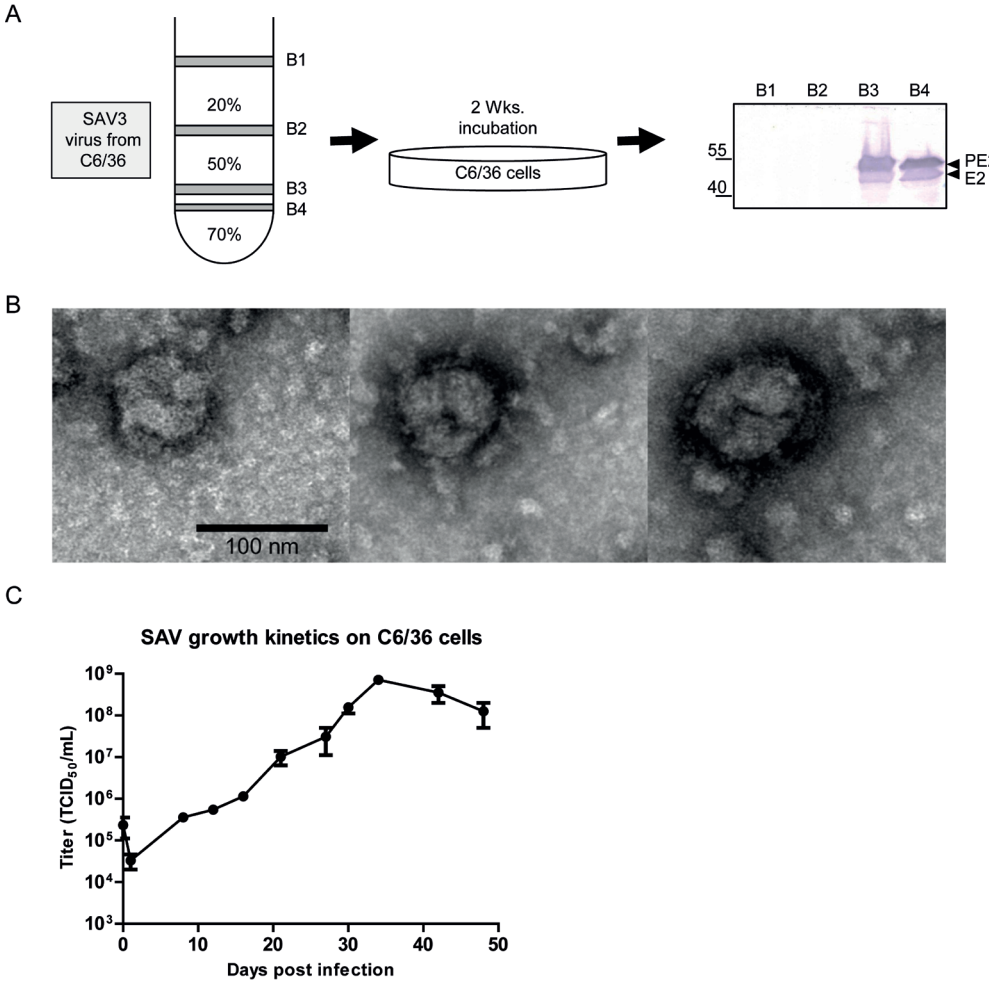



**Figure 3. SAV3 can be propagated on mosquito cells.** (A) Medium of a SAV3 infection of C6/36 cells (2 weeks, 15°C) was fractionated by discontinuous sucrose gradient (20-70%) ultracentrifugation (2h, 85000 x g, 4°C). The four visible bands were used to infect fresh C6/36 cells (2 weeks, 15°C). Infected cell fractions were analysed using Western blot analysis using α-E2 mAb (1:2000). (B) Transmission electron microscopy (JEOL JEM 1011) of the sucrose cushion (20% w/v) concentrated SAV3 infected C6/36 cell culture medium fraction (2h, 80000 x g, 4°C). Samples were spotted on copper coated grids and stained with 2% uranyl acetate. (C) SAV growth kinetics on C6/36 mosquito cells. WT SAV was used to infect C6/36 cells at an MOI of 0.1 and cells were incubated at 15°C. Medium samples were taken at several time points post infection and the titer was determined by an end-point dilution assay using CHSE214 cells. The experiment was performed in duplo. Bars shown indicate SEM.

C6/36 cells of $10^9$ $TCID_{50}$/ml, though it took approximately one month to reach this level (Fig. 3C). This delay in viral replication in C6/36 cells as compared to CHSE-214 cells is expected to be an effect of slower mosquito metabolism at 15°C than at 27°C (Delatte et al., 2009). It was concluded that, so far, mosquito cells cannot outcompete salmonid cells in SAV production speed.

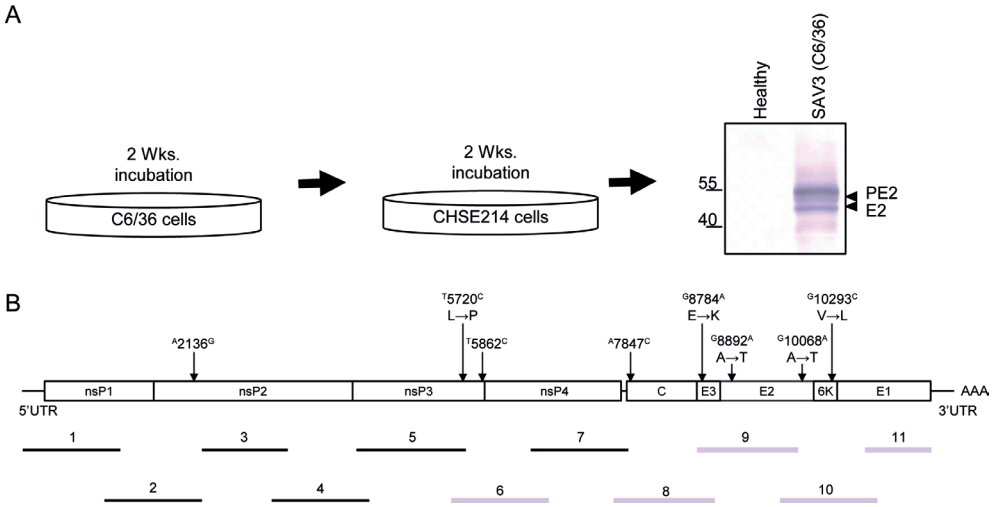



**Figure 4. SAV3 can alternate replication between invertebrate and vertebrate cell types without changes in its viral genome.** (A) Virus particles derived from C6/36 cells were used to infect Salmonid CHSE-214 cells. Infected cell fractions were analysed by Western blot analysis using α-E2 mAb (1:2000). Protein sizes in kDa are indicated at the left of the western blot. (B) Sequencing of mosquito cell-derived SAV viral RNA. Indicated are the 11 overlapping amplicons generated by RT-PCR, before sequence analysis. Nucleotide (nt) and amino acid differences to the reference strain are indicated on top. Regions where amino acid changes were suspected to originate from RNA replication in mosquito cells, were also sequenced on viral RNA derived from infected CHSE-214 cells (purple amplicons).

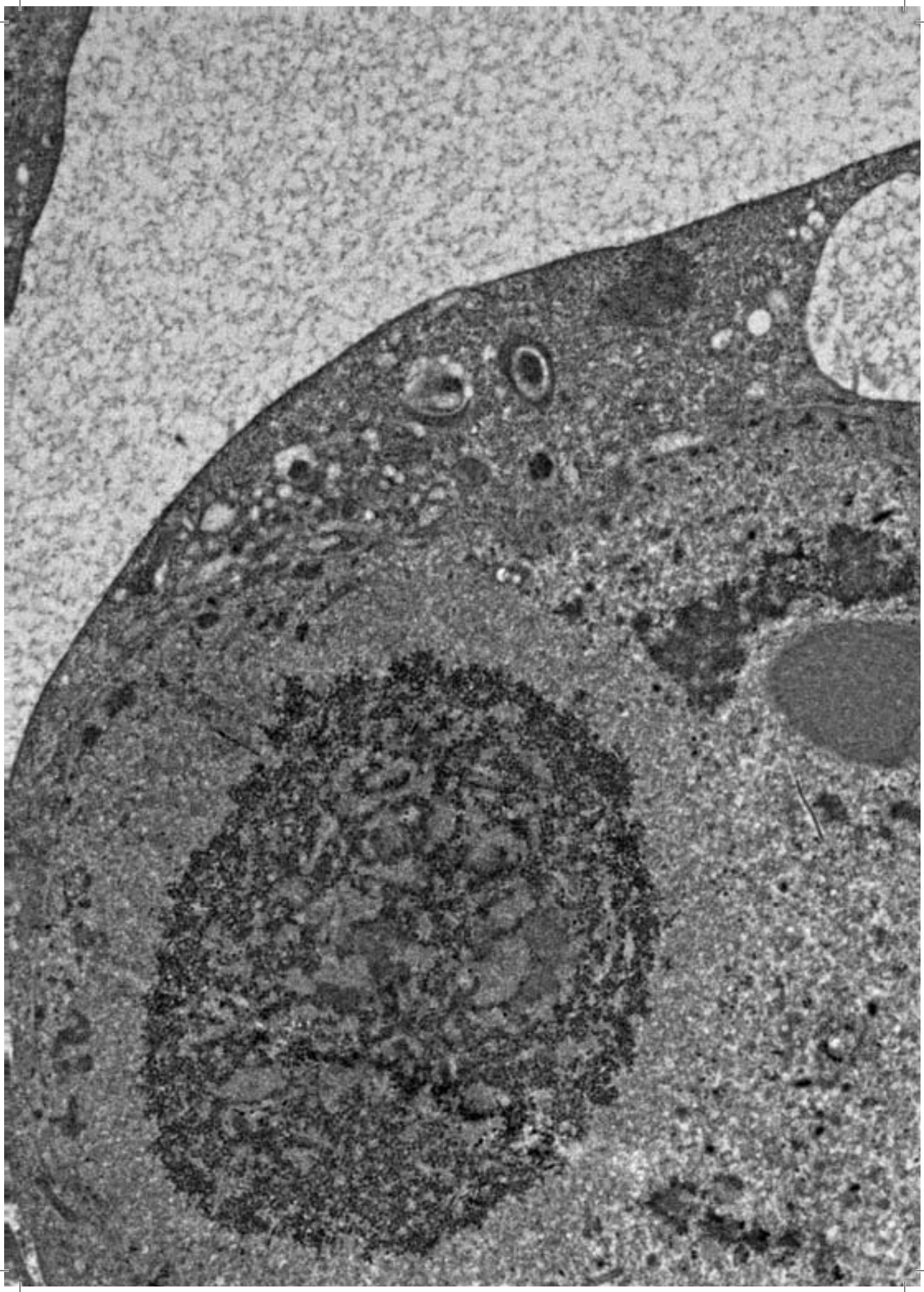
Mosquito cells were incubated with SAV3 for a long period of time (>4 weeks). Therefore, it is possible that upon replication mutations may have arisen or been selected that allowed easier viral entry into the cell and/or budding from the cell. Initially, to examine if C6/36-derived SAV3 particles were still infectious to CHSE-214 cells, the virus inoculum was used to infect fresh CHSE-214 cells at 15°C. Two weeks post infection, cells were harvested and analysed on SDS-PAGE and WB, resulting in E2 detection in SAV3 infected cells (Fig. 4A). Therefore, we concluded that SAV3 produced in mosquito C6/36 cells can still readily infect salmonid CHSE-214 cells. In addition, an important epitope on the E2 glycoprotein is still recognized by a neutralizing, conformational monoclonal antibody in an immunofluorescence assay and on Western blot. Although not tested, it is therefore likely that SAV produced in C6/36 or U4.4 cells is also infectious for salmon and trout. Thereafter, to analyse C6/36-derived SAV at nucleotide level, RNA was isolated from SAV3-infected C6/36 and CHSE-214 cells using TRIzol™ Reagent (Ambion). Next, cDNA from C6/36 derived RNA was obtained in 11 overlapping amplicons (Fig. 4B) by RT-PCR using SuperScript® III One-Step RT-PCR (Invitrogen) according to manufacturer's protocol. All amplicons were sequenced and compared to SAV3 reference strain H10 (JQ799139.1). Reliable sequence data was obtained from nt 73, until 52 nt upstream of the polyadenylation signal. The sequencing results showed 8 nucleotide differences to the reference strain, of which

5 led to a change in the encoded amino acid. To check if these mutations originated from viral RNA replication in C6/36 cells or whether they were already present in the virus inoculum, the corresponding amplicons derived from the CHSE-214-derived SAV viral RNA were also sequenced. These alignments showed that C6/36- and CHSE-214-derived SAV viral RNA were identical. Thus, no amino acid adaptations were necessary for SAV3 to replicate in mosquito cells. This result is in line with the hypothesis that alphaviruses limit mutations, to remain able to replicate in far divergent cell types and alternate hosts, rather than adapt to a specific cell type (Greene et al., 2005). The observation that SAV is able to replicate in mosquito cells without any adaptations is, besides applications in virus propagation for vaccine purposes, also interesting from an evolutionary point of view. Phylogenetic tree analysis of alphaviruses places SAV in its own clade far diverged from all other alphaviruses (Powers et al., 2001). Our results are in line with previous research though that suggested that alphaviruses may have a marine (Forrester et al., 2012) or invertebrate (Ventoso, 2012) origin.

In conclusion, we showed in our experiments here that SAV3, while not currently being classified as an arbovirus, also grows in invertebrate i.e. mosquito cells without mutations in the amino acid sequence. Similar viral titres are obtained in cell culture, albeit in a longer time-frame. Besides the biological importance of this observation, the use of invertebrate cells provides a putative alternative production platform for SAV vaccines.

## 3. ACKNOWLEDGEMENTS

We would like to thank Dr. Sjo Koumans (Boxmeer, The Netherlands) for providing SAV3 virus stock, monoclonal antibodies and the CHSE-214 cell line. We acknowledge Corinne Geertsema for cell culture maintenance. Additionally, we would like to express our gratitude to Dr. Stephane Villoing (Bergen, Norway) for his enthusiasm and many helpful discussions.



# Chapter 3

## Alphavirus capsid proteins self-assemble into core-like particles in insect cells: A promising platform for nanoparticle vaccine development

## ABSTRACT

The mosquito-borne chikungunya virus (CHIKV) causes arthritic diseases in humans, whereas the aquatic salmonid alphavirus (SAV) is associated with high mortality in aquaculture of Atlantic salmon and rainbow trout. Using modern biotechnological approaches, promising vaccine candidates based upon highly immunogenic, enveloped virus-like particles (eVLPs) have been developed. However, the eVLP structure (core, lipid membrane, surface glycoproteins) is more complex than that of non-enveloped, protein-only VLPs, which are structurally and morphologically 'simple'. In order to develop an alternative to alphavirus eVLPs, in this paper we engineered recombinant baculovirus vectors to produce high levels of alphavirus core-like particles (CLPs) in insect cells by expression of the CHIKV and SAV capsid proteins. The CLPs localize in dense nuclear bodies within the infected cell nucleus and are purified through a rapid and scalable protocol involving cell lysis, sonication and low-speed centrifugation steps. Furthermore, an immunogenic epitope from the alphavirus E2 glycoprotein can be successfully fused to the N-terminus of the capsid protein without disrupting the CLP self-assembling properties. We propose that immunogenic epitope-tagged alphavirus CLPs produced in insect cells present a simple and perhaps more stable alternative to alphavirus eVLPs.

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## 1. INTRODUCTION

Alphaviruses (family *Togaviridae*) cause fever, rash and arthritic or encephalitic disease in humans and animals and are predominantly transmitted by arthropod vectors, typically mosquitoes. At present, the most important alphavirus is chikungunya virus (CHIKV), which has re-emerged from Africa in 2004 as an invasive arbovirus causing large-scale epidemics in the Indian Ocean region, South East-Asia and Oceania (Paul et al., 2013; Suhrbier et al., 2012). CHIKV was also introduced in Europe with subsequent spread by autochthonous *Aedes albopictus* mosquitoes, in Italy (Rezza et al., 2007) and in France (Marc et al., 2011). As of December 2013, CHIKV is now also transmitted very efficiently by *Aedes aegypti* in the Americas, particularly in the Caribbean, and the epidemic is not expected to fade out any time soon (Leparc-Goffart et al., 2014). The only aquatic member within the genus *Alphavirus* is salmonid alphavirus (SAV), which infects fish in high-density aquaculture of Atlantic salmon and rainbow trout. Interestingly, SAV has no identified arthropod vector, yet it can replicate in invertebrate cells (Chapter 2; Olsen et al., 2013). Currently there is no licensed vaccine for CHIKV, whereas for SAV there is a commercial inactivated virus vaccine available.



The enveloped alphavirus particle encapsidates a positive, single-stranded RNA genome of approximately 12 kb, which is directly translated to yield the viral non-structural proteins (nsPs) necessary for replication (Jose et al., 2009). The viral structural proteins capsid (CP), envelope (E)3, E2, 6K and E1 are translated from a subgenomic messenger RNA. The envelope, consisting of a host-derived lipid bilayer from which E2-E1 spike complexes protrude, surrounds the nucleocapsid core containing the genomic RNA. The alphavirus core comprises 240 copies of a single CP, which self-assemble in the cytoplasm after autocatalytic, co-translational cleavage from the nascent structural polyprotein (Kuhn, 2007).

Previously, soluble CHIKV-E1 and -E2 structural protein subunits (sE1 and sE2) were expressed via baculovirus vectors in *Spodoptera frugiperda* (*Sf*) insect cells (Metz et al., 2011b). These subunits were shown to provide protection against chikungunya disease in an experimental mouse model (Metz et al., 2013b). Expression of all structural proteins resulted in the formation of enveloped virus-like particles (eVLPs) (Metz et al., 2013a), which were actively secreted from the cell and could be isolated by sucrose gradient centrifugation. These eVLPs consist of an alphavirus core, surrounded by a host-derived lipid membrane with associated glycoproteins E1 and E2 (envelope), of which the latter is surface-exposed and highly immunogenic. Compared to their sE1 and sE2 counterparts, the eVLPs provided superior protection against CHIKV challenge in vaccination studies in mice (Metz et al., 2013a; Metz et al., 2013b). CHIKV eVLPs were also produced in mammalian cells (Akahata et al., 2010) and this formulation has shown great promise in a recent human phase I clinical trial (Chang et al., 2014). The eVLPs from insect cells were found to have superior immunogenicity as compared to

similar doses of the subunits in mammalian produced eVLP, which underscores their vaccine potential (Metz et al., 2013b). SAV eVLPs were also produced using the same expression system, but a temperature shift from 27°C to 15°C was required for correct eVLP assembly and secretion (Metz et al., 2011a).

Whereas the insect-cell derived CHIKV eVLPs are amenable to large scale production in bioreactors (Metz and Pijlman, 2011), the purification of the eVLPs from the culture fluid remains tedious and involves multiple steps including high speed gradient centrifugation. In addition, baculovirus particles may be co-purified to varying levels as they share some intrinsic properties with the eVLPs. Furthermore, eVLPs have a complex structure, i.e. they are likely to be less stable over time than the morphologically more 'simple', non-enveloped, protein-only VLPs (Lua et al., 2014). Therefore, alternative approaches may be explored that address and resolve these important issues.

When we expressed the SAV structural polyprotein in *Sf*9 cells, dense nuclear bodies were observed by bright-field microscopy. Further examination by fluorescence and transmission electron microscopy (TEM) revealed that these nuclear bodies consist of assembled SAV nucleocapsid cores (Metz et al., 2011a). In New-World alphaviruses (e.g. Venezuelan equine encephalitis virus), the CP is responsible for host shut-off but depends on nuclear translocation for its action (Garmashova et al., 2007). In Old-World alphaviruses (e.g. CHIKV) host shut-off is regulated by the non-structural protein nsP2 and not by CP (Fros et al., 2013), yet nuclear and nucleolar localization signals are conserved within the CP amino acid sequence (Favre et al., 1994; Michel et al., 1990; Thomas et al., 2013). During Old-World alphavirus replication the CP still translocates to the nucleus, yet no function of the CP inside the nucleus is known (Jakob, 1994; Michel et al., 1990; Thomas et al., 2013).

In this study, we show that expression of the CP of two phylogenetically distant alphaviruses, CHIKV and SAV, by baculovirus vectors leads to the self-assembly and accumulation of core-like particles (CLPs) in the nucleus of infected *Sf* cells. Furthermore, we show that CLPs can be used as a protein carrier for incorporation of an immunogenic epitope of the homologous virus, without affecting the self-assembling properties of the CP or epitope conformation. Finally, we developed a simple purification method to isolate and concentrate these CLPs with high purity and protein recovery.

## 2. METHODS

### 2.1 Cloning and recombinant bacmid construction

The CHIKV (S27 strain, AF369024) CP sequence was amplified by PCR (primers listed in Table 1) and cloned in the pDONR207 vector using Gateway technology (Invitrogen). The eGFP gene was amplified by PCR and inserted at the N-terminus of the CHIKV CP sequence using conventional cloning procedures. A synthetic DNA sequence coding

for an immunogenic epitope of SAV (SAV3 isolate, DQ149204), the B-domain of the E2 glycoprotein (AA158-252) was ordered, flanked by Gateway compatible attB1/2 recombination sites and unique restriction sites EcoRI and BamHI. The SAV CP sequence was amplified by PCR and ligated either at the 5' ($CP^{E2BN}$) or 3' ($CP^{E2BC}$) prime end of the E2 sequence (primers listed in Table 1). By combining Gateway cloning with Bac-to-Bac (Invitrogen) technology, a recombinant *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) bacmid DNA was obtained, with the (fusion) protein encoding sequences under control of the very late polyhedrin promoter.

**Table 1.** Primers used for generation of the different expression constructs

| Primer name | Primer sequence (5'→ 3')[a] |
|---|---|
| CHIKV CP F | ATTB1-AAGCTTACC**ATG**GAGTTCATCCCAACCCAAACTTTTTA |
| CHIKV CP R | ATTB2-GGATCC**CTA**ATGGTGATGGTGATGGTGCATCCACTCTTCGGCCCCCT |
| SAV $CP^{E2BN}$ F | AAGGATCCGAGTTCATCCCAACCCAAAC |
| SAV $CP^{E2BN}$ R | TTGGATCC**CTA**CCACTCTTCGGCTCCCTCA |
| SAV $CP^{E2BC}$ F | AAGAATTCACC**ATG**GAGTTCATCCCAACCCAAAC |
| SAV $CP^{E2BC}$ R | TTGAATTCCCTCTCTTCGGCTCCCTCA |
| ATTB1 | GGGGACAAGTTTGTACAAAAAAGCAGGCTTA |
| ATTB2 | GGGGACCACTTTGTACAAGAAAGCTGGGTA |

[a] Restriction sites are underlined, and start and stop codons are printed in bold

### 2.2 Cell culture maintenance, transfection and infection

*Sf*9 were maintained in Sf900II medium as described earlier (Metz et al., 2013a). Transfection of *Sf* cells with recombinant bacmid DNA to generate baculovirus was carried out using Fectofly I (Invitrogen) and subsequent incubation at 27°C. Viral titers expressed as tissue culture infected dose 50 ($TCID_{50}$) per ml were determined by an end-point dilution assay (EPDA) using *Sf*9 Easy Titer cells (Hopkins and Esposito, 2009). Subsequent infections were performed by adding virus inoculum to the cells at a multiplicity of infection (MOI) of 10 $TCID_{50}$ units per cell. Cells were then incubated for 4 h at 27°C, after which medium was replaced by fresh medium and replaced at 27°C until capsid aggregations in the nucleus were visible. Microscopic images were captured using an Axio Observer Z1m inverted microscope (Zeiss), in combination with an X-Cite 120 series lamp.

### 2.3 SDS-PAGE and Western blot analysis

Cell protein samples were reduced using β-mercaptoethanol and loaded onto 10% bisacrylamide SDS-PAGE gels (Biorad). Subsequently, proteins were transferred onto an Immobilon-P polyvinylidene difluoride membrane (Millipore) by semi-dry blotting (Biorad), at a constant amperage of 0.05 A per gel. Membranes were blocked using 1% skimmed milk powder (Campina) in PBS containing 1% Tween20 (PBST) for 1 h

at room temperature (RT). Primary α-E2 (17H23 (Moriette et al., 2005) 1:2000) and alkaline phosphatase (AP)-labelled secondary antibodies were consecutively incubated with the membrane in 0.5% skimmed milk powder in PBST for 1 h at RT. The membrane was washed three times with PBST and once with AP buffer (100 mM NaCl, 5 mM $MgCl_{2,}$ 100 mM Tris-HCl, pH 9.5) Finally, NBT-BCIP substrate solution (Roche) was added and specific proteins became visible by purple coloration of the membrane upon substrate conversion.

### 2.4 Nucleocapsid isolation

Infected *Sf* cells were harvested from a T75 culture flask in a pre-chilled 10 ml tube and washed once with ice-cold PBS by centrifugation for 5 min at 3000 rpm (Thermo Scientific Heraeus Labofuge 200, rotor 2760), before gentle resuspension in 500 µl of ice-cold hypotonic buffer (20 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$; pH 7.4). The cell samples were incubated on ice for 15 min, after which 25 µl of 10% Nonidet P-40 (NP40) was added. The cell-NP40 mixtures were vortexed at maximum speed for 15 s. Nuclei were harvested by centrifugation for 10 min at 800 x g at 4°C. The nuclei were suspended in 500 µl cold buffer (20 mM HEPES, 20 mM EDTA, 150 mM NaCl; pH 7.6) and sonicated on ice for 6 sonication rounds of 10 s (Sonics Vibracell, output 2 W), interrupted by 10 s breaks. The nucleocapsids were harvested by centrifugation at 1700 x g for 15 min at 4°C, after which they were suspended in 500 µl PBS buffer (1.37 M NaCl, 27 mM KCl, 43 mM $Na_2HPO_4$, 14.7 mM $KH_2PO_4$; pH 7.4).

### 2.5 Antibody binding to CLP

Purified CLP, $CLP^{E2BN}$ and $CLP^{E2BC}$ were spotted (two times 2 µl) in a dilution range (1x, 10x, 50x) on two nitrocellulose membranes (Millipore). Both membranes were blocked with 1% skimmed milk powder (Campina) in PBST during 1 h incubation at RT. Thereafter, one membrane was incubated with a neutralizing, monoclonal antibody against the E2 antigenic peptide (17H23, 1:1000). The membrane with loading controls was incubated with serum derived from SAV-infected salmon (1:500). After three washes with PBST, the secondary antibodies, goat-anti-mouse (AP conjugated; 1:2500) and rabbit-anti-salmon (1:1000), were added to the membranes and incubated for 1 h at RT. The membrane that was initially incubated with the salmon-anti-SAV serum, was subjected to a third antibody, goat-anti-rabbit (AP-conjugated; 1:2500). Both membranes were developed by adding NBT-BCIP in AP buffer and incubation until purple coloration of the dots appeared.

### 2.6 Electron microscopy

CHIKV and SAV CP were expressed in *Sf*9 cells by recombinant baculovirus vectors at 27°C until dense nuclear bodies in the nucleus were noticeable by light microscopy, typically 24-48 h post infection (hpi). Cells were harvested, washed in PBS and fixed

during a 1 h incubation in fixative 1 (2% paraformaldehyde and 3% glutaraldehyde in buffer 2; 0.1 M $Na_2HPO_4.2H_2O$, 9.7 mM $C_6H_8O_7.2H_2O$ and 1.5 mM $CaCl_2$, pH 7.2). The cells were pelleted and washed twice with buffer 2. Subsequently, cells were pelleted for 5 min at 2700 x g through a layer of 5% (w/v) gelatin in $H_2O$. The remaining gelatin was removed from the cell pellet and the sample was solidified on ice for 10 min. The gelatin cell pellet was taken up in fixative 1 and cut in pieces of approximately 0.5 $mm^2$, before six times 10 min washes in buffer 1 (0.1 M $Na_2HPO_4.2H_2O$ and 9.7 mM $C_6H_8O_7.2H_2O$, pH 7.2). Further fixation of the specimens was obtained by incubation in fixative 2 (1% $OsO_4$ in buffer 2) for 1 h at RT. Cells were washed three times in distilled water before dehydration was obtained by successive incubation with 30%, 50%, 70%, 80%, 90%, 96% (all 5 min) and 100%, 100% (both 10 min) ethanol. Embedding in LR-White resin (LRW, EMS, Aurion) took place during successive incubations in 1:1 ethanol:LRW, 1:2 ethanol:LRW, LRW (all 1 h), LRW (overnight 4°C), LRW (1 h). Each specimen was placed in a separate gelatin capsule and the resin was polymerized during 48 h incubation at 55°C. After polymerization the samples were sectioned by an ultra-microtome (Leica FCS). Sample sections were captured on copper grids and stained by lead citrate, before analysis by transmission electron microscopy (JEOL JEM 1011, JEOL, MA, USA).



## 3. RESULTS AND DISCUSSION

### 3.1 Nuclear translocation of alphavirus capsid protein

Upon infection of *Sf* cells with baculovirus vectors expressing the complete CHIKV or SAV structural polyprotein (SP), dense nuclear bodies became visible from 24 hpi (Fig 1A) (Metz et al., 2011a). Similar results were obtained upon expression of the CHIKV CP, suggesting that the CPs of these viruses accumulated in the nucleus (Fig. 1B, left). Staining with Hoechst dye showed that the dense nuclear bodies co-localize with nucleic acid (Fig. 1B, right). To confirm that the dense nuclear bodies were indeed accumulations of the CP, eGFP was fused to the N-terminus of the CHIKV CP (eGFP-CP). The fusion protein was expressed in *Sf* cells by baculovirus vectors and its intracellular localization was analysed by fluorescence microscopy. The eGFP-CP fusion protein was visible only in a confined area within the nucleus (Fig 1C, top), whereas the GFP control protein freely diffused throughout the cell (Fig 1C, bottom). The dense nuclear bodies completely overlapped with the Hoechst staining (nucleic acid) and with the eGFP-CP signal, confirming that CP translocates to the nucleus and forms aggregates in a specific location within the nucleus, possibly the nucleolus.

### 3.2 CHIKV and SAV CP assemble in the nucleus as core-like particles

Both SAV and CHIKV dense nuclear CP aggregates were analysed by transmission electron microscopy. Following infection for 24-48 h, *Sf*9 cells were fixed and prepared for TEM analysis. The nuclei of all infected cells were enlarged (Fig 2B-D) in comparison

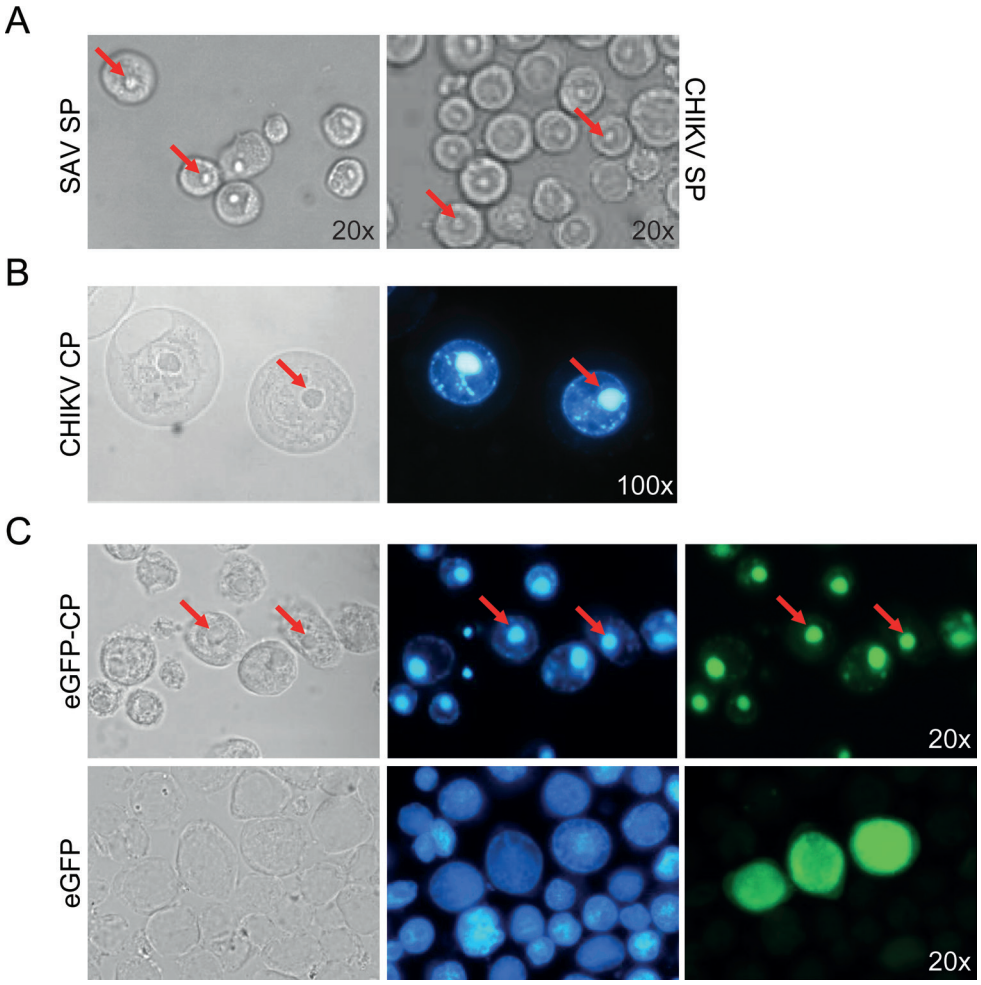


**Figure 1. Alphavirus capsid protein translocates to the nucleus.** (A) Brightfield image of (left) SAV and (right) CHIKV structural polyprotein (SP) cassette (20x magnification). (B) Brightfield and immunofluorescence image of CHIKV capsid protein (CP) expressed in *Sf*9 cells (100x magnification). (C) Brightfield and (immuno)fluorescence images of CHIKV CP with an eGFP fusion (upper panel), or eGFP control (lower panel), expressed in *Sf*9 cells (20x magnification). Red arrows indicate dense nuclear aggregates.

to those from uninfected *Sf*9 cells (Fig 2A) due to baculovirus DNA replication (Blissard and Rohrmann, 1990). In addition, in those cells expressing either SAV (Fig 2C) or CHIKV (Fig 2D) CP, an electron dense area in the nucleus was visible. Upon magnification (Fig 2E and F), very high numbers of spherical particles of approximately 30 nm in diameter were visible, similar to the predicted size of alphavirus nucleocapsid cores (Acheson and Tamm, 1967). This indicated that alphavirus CPs were able to assemble into CLPs in the nucleus of baculovirus-infected insect cells.

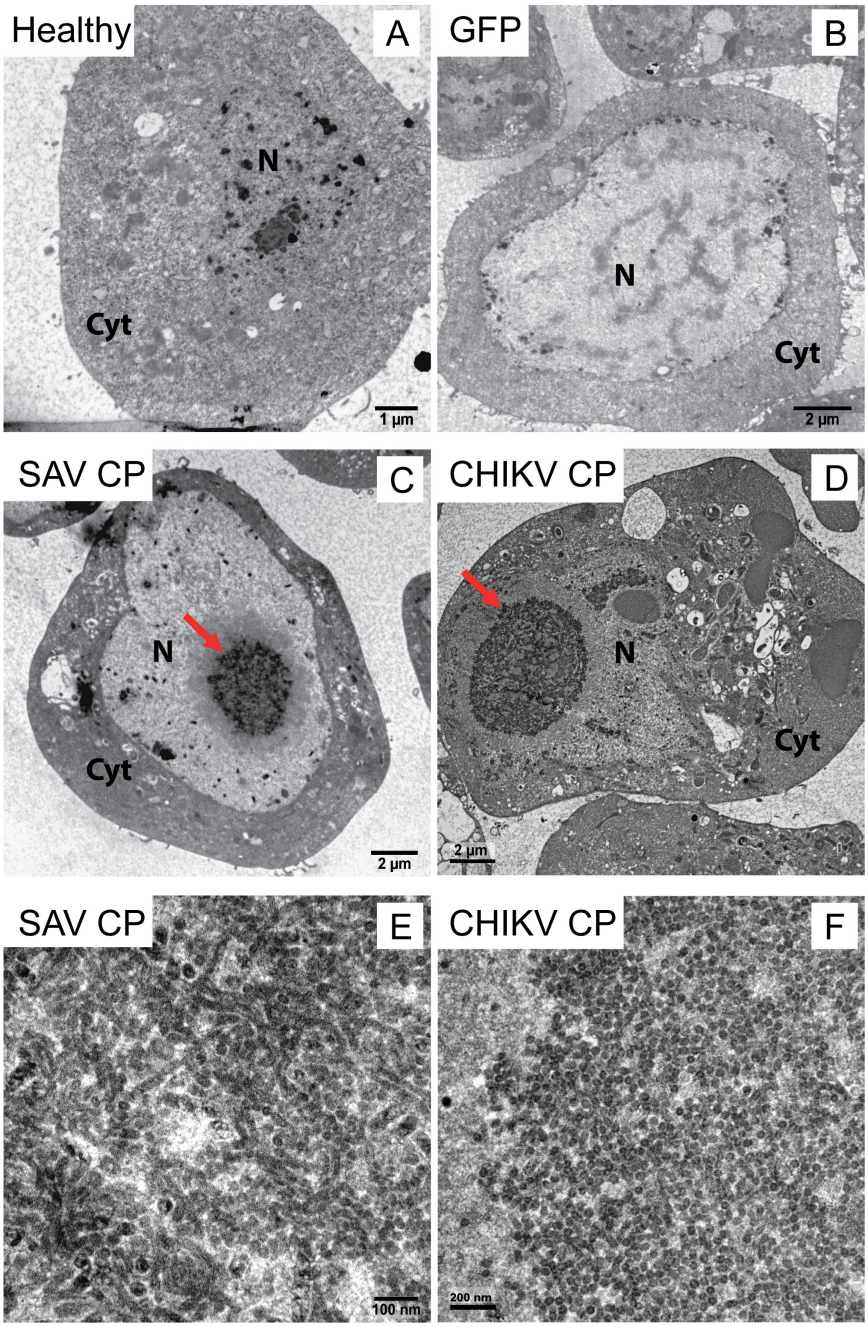


**Figure 2. Alphavirus nucleocapsid formation in the nucleus.** Transmission electron microscopy images of *Sf* cells. (A) Healthy *Sf* cells. Baculovirus expression of (B) eGFP, (C) SAV capsid protein (CP) and (D) CHIKV CP. (E) Magnification of the dense area in the nucleus following recombinant baculovirus expression of SAV CP or (F) CHIKV CP. Red arrows indicate dense nuclear aggregates.

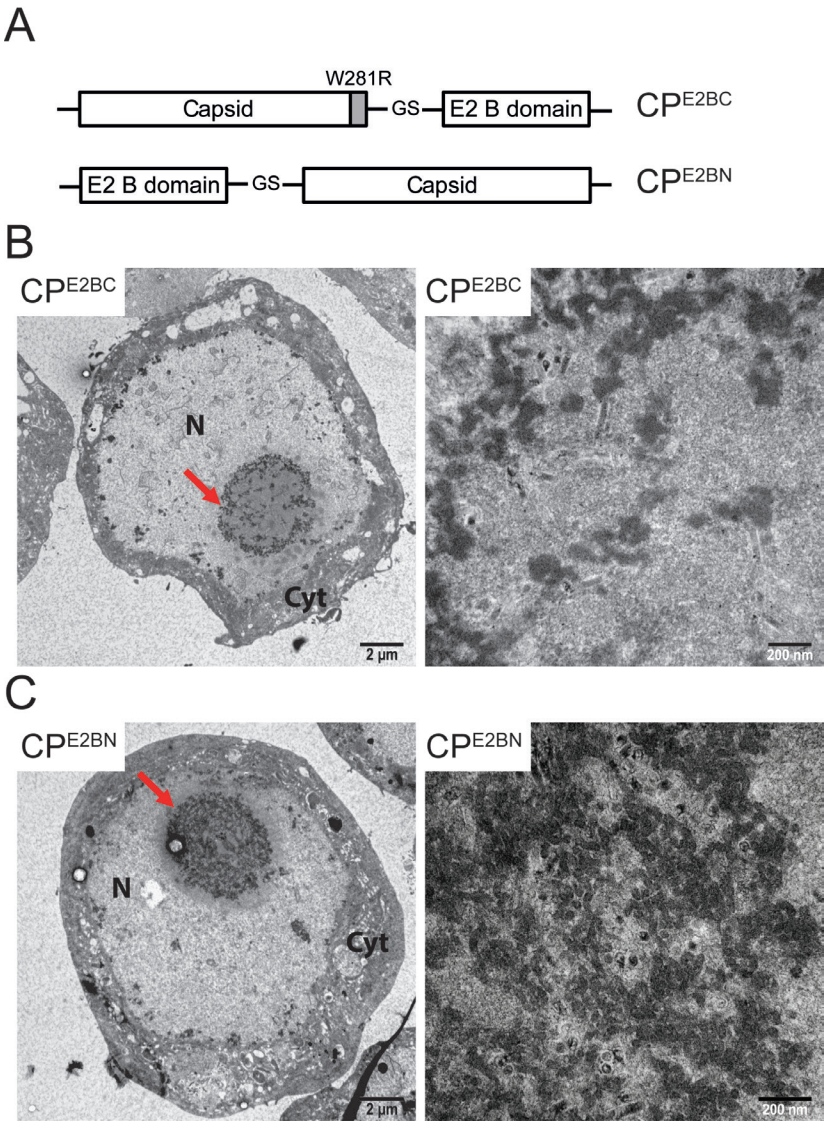


**Figure 3. Assembly of CLP with C- and N-terminal fusions.** (A) Schematic representation of the C- and N-terminal fusion proteins (B) Sf cells were infected with baculovirus vectors expressing the SAV CP with an C-terminal fusion of the SAV E2 protein B-domain region ($CP^{E2BC}$) or (C) N-terminal fusion of the SAV E2 protein B-domain ($CP^{E2BN}$). The right panels are magnifications of the dense nuclear areas in the left panels. Red arrows indicate dense nuclear aggregates.

### 3.3 Assembly of alphavirus CLPs with N- and C-terminal epitope fusions

To examine the possible use of alphavirus CLPs as protein/peptide/antigen carriers, an important alphavirus immunogenic epitope was fused to the N- or C-terminus of the CP (Fig 3A). The most immunogenic epitope against which antibodies are raised during wild type alphavirus infections, lies within the distal tip, or B-domain, of the

E2 glycoprotein (Hunt et al., 2010; Porta et al., 2014). Antibodies raised against this region are often sufficient to neutralize an infection and mutations of neutralization escape variants map within this region (Coffey and Vignuzzi, 2011; Voss et al., 2010). In addition, peptide vaccination of this region alone may be sufficient to raise a substantial neutralizing immune response (Moriette et al., 2005; Snijders et al., 1991). Therefore, fusion of this 96 amino acid-long peptide to the CLPs may generate a promising vaccine candidate, although it is not clear beforehand whether or not such chimeric CP will still be capable to assemble as CLPs. To test this, the B-domain of the SAV E2 glycoprotein was fused to the N- ($CP^{E2BN}$) or C-terminus ($CP^{E2BC}$) of the SAV CP. This region lacks glycosylation sites that might influence the immunogenicity of the peptide (Karlsen et al., 2015). Upon expression of both $CP^{E2BN}$ and $CP^{E2BC}$ fusion proteins by baculovirus vectors in *Sf* cells, dense nuclear bodies were visible by light microscopy as shown before (Fig 1A). However, TEM analysis of the *Sf* cells expressing the $CP^{E2BC}$ fusion protein showed that in this case CLP formation seemed to be impeded and only protein aggregates were visible (Fig 3B). We expect the inability of the C-terminal fusion to form CLPs to be caused by steric hindrance. However, expression of $CP^{E2BN}$ (Fig 3C) did lead to the assembly of authentic CLPs in the nucleus of *Sf* cells.



### 3.4 Purification of chimeric alphavirus CLPs with high protein recovery

Because the chimeric CLPs assembled as defined aggregates within the nucleus, attempts were made to purify these aggregates by simple, scalable purification methods (Fig 4A). AcMNPV-$CP^{E2BN}$ infected *Sf* cells were washed in cold PBS, before lysis in a hypotonic buffer to disrupt the plasma membrane. This lysis step separated the nucleus from the cytoplasm without substantial $CP^{E2BN}$ protein loss, since similar epitope detection intensities between the start material and the nuclear fraction are observed (Fig 4B, middle panel, lane 3 and 5) and almost no proteins were detected in the cytoplasmic fraction (Fig 4B, middle panel, lane 4). The nuclear fraction was sonicated on ice to disrupt the nuclear membrane. Subsequent low-speed centrifugation resulted in a pellet containing the CLPs. From the CBB staining (Fig 4B, top panel, lane 7) it can be concluded that the major components in this fraction are CLPs and that there is a relatively low amount of background protein. The high CLP recovery upon low speed centrifugation suggests that the integrity of the CLPs is largely unaffected after the sonication procedure, since dissociated capsid monomers would not be pelleted. As expected, no baculoviral envelope surface glycoprotein (GP64) could be detected in this fraction (Fig 4B, lower panel, lane 7), suggesting that the CLPs already have a high degree of purity. In addition, the purification method does not contain detergents in the final purification steps, which facilitates vaccine formulation.

Finally, to confirm the correct conformation of the fusion peptide after the purification process, a binding assay was performed in which purified chimeric CLP and authentic CLP were spotted on a nitrocellulose membrane. The membrane was stained with a

neutralizing, monoclonal antibody against the E2 antigenic peptide. A clear signal was observed in the $CLP^{E2BN}$ purified fraction, which was also more intense than in the $CLP^{E2BC}$ purified fraction (Fig 4C, upper panels). The control CLP did not show specific binding. A loading control shows that equal amounts of each $CLP^{E2BN}$, $CLP^{E2BC}$, and CLP were loaded (Fig 4C, lower panels). It was concluded that the fusion peptide in the $CLP^{E2BN}$ purified fraction retains the correct conformation after purification, which increases the potential of chimeric CLPs as successful vaccine candidate.

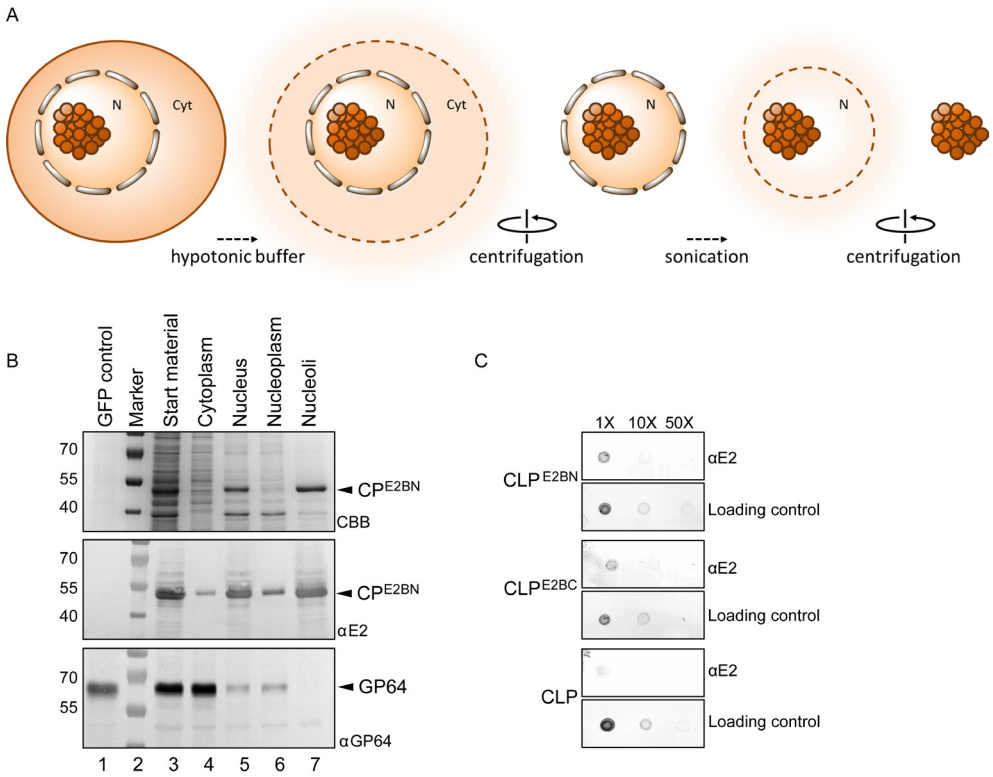


**Figure 4. Efficient purification of CLPs.** (A) Schematic representation of the CLP purification process; Cyt = cytoplasm, N = nucleus. (B) Coomassie Brilliant Blue (CBB) staining (top panel), Western Blot detection of the SAV E2 B-domain (17H23, middle panel), and Western blot detection of the baculoviral surface glycoprotein GP64 (lower panel) of $CP^{E2BN}$ infected Sf cells and fractions during CLP purification. (C) Dot blot of the purified chimeric CLP and CLP fraction. The fusion peptide is detected by a peptide-specific, monoclonal antibody (17H23).

## 4. CONCLUDING REMARKS

VLPs in general have self-adjuvanting properties and are highly effective immunogens due to their particulate nature and display of epitopes in repeated regular patterns (Roy and Noad, 2009). Most VLPs used as protein carriers for vaccination purposes display heterologous peptides. The use of particulate alphavirus CLPs with immunogenic

epitopes of the homologous virus (e.g. the B-domain of E2) may contribute to increased antigenic properties of the chimeric CLPs (Kam et al., 2014). However, the true immunogenicity of the chimeric CLPs needs to be experimentally validated in animal models (Metz et al., 2013b).

We have shown that a fusion of an immunogenic peptide to the N-terminus of CP in the case of SAV is experimentally feasible and straight forward and that the self-assembling properties and the particulate nature of the chimeric CLPs are retained. In conclusion, this study shows that alphavirus CLPs produced in insect cells forms a promising platform for nanoparticle vaccine development, which may find application in other industrial processes due to the easy purification process. The CLPs could be attractive, arguably more stable, alternatives to the highly immunogenic, but complex and less stable eVLPs. Yet, their immunogenicity should be evaluated in comparative vaccination trials.



## ACKNOWLEDGEMENTS

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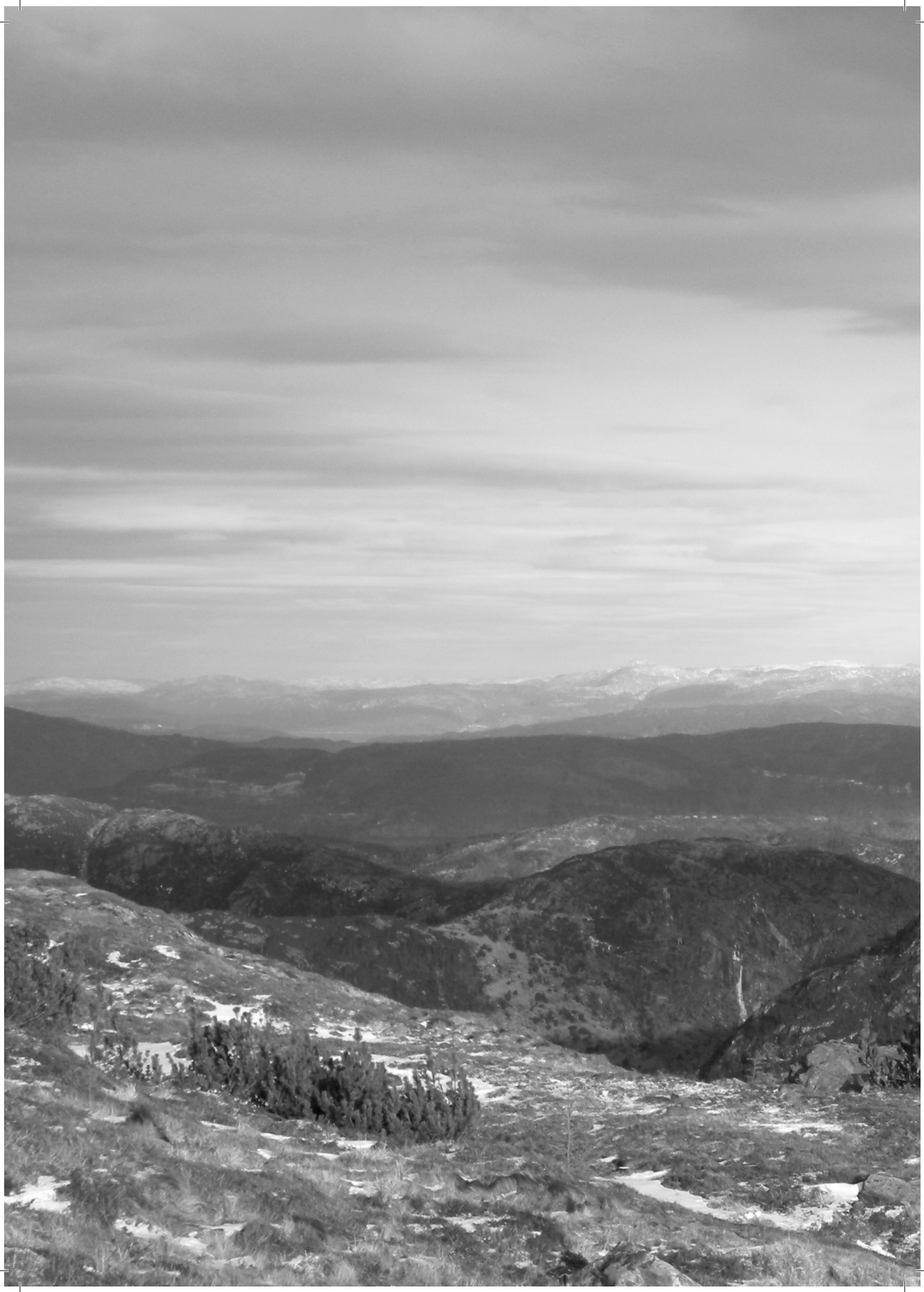

# Chapter 4

## Salmonid alphavirus glycoprotein E2 requires low temperature and E1 for virion formation

## ABSTRACT

Salmonid alphavirus (SAV; also known as Salmon pancreas disease virus; family *Togaviridae*) causes pancreas disease and sleeping disease in Atlantic salmon and rainbow trout, respectively, and poses a major burden to the aquaculture industry. SAV infection in vivo is temperature-restricted and progeny virus is only produced at low temperatures (10-15°C). Using engineered SAV replicons we show that viral RNA replication is not temperature-restricted suggesting that the viral structural proteins determine low-temperature dependency. The processing/trafficking of SAV glycoproteins E1 and E2 as a function of temperature was investigated via baculovirus vectors in *Sf*9 insect cells. We identified SAV E2 as the temperature determinant by demonstrating that membrane trafficking and surface expression of E2 occurs only at low temperature and only in the presence of E1. This is the first study that identifies E2 as the critical determinant of SAV low-temperature dependent virion formation.

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## 1. INTRODUCTION

Salmonid alphavirus (SAV) is the causative agent of pancreas disease (PD) in Atlantic salmon (*Salmon salar* L.) and sleeping disease (SD) in rainbow trout (*Oncorhynchus mykiss* W.). SAV outbreaks are associated with major economic losses in the European aquaculture of these high value fish species (McLoughlin and Graham, 2007). SAV, also known as Salmon pancreas disease virus (SPDV; ICTV 9th report, 2011), is a unique member of the genus *Alphavirus* (family *Togaviridae*) as most alphaviruses are transmitted via invertebrate vectors, typically mosquitoes. So far, no vector has yet been implicated in SAV transmission, although the virus was detected in the salmon louse *Lepeophtheirus salmonis* (Petterson et al., 2009) and we recently showed for the first time that SAV can complete its infectious cycle in invertebrate cells, i.e. in cells of the mosquito *Aedes albopictus* (Chapter 2). Progeny SAV was produced, yet only in a temperature-dependent manner, at 15°C but not >18°C (Chapter 2). Similarly, SAV infection in fish has the same replication optimum between 10°C and 15°C and virion formation is lost at temperatures above 18°C (Graham et al., 2008b; McLoughlin and Graham, 2007), a phenomenon that is not well understood.



The infectious cycle of SAV is expected to consist of general alphavirus 'life' cycle elements (Villoing et al., 2000; Weston et al., 2002; Weston et al., 1999). The viral genome consists of a single, positive stranded RNA, which produces a subgenomic mRNA that encodes the viral structural proteins capsid (C), envelope (E)3, E2, 6K and E1. Upon translation, the C protein is autocatalytically cleaved off from the nascent polyprotein. The signal sequence at the N-terminus of E3 translocates the envelope cassette (precursor E2 (PE2), 6K and E1) into the endoplasmic reticulum (ER). The trimeric alphavirus spikes consisting of three PE2-E1 heterodimers are then formed and travel to the trans-Golgi network (TGN). Here PE2 is processed by the host enzyme furin, which separates E3 from E2 and renders the spike sensitivity for low-pH induced activation. Finally, the glycoprotein spikes appear on the cell surface and by association with the viral nucleocapsid the enveloped virions then leave the cell by budding (Garoff et al., 2004; Kielian, 2010; Kuhn, 2007).

In contrast to other well-studied alphaviruses, the requirements for SAV glycoprotein surface translocation, and the putative role of temperature therein, are not known. Of note, temperature restriction was also observed during baculovirus expression of SAV virus-like particles (VLPs) in *Spodoptera frugiperda* (*Sf*) 9 insect cells, which was efficient at 15°C but restricted >18°C (Metz et al., 2011a). In the present study, we use this model to further investigate the processing and trafficking of SAV glycoproteins E2 and E1 as a function of temperature. Our experiments uncover that the critical determinant for low-temperature dependent SAV virion formation lies within the E2 glycoprotein, which needs both low temperature and co-expression of E1 for proper translocation and presentation at the cell surface.

## 2. MATERIALS AND METHODS

### 2.1 Plasmid construction

A synthetic, full-length infectious clone was designed based on the SAVH20/03 isolate, a salmonid alphavirus subtype 3 (SAV3, accession number DQ149204.1) and ordered (Blue Heron Biotech, Bothell, WA, USA). A hammerhead ribozyme (HHr) was inserted upstream of the 5' untranslated region (UTR) to generate full infectious SAV3 isolate with HHr (pFISH) (Fig. 1B). Self-ligation via AscI removed the second subgenomic promoter plus structural polyprotein cassette and gave the replicon pSAV/eGFP (Fig. 1B). Recombinant baculoviruses were generated by Gateway technology combined with Bac-to-Bac® (Invitrogen) as described (Metz et al., 2011) (Fig. 2A). A furin cleavage site mutation (RKKR → RKAA) was engineered by Quick-change PCR to generate Ac-SAV3$^{Fmut}$. Restriction enzymes were from New England Biolabs (Ipswich, MA), primers are listed in Table 1.

**Table 1. Primers used for generation of the different expression constructs and point mutations.**

| Name Primer | Primer sequence (5'→ 3')* |
|---|---|
| SAV3_C_F | ATTB1-GGATCCACC**ATG**TTTCCCATGCAATTCACAAA |
| SAV3_C_R | ATTB2-GGATCC**TGA**CCAAGGTATGGCCTCGCTG |
| SAV3_E3_F | ATTB1-GGATCCACC**ATG**ACACGTGCTCCAGCCCTCC |
| SAV3_E2_R | ATTB2-GGATCC**TGA**CGCACGAGCCCCAGGTATG |
| SAV3_E1_R | ATTB2-GGATCC**TGA**GCTCTTGACTATCCGGATTCT |
| SAV3$^{Fmut}$_F | CACCTGCAATTCCGCCCGGAAAGCGGCGGCTGTGTC |
| SAV3$^{Fmut}$_R | GGCAGGCGACGCAGACACAGCCGCCGCTTTCCGGGCG |
| ATTB1 | GGGGACAAGTTTGTACAAAAAAGCAGGCTTA |
| ATTB2 | GGGGACCACTTTGTACAAGAAAGCTGGGTA |

* Restriction sites are displayed in italics, and start/stop codons are printed in bold. Nucleotides changed during quick-change PCR are underlined.

### 2.2 Cells and viruses

*Spodoptera frugiperda Sf*9 cells (Invitrogen) and Chinook salmon embryo CHSE-214 cells were cultured as described (Hikke et al., 2014; Metz et al., 2011a). Viral titers were determined by an end-point dilution assay (EPDA) using *Sf*9 Easy Titration cells (Hopkins and Esposito, 2009) for baculoviruses or CHSE-214 cells for SAV. CHSE-214 cells were transfected at 12°C for 4 h using FuGene (Promega) according to manufacturer's protocol, then placed at 12°C or 20°C.

### 2.3 Immunofluorescence and protein analysis

Cells were fixed with 4% paraformaldehyde in PBS for 10 min at RT and optionally

permeabilized with ice-cold acetone/methanol for 10 min. Immunofluorescence analysis with mAbs α-E1 (4H1 (Todd et al., 2001), 1:500) or α-E2 (17H23 (Moriette et al., 2005), 1:1000) was conducted as described (Hikke et al., 2014).

VLPs were expressed by infecting *Sf*9 cells with baculovirus Ac-SAV3 or the furin cleavage site mutant Ac-SAV3$^{Fmut}$ for 1 d at 27°C, followed by 5 d at 15°C. The chimeric Ac-CHIKV/SAV constructs were expressed at 27°C. VLPs were precipitated as described (Metz et al., 2011). For the deglycosilation assay, cell samples were treated with PNGase or EndoH (New England Biolabs) at 37°C for 2 h (Metz et al., 2013). SDS-PAGE and western blot analysis were conducted as described (Hikke et al., 2014).

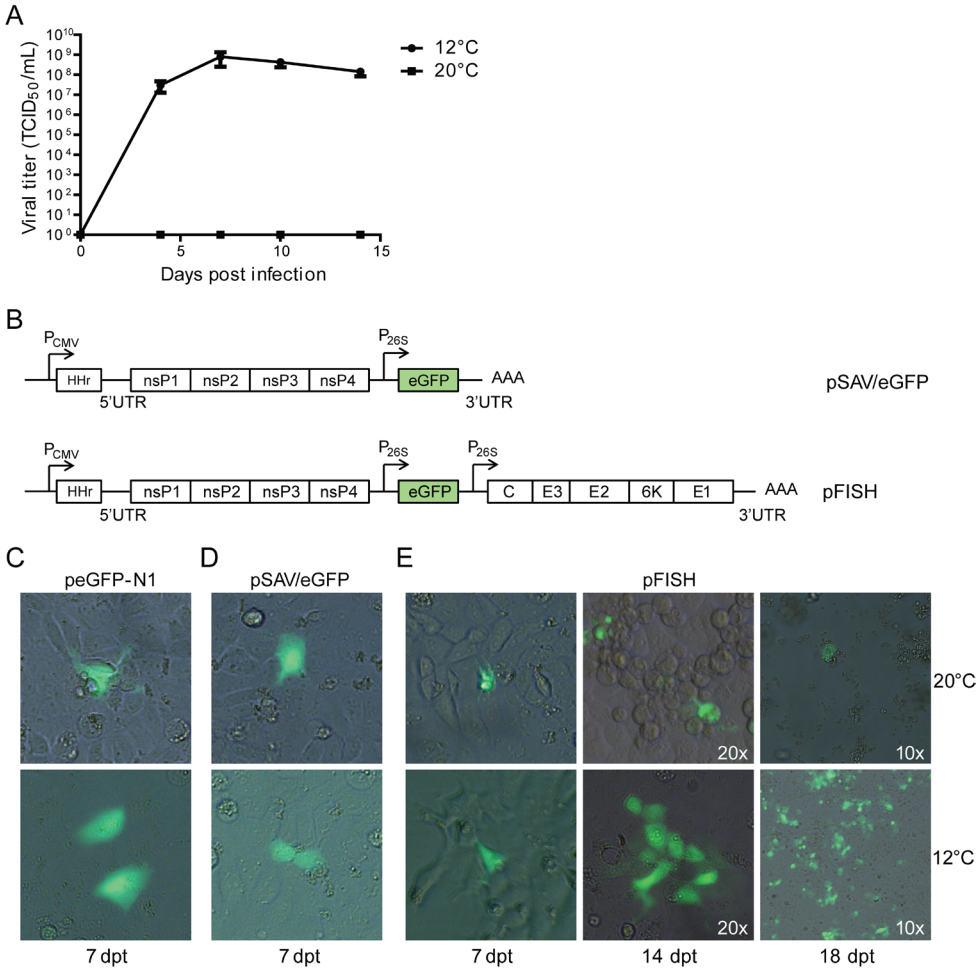


**Figure 1. SAV virion production but not RNA replication is dependent on low temperature.** (A) SAV infections of CHSE-214 cells were incubated at 12°C and 20°C and viral titers measured at different time points post infection. Error bars indicate standard deviation of triplicates. (B) Schematic representation of the replicon and full infectious SAV clone used. (C) Transfection of CHSE-214 cells with the positive control plasmid peGFP-N1 and (D) the replicon pSAV/eGFP. (E) Transfection of CHSE-214 cells with pFISH, followed in time.

## 3. RESULTS AND DISCUSSION

### 3.1 SAV virion formation but not RNA replication is restricted at 20°C

As previous studies had shown that SAV reproduction (Graham et al., 2008a; McLoughlin and Graham, 2007) and VLP production (Metz et al., 2011a) were completely absent at temperatures above 18°C, we set out to determine the viral factors responsible for this low-temperature dependency. SAV growth curve analysis at 12°C and at 20°C was performed on salmonid CHSE-214 cells. *De novo* virion formation was only observed at 12°C, with titers that steadily increased to $8*10^8$ $TCID_{50}$/ml at 7 days post infection (dpi), whereas no virus was produced at 20°C (Fig. 1A).

To determine whether or not SAV RNA replication occurred at 20°C, CHSE-214 cells were transfected with pSAV/eGFP replicon DNA (Fig. 1B) and incubated at either 12°C or 20°C. GFP expression was visible in replicon transfected cells incubated at both 12°C and 20°C (Fig. 1D), suggesting that RNA replication was not inhibited at 20°C, as in agreement with recent data (Olsen et al., 2013). In addition, a synthetic SAV infectious clone (pFISH, Fig. 1B), was used to transfect CHSE-214 cells and GFP expression was followed in time. pFISH-transfected CHSE-214 cells incubated at 12°C showed viral spread at 14 and 18 days post transfection (dpt) (Fig. 1E). In contrast, cells transfected with pFISH and incubated at 20°C only showed single GFP-positive cells, indicating that replication was restricted to single cells and viral spread did not occur (Fig. 1E). We conclude that SAV structural proteins, rather than viral RNA replication, are likely to be the critical determinants for low-temperature dependent virion formation.

### 3.2 SAV structural glycoprotein processing and surface localization

Recently, we showed that SAV is able to complete its infection cycle within *A. albopictus* mosquito cells (Chapter 2) and that SAV replicon RNA replicates in *S. frugiperda* (*Sf*) insect cells (Olsen et al., 2013). Furthermore, *Sf* cells are capable of expression, correct folding and posttranslational modification of vertebrate (viral) proteins (van Oers et al., 2006) and have been used to successfully produce SAV VLPs at low temperature (Metz et al., 2011a). Thus, insect cells are a good proxy model to study the posttranslational processing and trafficking of SAV glycoproteins E2 and E1 as a function of temperature. SAV structural genes were cloned, in different combinations, in baculovirus expression vectors (Fig. 2A) and were expressed in *Sf*9 cells at 27°C or 15°C (Fig. 2B-D). Infected cells were analysed for protein expression and PE2 processing by western blot (WB) (Fig. 2BC) and for surface localization of E1 and E2 by immunofluorescence (Fig. 2D).

At 15°C, all SAV constructs produced a protein of ~55 kDa, which corresponds to the uncleaved PE2 precursor (Fig. 2B). Furthermore, Ac-SAV3 and Ac-Env produced an additional lower band of ~48 kDa, corresponding to the furin-cleaved, mature form of E2 (Fig. 2B, lanes 1 and 3). In contrast, Ac-PE2 failed to produce mature E2 and only expressed the larger, uncleaved PE2 (Fig. 2B, lane 4). This suggests that 6KE1 is required

for processing of the PE2 precursor by host furin into mature E2. At 27°C, however, none of the constructs produced mature, furin-cleaved E2, but only the PE2 precursor (Fig. 2C). This suggests that in addition to the requirement for E1, low temperature is indispensable for correct processing of PE2 to yield mature E2.

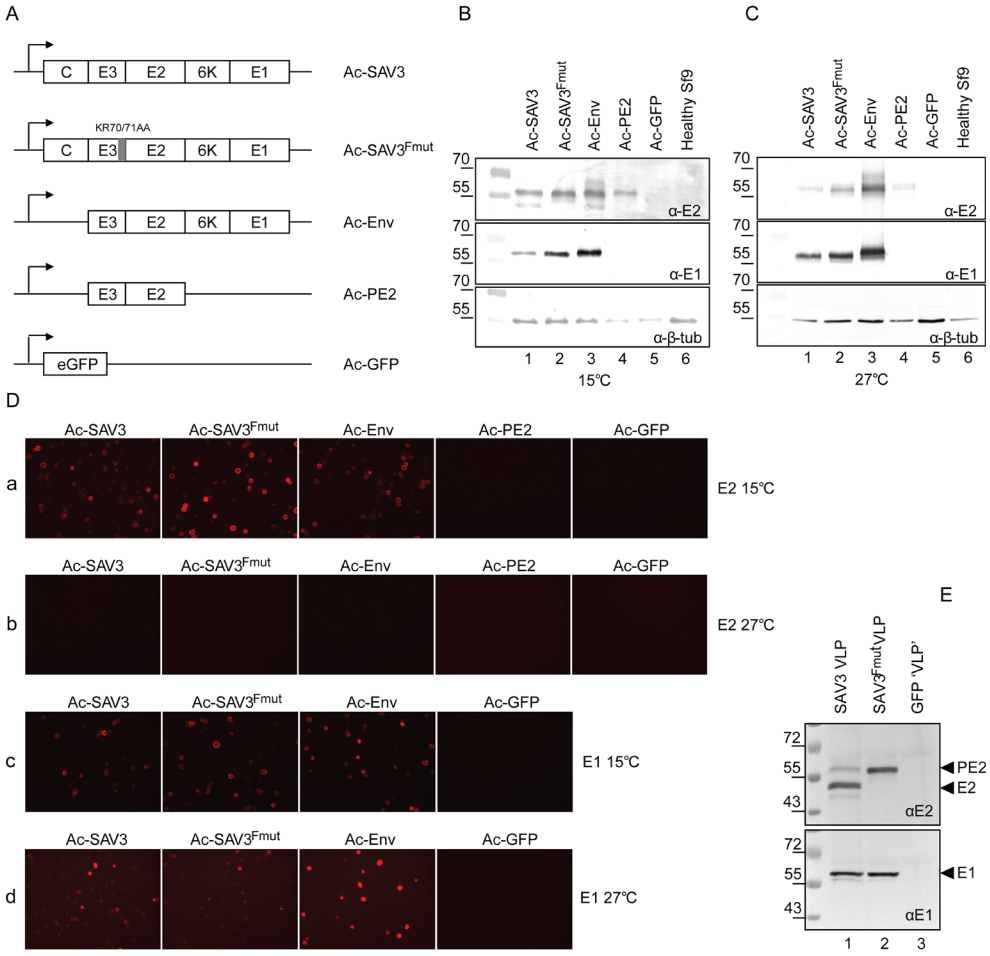


**Figure 2. SAV structural protein processing and surface translocation.** (A) Schematic representation of the different SAV structural protein combinations used in this study. Recombinant baculoviruses expressed the proteins under control of a late baculovirus promoter (arrow). Env: envelope cassette, PE2: precursor E2, C: capsid protein. (B,C,D) Different SAV structural subunits were expressed via baculovirus vectors in *Sf*9 insect cells and incubated at 15°C or 27°C. Western blot detection of SAV E2 and E1 glycoproteins in *Sf*9 cell fractions infected with SAV structural proteins incubated at 15°C (B) and (C) 27°C. Protein sizes in kDa are indicated at the left. (D) SAV structural protein surface translocation at 15°C and 27°C. Cells were surface stained in an immuno fluorescence assay for the presence of E2 and E1. (a) Detection of E2 glycoprotein after incubation at 15°C or (b) 27°C. (c) Detection of E1 glycoprotein after incubation at 15°C or (d) 27°C. (E) Furin cleavage is not required for SAV VLP secretion. Media of baculovirus infected *Sf*21 cells, incubated at 15°C, was analysed for the presence of SAV glycoproteins. Protein sizes in kDa are indicated at the left.

Next, we analysed the surface localization of SAV envelope glycoproteins E1 and E2 as function of the temperature. Cells infected with Ac-SAV3 displayed E2 at the surface at 15°C (Fig. 2D, panel a) but not at 27°C (Fig. 2D, panel b). Interestingly, and in sharp contrast to E2, E1 was detected at the surface of cells infected with Ac-SAV3 at both 15°C (Fig. 2D, panel c) and 27°C (Fig. 2D, panel d). This indicates that E1 surface translocation is independent of E2 and low temperature. Next, we determined that the capsid protein was not required for surface expression of E1 and E2 glycoproteins by observing that cells infected with Ac-Env displayed both E2 and E1 glycoproteins on the cell surface of (Fig. 2D, panels a, c, d). Again, E2 was only detected on the cell surface at 15°C but not at 27°C (Fig. 2D, panel b). Since E1 could surface translocate independently (Fig. 2D, panel d), it was investigated whether also E2 could travel to the cell surface on its own. However, when the precursor E2 (Ac-PE2) was expressed at 15°C or 27°C (Fig. 2D, panels a, b), no E2 protein was detected at the cell surface, confirming that surface translocation of E2 not only requires low temperature, but E1 as well.

### 3.3 Furin cleavage is not required for E2 surface translocation or VLP secretion

SAV E2 detection at the cell surface was correlated with the presence of mature, furin-processed E2 (Fig. 2)(Metz et al., 2011a). A furin cleavage site knock-out mutant was generated (SAV3$^{Fmut}$) to determine whether or not furin processing of PE2 was required for surface translocation and/or VLP secretion. First, Western blot analysis confirmed that cells infected with Ac-SAV3Fmut at 15°C only produced PE2 and no furin-processed E2 (Fig. 2B, lane 2). However, the mutant Ac-SAV3$^{Fmut}$ did not block the surface expression of E2 at 15°C as observed by intense staining of the cell surface in the IFA (Fig. 2D, panel a), suggesting that E2 surface translocation is furin-independent.

VLPs are secreted in the medium upon expression of SAV structural proteins (Ac-SAV3) at 15°C, but not at 27°C. Both E1 and E2 were found in the medium fraction of cells infected at 15°C with the positive control virus Ac-SAV3 (Fig. 2E, lane 1), as well as the furin mutant Ac-SAV3$^{Fmut}$ (Fig. 2E, lane 2) but not the negative control virus Ac-GFP (Fig. 2E, lane 3). This indicates that VLP formation does not depend on furin cleavage as well.

### 3.4 ER arrest of SAV PE2 at low temperature or in the absence of E1

It is now clear that correct furin processing and surface translocation of E2 are correlated (but not causal) events and both are dependent on i) low temperature and ii) the presence of E1. We hypothesized that PE2 is arrested in the secretory pathway at 27°C or at 15°C in the absence of glycoprotein E1. Cell fractions containing the expressed PE2 were treated with the endoglycosidases PNGase F (which removes all glycans) or EndoH (which can only remove immature glycans from glycoproteins arrested in the ER), and were analysed on WB (Fig. 3).

In Ac-SAV3 infected cells, both PE2 and E2 were detected (Fig. 3, lane 1), in line with our previous study (Metz et al., 2011a). PNGase F (Fig. 3, lane 2), but not EndoH (Fig. 3,

lane 3), was able to remove glycans from the mature, furin-processed E2 protein when expressed at 15°C as visualized by a ~2 kDa reduction in protein size. This showed that E2 was glycosylated, left the ER and travelled to the Golgi, where the glycans were modified to render them resistant against EndoH treatment. In contrast, the larger PE2 precursor (~55 kDa) produced at 15°C appeared susceptible to cleavage by both PNGaseF and EndoH, as illustrated by a down shift of this protein by about 2 kDa. This suggests that PE2 was glycosylated, but remained localized to the ER. When the proteins were expressed at 27°C, only PE2 was observed (Fig. 3, lane 1) that was sensitive to both endoglycosidases (Fig. 3, lanes 2, 3). Specific ER staining in the baculovirus-infected *Sf*9 cells was unfortunately unsuccessful due to the enlarged nucleus that strongly condensed the cytoplasm including the ER.

In the absence of E1 (Ac-PE2), at both 15°C and 27°C only the PE2 protein was detected (Fig. 3, lane 4) that was sensitive to both endoglycosidases (Fig. 3, lanes 5, 6). Therefore, we conclude that at high temperature (27°C) or in the absence of E1, PE2 is arrested in the ER and cannot participate in virion formation.



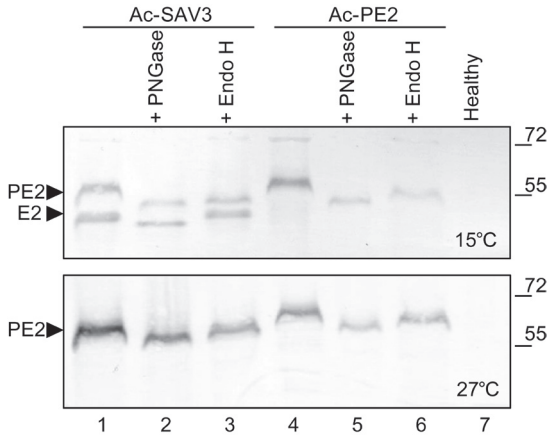


**Figure 3. ER arrest of PE2.** Deglycosylation assay of Ac-SAV3 and Ac-PE2 infected *Sf*9 cell samples. SAV structural cassette and PE2 subunit were expressed by recombinant baculoviruses in *Sf*9 cells at both 27°C and 15°C. Cell fractions were treated with PNGase (removes all glycans) and EndoH (removes only immature glycans of glycoproteins arrested in the ER) and analysed on Western blot. Protein sizes in kDa are indicated at the right.

## 4. CONCLUSIONS

In this study we showed that SAV spread is completely arrested at higher temperatures (20°C in CHSE-214 cells), but that viral RNA replication is not compromised at these higher temperatures. Furthermore, we showed that E1 translocated to the cell surface at all temperatures tested and independently of E2. In contrast, PE2 was unable to exit the ER at 27°C or in the absence of E1. Furin cleavage of PE2 was not required for E2 cell surface translocation and VLP production. Therefore, the ability to produce virions is

dependent on the correct surface translocation of glycoprotein E2, which requires low-temperature and the presence of E1 (Fig. 4). Our findings lead to a better understanding of the low-temperature dependent 'life' cycle of this unique, aquatic alphavirus and may have important implications for future vaccine design.

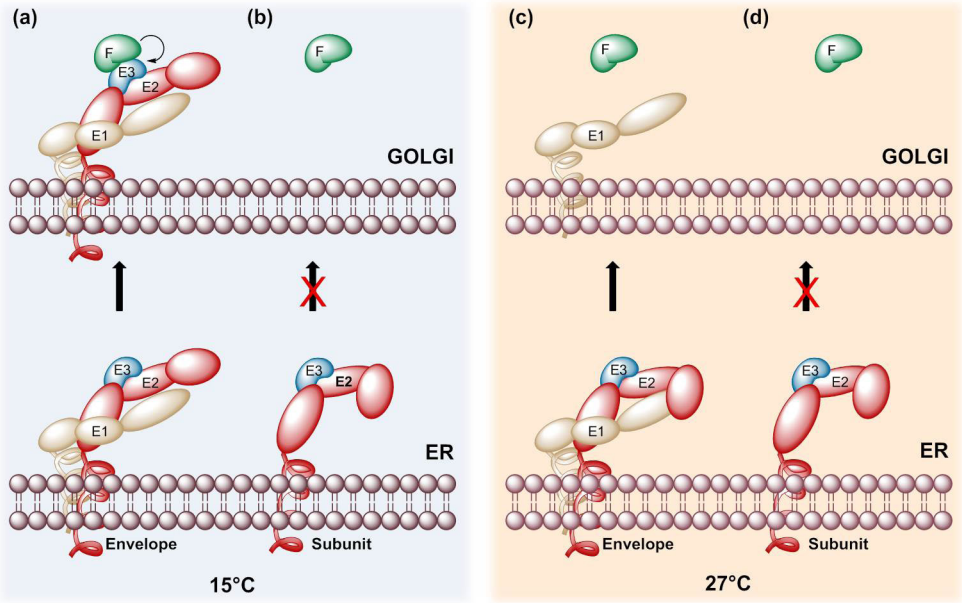


**Figure 4. Proposed model for low-temperature dependent SAV glycoprotein trafficking.** Expression of the (a) SAV envelope cassette and (b) PE2 subunit at 15°C and expression of the (c) SAV envelope cassette and (d) PE2 subunit at 27°C. F: furin, cleavage indicated by arrow, ER: endoplasmic reticulum.

## ACKNOWLEDGEMENTS

We thank Dr. Sjo Koumans (Boxmeer, The Netherlands) for providing SAV3 virus, monoclonal antibodies and the CHSE-214 cell line. We acknowledge Dr. Richard Kormelink for helpful discussions.


IP130012.1
SAV-VLP
Prod date 20-nov
Store 2-8°C
IP130012.2
VLP-CTRL
Prod date 20-nov
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CHICK/S
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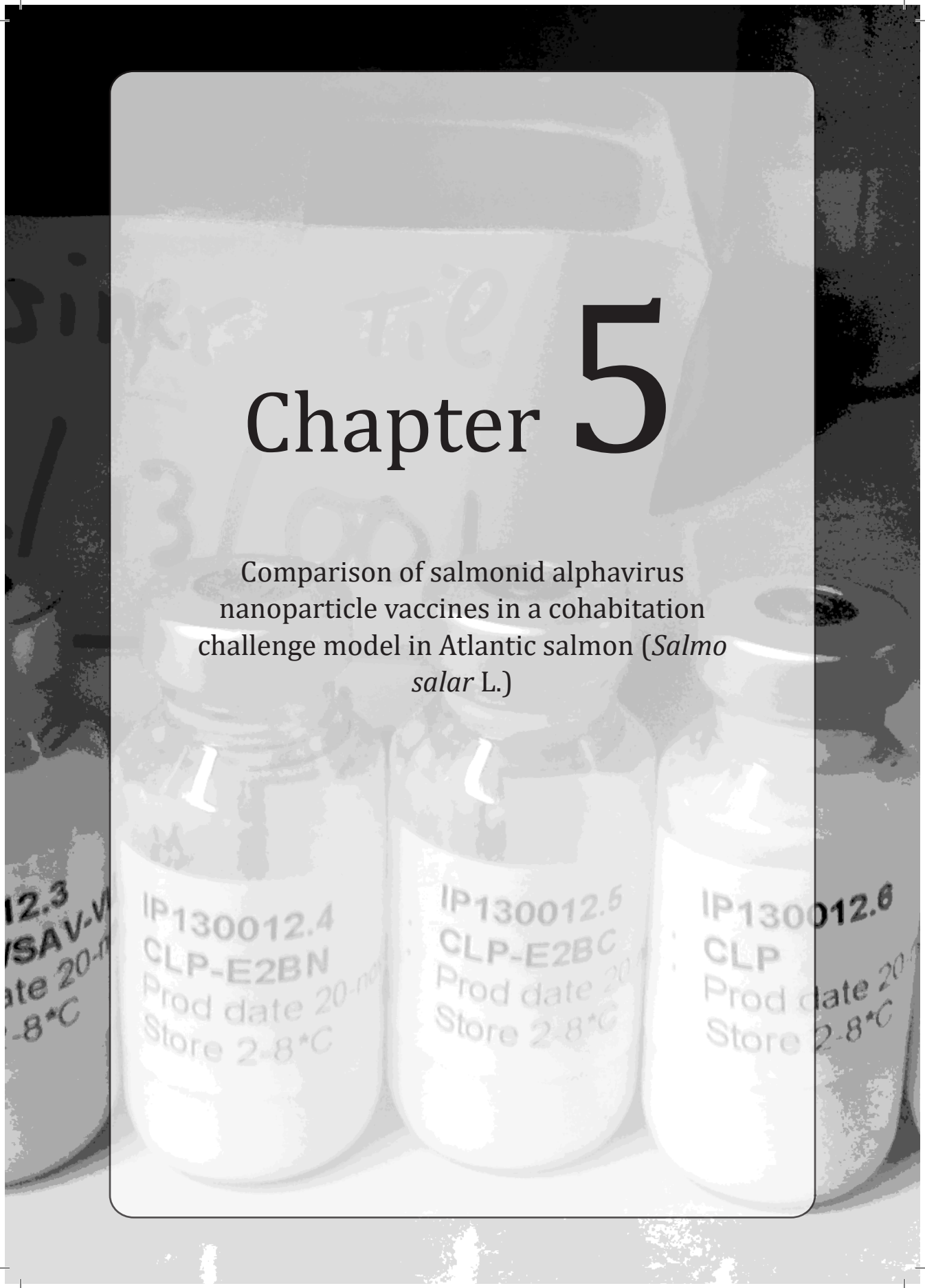
# Chapter 5

Comparison of salmonid alphavirus nanoparticle vaccines in a cohabitation challenge model in Atlantic salmon (*Salmo salar* L.)



## ABSTRACT

Salmonid alphavirus is the causative agent of pancreas disease and sleeping disease in Atlantic salmon and rainbow trout and poses a major burden to aquaculture farming of these high value fish species. The current vaccine is an inactivated virus vaccine, which is grown on a salmonid cell line at low temperature. Previously, alternative vaccine candidates in the form of SAV enveloped virus-like particles (eVLPs) and core-like particles (CLPs) were developed. SAV eVLPs can only be formed at low temperature (<18°C), whereas eVLPs of a related alphavirus, chikungunya virus (CHIKV), can be produced at 27°C. In the present study, a chimeric SAV/CHIKV eVLP is produced at 27°C. The alphavirus E2 B-domain is considered to be an immunodominant epitope, resulting in neutralizing antibody generation. Therefore, the CHIKV E2 B-domain was exchanged with the corresponding SAV E2 B-domain within the structural protein cassette and the resulting chimeric eVLP was successfully expressed by recombinant baculoviral vectors at 27°C. CHIKV/SAV E2 cell surface translocation was found not to be low-temperature restricted, which showed that the E2 B-domain of SAV is not temperature-sensitive. All vaccine candidates, SAV eVLP, SAV peptide-fused CLP ($CLP^{E2BN}$ and $CLP^{E2BC}$) and CHIKV/SAV chimeric eVLP ($CHIKV/SAV^{Bdom}$), were tested in a vaccination/cohabitation challenge trial of Atlantic salmon parr. High mortality occurred upon viral challenge, except in the group of fish vaccined with SAV inactivated antigen, and PCR analysis confirmed high viremia in all other test groups. It was concluded that eVLP or CLP vaccination does not result in the establishment of a protective immune response in salmon against SAV challenge. The results shed light on the vaccine prerequisites for a potent immune response in salmon and may aid in future vaccine design against salmonid alphavirus infections.

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## 1. INTRODUCTION

Salmonid alphavirus (genus *Alphavirus*, family *Togaviridae*) is the causative agent of pancreas disease and sleeping disease in Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* W.). SAV infections may result in growth retardation, muscle myopathy, lower fillet quality and up to 48% mortality. Therefore, SAV infections pose a major burden on aquaculture of these high value fish species (Hodneland et al., 2005; Lerfall et al., 2012; McLoughlin and Graham, 2007). Vaccination of young salmon parr with a commercial inactivated whole virus vaccine provides effective protection against SAV infection. The current vaccine is propagated on a salmonid cell line at low temperature, which is a time-consuming process.

Recently, a prototype nanoparticle vaccine against SAV in the shape of an enveloped virus-like particle (eVLP) was successfully developed. This eVLP was produced by expression of the structural proteins in *Spodoptera frugiperda* (*Sf*) insect cells by recombinant baculoviral vectors (Metz et al., 2011a). Normally, baculovirus protein expression is performed at 27°C (King and Possee, 1992). However, at this temperature, no SAV eVLPs were found in the medium fraction (Metz et al., 2011a) because the precursor E2 (PE2) subunit was retained in the ER (Chapter 4). Lowering the infection incubation temperature to 15°C rescued eVLP secretion (Metz et al., 2011a). The low-temperature dependent eVLP/virion production could be allocated to the glycoprotein E2, which needs both low temperature and E1 for correct folding (Chapter 4). However, the amount of SAV protein produced at 15°C, and thereby the eVLP yield, was much less than at 27°C, but in the latter case no eVLPs were formed (Metz et al., 2011a). Therefore, we aimed to generate an immunogenic SAV nanoparticle vaccine at 27°C.



First, a SAV nanoparticle vaccine was developed in the shape of a core-like particle (CLP) (Chapter 3). Upon baculovirus expression of either the full structural protein cassette or solely the SAV capsid protein (CP) at 27°C in *Sf* cells, nuclear aggregates were observed. Upon further examination by electron microscopy, these aggregates were shown to consist of capsid proteins that had self-assembled into CLPs with high resemblance to alphavirus nucleocapsids. It was hypothesized that these CLPs could be used as carriers for an immunogenic epitope of SAV, the B-domain of glycoprotein E2. Thus, the SAV E2 B-domain was fused to either the N- or C-terminus of the SAV CP ($CP^{E2BN}$ and $CP^{E2BC}$). Baculovirus $CLP^{E2BN}$ expression still resulted in CLP formation and production was efficient at 27°C. In addition, a simple purification method was developed to obtain the CLPs at high purity (Chapter 3).

Second, to combine the advantages of efficient CLP vaccine production at 27°C with the structural resemblance between eVLPs and the infectious virion, a chimeric eVLP was designed. Chikungunya virus (CHIKV) is a related alphavirus, and infection in humans is associated with symptoms including fever, rash and arthralgia. Currently, no commercial vaccine against CHIKV is available (Suhrbier et al., 2012). However, a prototype CHIKV

eVLP vaccine produced by the baculovirus expression system in *Sf*9 cells was found to protect against CHIKV infection in different vaccination-challenge mouse models (Metz et al., 2013a; Metz et al., 2013b). CHIKV eVLPs are produced by expression of the complete structural polyprotein at the baculovirus expression system's optimum temperature of 27°C. Therefore, the CHIKV structural polyprotein could serve as a 'scaffold' to produce SAV immunogenic, chimeric eVLPs at 27°C.

In the present study, the immunogenic CHIKV E2 B-domain was exchanged with the corresponding SAV E2 B-domain, and the complete CHIKV/SAV chimeric structural cassette was expressed by recombinant baculoviral vectors at 27°C. The E2 B-domain is considered to be an immunodominant epitope resulting in neutralizing antibodies, as shown in chimeric virus studies in mice (Weger-Lucarelli et al., 2015). To identify the most promising lead candidates for further development, all prototype vaccines; SAV eVLP, SAV peptide-fused CLP (CLP$^{E2BN}$ and CLP$^{E2BC}$) and CHIKV/SAV chimeric eVLP (CHIKV/SAV$^{Bdom}$), were tested in a vaccination/cohabitation challenge trial of Atlantic salmon parr.

## 2. MATERIALS AND METHODS

### 2.1 Cloning and recombinant bacmid construction.

Chimeric structural polyproteins were generated by exchanging SAV E2 peptides with the corresponding regions in the chikungunya virus (CHIKV) structural polyprotein backbone (Metz et al., 2013a). A DNA sequence encoding the SAV E2 B-domain (aa158-252) or extended sequences, flanked by CHIKV E2 protein coding sequences until unique restriction sites SphI and XhoI, was designed and synthesized (IDT DNA technologies). The chimeric sequence was cloned into the pDONR207-CHIKVS27 (accession number AF369024) structural polyprotein using restriction enzyme digestion and ligation, resulting in CHIKV/SAV$^{Bdom}$. In the synthetic sequence a new unique restriction site NdeI was generated upstream of the SAV E2 B-domain without disrupting the amino acid sequence. A SAV C-terminal extension of the B-domain was generated by fusion PCR, adding NdeI/XhoI restriction site sequences to the PCR product. These were then used for replacement of the B domain from the CHIKV/SAV$^{Bdom}$ construct with the elongated SAV domain (CHIKV/SAV$^{C\text{-}Bdom}$). Additionally, a 'reversed' chimeric construct was made, in which the E2 C-domain of CHIKV replaced the corresponding region in the SAV structural polyprotein backbone (SAV/CHIKV$^{Cdom}$). Using Gateway™ technology combined with Bac-to-Bac (Invitrogen) technology, recombinant bacmids were obtained. A schematic presentation of the generated constructs is given in Fig. 1A.

### 2.2 Cell culture maintenance, transfection and infection.

*Sf*9 and *Sf*21 cells were maintained as a monolayer culture in Sf900-II medium supplemented with 5% Fetal Bovine Serum (FBS), or Grace's medium supplemented

with 10% FBS, at 27°C. Twice a week, cells were dislodged by mechanical detachment and passaged. Transfection of *Sf* cells with recombinant bacmid DNA was obtained using ExpreS$^2$ Insect-TR (ExpreS$^2$ion® Biotechnologies) transfection reagent according to manufacturer's protocol. Recombinant baculoviruses were titrated using *Sf*9 'easy titration' cells (Hopkins and Esposito, 2009) and the titer was determined as tissue culture infectious dose 50 ($TCID_{50}$) per mL. Infection of *Sf* cells with recombinant baculovirus was performed at a multiplicity of infection (MOI) of 10 $TCID_{50}$ units per cell.

### 2.3 SDS-PAGE and western blot analysis

Protein samples were loaded onto sodium dodecyl sulfate (10%) polyacrylamide gel electrophoresis (SDS-PAGE) gels and run at constant amperage of 240 mA until a clear separation of the marker proteins was visible. Some samples were treated with the deglycosylation enzyme PNGase F (New England Biolabs), to remove carbohydrate moieties, according to manufacturer's protocol prior to loading on the SDS-PAGE gel. Following protein separation, the proteins were transferred from the gel onto Immobilon-P polyvinylidene difluoride membranes (Millipore) by semi-dry blotting (Biorad) for 1 h at constant amperage of 0.05 A per gel. Following protein transfer, the membranes were treated for 1 h with 1% skimmed milk in PBS containing 0.1% Tween 20 (PBS-T) to prevent a-specific binding. Primary antibodies against the E2 B-domain (monoclonal antibody (Mab) 17H23, 1:2000; Moriette et al., 2005), E1 (4H1, 1:1000; Todd et al., 2001) and β-tubulin (Abcam AB6046, 1:8000) were diluted in PBS-T and incubated with the membranes for 1 h in PBS-T. The membranes were washed 3 times with PBS-T, before 1 h incubation with an alkaline phosphatase (AP) conjugated secondary antibody. Then, the membrane was washed 3 times with PBS-T, once with AP buffer (15mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$; pH 9.5) and then incubated with NBT-BCIP, a substrate for AP, which started a colorimetric substrate conversion. To stop the reaction, membranes were washed with milliQ water and bands were captured by a Geldoc, using Image Lab 5.0 software (Biorad).

### 2.4 Immunofluorescence assay

Recombinant baculovirus infected *Sf*9 cells were fixed with 4% paraformaldehyde (PFA) in PBS (1.37M NaCl, 27mM KCl, 43mM $Na_2HPO_4$, 14.7mM $KH_2PO_4$; pH 7) for 10 min at room temperature (RT). For permeabilization, an ice-cold 50/50 mix of acetone and methanol was added to the cells for 20 min. Then, excess epitopes were blocked by adding 1% skimmed milk in PBS and incubation for 1 h at RT. A primary mAb against E2 was added (17H23, 1:1000) and incubated with the cells for 1 h at RT. After 3 washes of 5 min with PBS, a secondary antibody conjugated with an Alexa546 fluorophore (Life Technologies) was added and allowed to bind during 1 h incubation at RT. The cells were washed 3 times for 5 min, with the addition of Hoechst 33258 nuclear staining

during the second wash. Presence of the E2 protein on the surface and intracellularly was analyzed using a Zeiss fluorescence microscope using AxioVision software.

### 2.5 Vaccines

**Core-like particles**. *Sf* insect cells were infected with AcMNPV-CLP$^{E2BN}$ and AcMNPV-CLP$^{E2BC}$ and incubated for 3 days at 27°C. The core-like particles with an N-terminal fusion of the E2 B-domain to the capsid protein (CLP$^{E2BN}$) were prepared as described earlier (Chapter 3).

**SAV eVLP.** *Sf* insect cells were infected with AcMNPV-SAV SP and incubated for 1 day at 27°C and 3 days at 15°C (Metz et al., 2011a). Then, cell fraction and medium were harvested and separated by centrifugation for 5 min at 1600 rpm. The cell pellets were sonicated on ice for three consecutive rounds of 30 s sonication (continuous pulse, output 2 W) and 30 s recovery on ice. To obtain a higher concentration of eVLP in the medium fraction, the medium was filtered through a 0.2 µm filter and then concentrated using Amicon® Ultra-15 (10K) centrifugal filters at 5000 x g, until the volume was reduced 5 times. As vaccine, the sonicated cell material together with concentrated medium fraction was used.

**CHIKV/SAV$^{Bdom}$ eVLP.** *Sf* cells were infected with AcMNPV-CHIKV/SAV$^{Bdom}$ and incubated at 27°C for 3 days. Further vaccine preparation was identical to SAV eVLP vaccine preparation.

An overview of the recombinant baculoviruses used for vaccine generation is given in Figure 2A.

### 2.6 Immunization, sampling and challenge

The animal experiment was conducted at the Industrielaboriet (ILAB) facilities, Bergen, Norway. Unvaccinated Atlantic salmon (*Salmo salar* L.) presmolts (30-40 g) were kept in UV-radiated freshwater at 12°C (500 L tank) and fed according to appetite using commercial fish feed. Before vaccination, the fish were starved for 36 h, anesthetized by bath immersion in Finquel (0.1 mg/mL for 2–3 min) and divided in 8 groups of 62 fish. All test vaccine groups were GNE oil-based formulated and doses of 0.1 mL were intraperitoneally (i.p.) injected. As a positive control for protection, a group vaccinated with inactivated SAV antigen was included. As a negative control, a similar sized group of salmon was injected with a saline solution (Table 1).

All 8 groups were kept together in a 3000 L challenge tank. At 6 weeks post-vaccination, the vaccine efficacy was tested in a cohabitation challenge experiment, by releasing SAV infected shedders (20% of the total number of fish) amongst the vaccinated fish. At 3 weeks post-challenge (wpc), serum samples of 20 fish per group were collected and analyzed for the presence of nsP1 transcripts by quantitative real-time PCR (Applied Biosystems). The relative percentage protection at 3 wpc was calculated (RPP= [1-(%

PCR positive fish in vaccinated group/% PCR positive fish in unvaccinated group)] x 100). Cumulative mortality was followed over time for all test groups from 24 days post challenge (dpc) onwards. Because of high mortality rates in vaccinated cohorts, it was decided to terminate the trial at 38 dpc. The relative percentage survival (RPS) at 38 dpc was calculated [RPS = (1 – cumulative mortality vaccinated group/cumulative mortality non-vaccinated group) × 100].

### 2.7 Detection of antibodies in post-challenge serum

Vero cells were infected with CHIKV (MOI of 1) and fixed at 1 dpi using 4% PFA in PBS. The cells were permeabilized using 0,1% SDS in PBS and washed once with PBS. Sera from the CHIKV/SAV$^{Bdom}$ vaccinated group (n=20) and inactivated SAV antigen vaccinated group (n=20) were pooled and used as primary antibody (1:200). A secondary antibody rabbit-anti-salmon IgM was added for 1 h at RT (1:3000). After 3 washes with PBS, a third antibody (goat-anti-rabbit, 1:500), conjugated with an Alexa-488 fluorophore was added to the cells. As a positive control for infection, one well was subjected to a primary antibody against the CHIKV E2 protein.

## 3. RESULTS



### 3.1 Chimeric eVLP production at 27°C

Previously it has been shown that SAV eVLPs can be produced in insect cells using baculovirus vectors, however, the temperature needs to be lowered during eVLP production (Metz et al., 2011a). Preferably, a SAV-immunogenic eVLP is produced at 27°C, at which temperature the protein expression level in *Sf* insect cells reaches its optimum. Expression of the structural proteins of a related alphavirus, CHIKV, at 27°C in *Sf* cells leads to the secretion of eVLPs into the medium fraction. Therefore, CHIKV structural proteins were used as a backbone for the generation of chimeric CHIKV/SAV eVLPs at 27°C (Fig. 1A). Initially, the most immunogenic region of CHIKV E2, the B-domain (Fig. 1B), was replaced by the corresponding region of SAV E2 (CHIKV/SAV$^{Bdom}$). This exchange would result in an increase in weight/size of the PE2 subunit from 55 kDa to 59 kDa. Normally, SAV E2 would only translocate to the cell surface at low temperatures (<18°C) but not at an incubation temperature of 27°C (Fig. 1D, 2$^{nd}$ panel). Surprisingly, when placed within the CHIKV structural polyprotein scaffold, the SAV E2 B-domain region was detected at the cell surface of the insect cells at 27°C (Fig. 1D, 3$^{rd}$ panel). WB detection of the SAV E2 protein also showed that in this context, PE2 was furin-processed into mature E2 at both temperatures (Fig. 1C, lane 5). In addition, secreted eVLPs could be detected (data not shown). This indicates that folding of the SAV E2 B-domain is not temperature sensitive and that a chimeric eVLP vaccine candidate can be successfully produced at 27°C.

Next, the SAV segment within the CHIKV structural polyprotein was extended, by

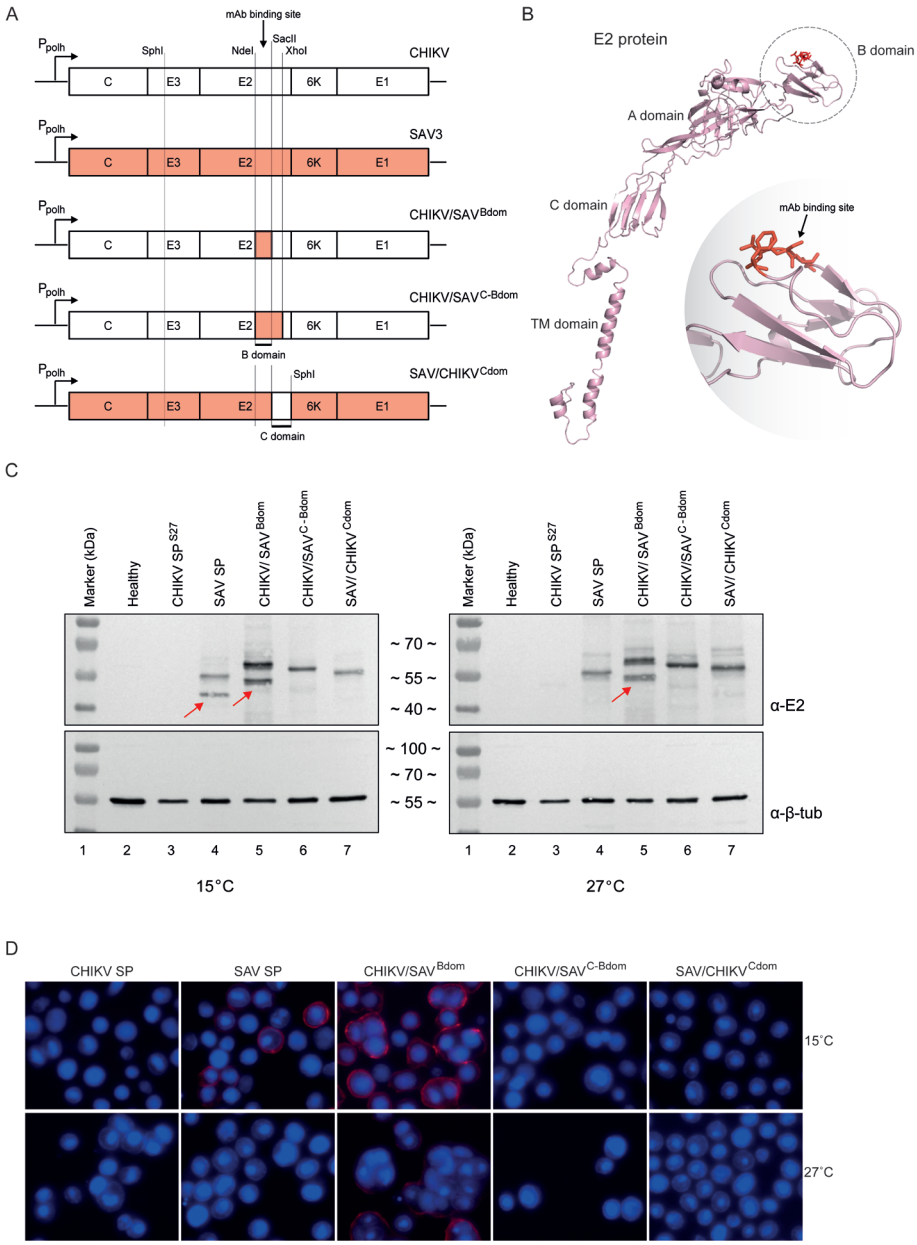


**Figure 1. A chimeric chikungunya/salmonid alphavirus polyprotein is successfully processed at 27°C.** (A) Schematic presentation of the chimeric constructs made. All constructs were used to generate recombinant baculoviruses. Restriction sites indicated are those used for cloning. (B) Schematic presentation of the glycoprotein E2 (model from S. Villoing). Encircled is the E2 B-domain, with the binding motif of the mAb 17H23 coloured in red. Inset: enlargement of the E2 B-domain. (C) Deglycosylated, recombinant baculovirus infected *Sf*21 cell fractions were analysed for differences in the maturation status of SAV E2 between the various chimeras. Red arrows indicate furin-processed E2. (D) Immunofluorescence staining against the E2 protein on the cell surface of baculovirus-infected *Sf*21 cells at 15°C (upper row) and 27°C (lower row).

replacement of both the E2 B-domain and partial E2 C-domain (CHIKV/SAV$^{C\text{-}Bdom}$). Expression of this chimeric polyprotein at 27°C resulted in loss of E2 detection at the cell surface (Fig. 1D, 4$^{th}$ panel). Only minor amounts of furin-processed E2 were visible on western blot (WB) (Fig. 1C, 27°C, lane 6). In addition, PE2 furin-processing was also inhibited at low temperature (Fig. 1C, 15°C, lane 6) in comparison to the SAV (Fig. 2C, 15°C, lane 4) and CHIKV/SAV$^{Bdom}$ (Fig. 2C, 15°C, lane 5) structural polyprotein samples. Therefore, it is possible that the low-temperature dependency resides in the C-domain region, more specifically at the C-terminus of the B-domain.

To analyse whether replacement of the SAV E2 C-domain with the corresponding CHIKV E2 C-domain would be sufficient to rescue SAV eVLP formation at 27°C, a 'reverse' chimera was produced (SAV/CHIKV$^{Cdom}$). However, expression of this chimeric protein did not lead to surface translocation of the chimeric E2 glycoprotein (Fig. 1D, 5$^{th}$ panel). Also no furin cleavage of PE2 occurred (Fig. 1C, lane 7). Most likely more factors than solely the E2 C-domain are involved in the low-temperature dependent virion production and eVLP production. It was decided to carry out vaccination experiments with the initial chimeric CHIKV/SAV$^{Bdom}$ eVLP vaccine candidate, which could successfully be produced at 27°C.

## 3.2 Vaccine candidates contain high amounts of antigen

Before the onset of the vaccination trial, all vaccine materials were tested by western blot analysis. Samples were taken from the vaccine material, equally diluted, and subjected to SDS-PAGE, next to a SAV protein sample with a known antigenic mass (10

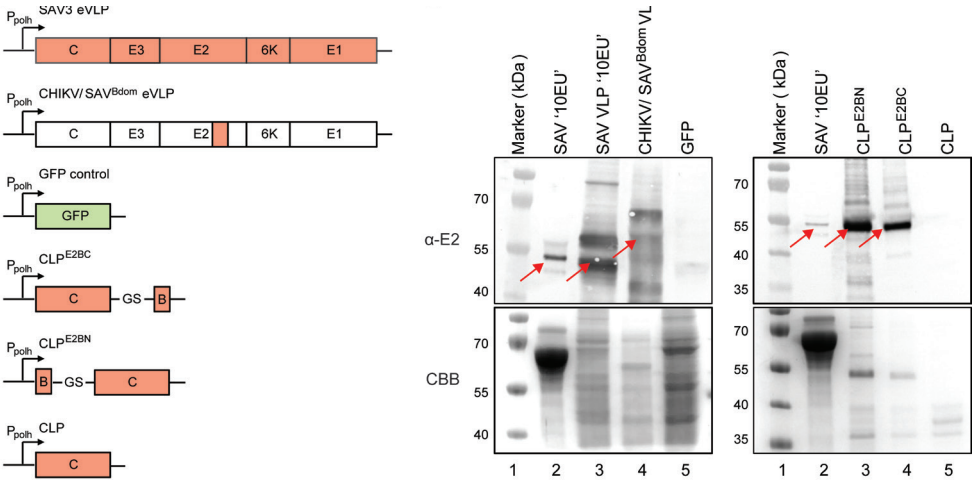


**Figure 2. Vaccine groups.** (A) Inserted sequences in recombinant baculovirus constructs (bacmids) used for vaccine generation. (B) SDS-PAGE and E2 WB of vaccine material samples. Left: eVLP vaccine groups of are compared to a reference SPDV standard. Equal amounts of ELISA units (EU) are loaded. Right: CLP vaccine groups are compared to a SAV reference sample. Red arrows indicate (furin-processed) E2. Dilutions were prepared in such a way that similar volumes would be used for vaccination.

ELISA units). In this way it was possible to estimate the amount of antigenic protein present in the respective candidate vaccine preparations. The gel that was subjected to Coomassie Brilliant Blue staining showed that all vaccine materials contained high amounts of protein. The three CLP vaccine preparations showed a relatively high purity of the antigenic protein (Fig. 2B, right panel, lane 3-5). A thick protein band was visible in the SAV standard sample (Fig. 2B, lane 2), which represents bovine serum albumin from the fetal calf serum in the cell culture medium. Western blot analysis resulted in detection of the antigen of interest, SAV E2, in both the tested vaccine samples (Fig. 2B, lane 3-5) as well as the positive control (Fig. 2B, lane 2). From this western blot it was concluded that all vaccine candidate preparations contained substantially more SAV antigenic protein than the standard sample, which was considered sufficient to initiate a vaccination trial.

### 3.3 Vaccination trial with CLPs and (chimeric) eVLPs

To test the efficacy of the vaccine candidates, a vaccination/cohabitation animal trial was performed. Per group, 62 fish were vaccinated according to the trial setup (Table 1). After 6 weeks, the fish were challenged by releasing SAV-infected shedders in the tank. In all test groups, except the positive control group (inactivated SAV antigen), salmon started to die around 27 dpc (Fig. 3). Mortality continued to occur and at 38 dpc it was decided to terminate the animal trial. In the positive control group, only one fish died. SAV viremia was analyzed by PCR at 3 wpc. Since it was clear that the salmon suffered from SAV infection, no histological analysis of pancreas disease-specific lesions was performed on the remaining fish in the test groups, solely in the positive and negative

**Table 1. Vaccine trial experimental setup and outcome.**

| Vaccine name | Inactivation of antigen | Dose (ml) and route | SAV positive sera samples | $RPS_{endpoint}$ (%) 38 dpc |
|---|---|---|---|---|
| SAV VLP | yes[a] | 0.1, i.p. | 19/20 | 28.0 |
| CHIKV/$SAV^{Bdom}$ VLP | yes[a] | 0.1, i.p. | 20/20 | 28.0 |
| VLP control (GFP) | yes[a] | 0.1, i.p. | 20/20 | 48.0 |
| $SAV\ CLP^{E2BN}$ | no | 0.1, i.p. | 20/20 | 32.0 |
| $SAV\ CLP^{E2BC}$ | no | 0.1, i.p. | 19/20 | 20.0 |
| SAV CLP | no | 0.1, i.p. | 19/20 | 49.2 |
| Inactivated antigen | yes[b] | 0.1, i.p. | 0/20 | 95.9 |
| Saline | no | 0.1, i.p. | 19/20 | - |

[a]Binary ethylenimine (BEI) inactivated, [b]Formalin inactivated

dpc = days post-challenge, i.p. = intraperitonial injection, $RPS_{endpoint}$ = relative percentage survival = {(1 − cumulative mortality vaccinated group/cumulative mortality non-vaccinated group) × 100}

control groups. The saline vaccinated group showed specific heart lesions in 89% of the fish (16/18), which, in addition to high mortality, confirmed a high challenge pressure. The inactivated SAV antigen vaccinated group only showed specific heart lesions in 30% of the fish (6/20) and therefore was shown to give significant protection. All groups, except the group vaccinated with inactivated SAV antigen, showed positive presence of SAV (Table 1). At 38 dpc, the RPS was calculated for all groups. As the $RPS_{endpoint}$ of the VLP- and CLP control group were higher than those of the actual test groups, it was concluded that none of the CLP or eVLP vaccine candidates provided protection against SAV infection.

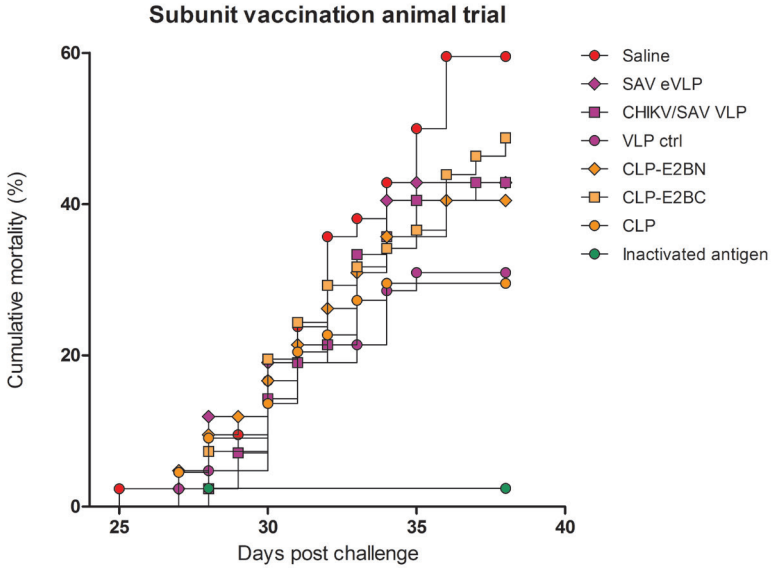


**Figure 3. Cumulative mortality versus dpc of the test groups during the vaccination/co-habitation challenge trial.** Test groups are depicted in purple (eVLP vaccine groups) and orange (CLP vaccine groups). The negative control (saline) is shown in red, and the positive control in green (inactivated SAV antigen).

### 3.4 Vaccination does not induce a specific antibody response.

The efficacy of subunit or nanoparticle vaccination depends largely on the generation of neutralizing antibodies. Because all test groups were exposed to SAV during challenge, all test groups were expected to have generated SAV-specific antibodies. Therefore, post-challenge detection of antibodies against SAV was not a correct measure for vaccine-induced antibody generation. To detect if an antibody response was generated upon vaccination, the serum collected from the salmon vaccinated with CHIKV/$SAV^{Bdom}$ chimeric eVLP was used as primary antibody in an immune fluorescence assay on CHIKV-infected Vero cells. In this case it was possible to see if antibodies against CHIKV structural proteins, rather than to SAV structural proteins, were formed as a result of vaccination. As a negative control, serum of inactivated SAV antigen vaccinated fish was used as primary antibody. In addition, as a positive control for CHIKV infection,

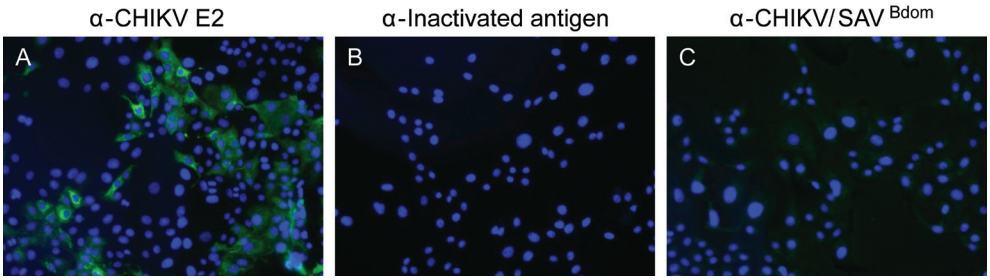


**Figure 4. Detection of vaccine-induced antibody production in post-challenge sera.** Vero cells were infected with CHIKV and exposed to (A) a polyclonal antibody against CHIKV E2 as positive control for infection, (B) sera from inactivated SAV antigen vaccinated fish as negative control and (C) CHIKV/SAV$^{Bdom}$ vaccinated fish (20x magnification).

a polyclonal antibody against the CHIKV E2 glycoprotein was used. The positive control showed that around 60% of cells were CHIKV infected (Fig. 4A). Sera from the inactivated SAV antigen vaccinated fish did not lead to detection as expected (Fig. 4B). The use of CHIKV/SAV$^{Bdom}$ sera as primary antibody, however, did not result in a detectable fluorescence signal (Fig. 4C). It could be concluded that no detectable amount of antibodies was generated in response to eVLP vaccination.

## 4. DISCUSSION

Current vaccination strategies against SAV involve intraperitoneal injection with inactivated virus, grown on a salmonid cell line at low temperature (10-15°C). In the current study, alternative nanoparticle vaccines based on CLPs, eVLPs and chimeric eVLPs were developed and tested in vivo. Previous studies had shown that SAV eVLPs could be produced by baculovirus expression of the structural polyprotein in *Sf* insect cells (Metz et al., 2011a). A decrease in temperature from 27°C to below 18°C was needed to obtain secreted eVLP, and processing of PE2 by host furin into mature E2 (Metz et al., 2011a). During infection, dense nuclear aggregates were observed in the *Sf* cells, which appeared to be accumulated CLPs. These CLPs also formed during incubation at 27°C, and an N-terminal fusion of the highly antigenic B-domain of the SAV glycoprotein E2 (Coffey and Vignuzzi, 2011; Voss et al., 2010; Weger-Lucarelli et al., 2015) did not disrupt CLP formation (Chapter 3).

In the present study, an additional vaccine candidate was developed that combined the structural advantages of an eVLP with the production temperature (27°C) of the CLP vaccine. Previous work allocated the low-temperature dependent virion formation to the E2 glycoprotein, that needs both low temperature and the presence of E1 for cell surface translocation and PE2 maturation (Chapter 4). With the aim to generate an eVLP at 27°C, chimeric E2 proteins based on CHIKV and SAV were successfully generated and expressed in a complete structural polyprotein in *Sf* cells by recombinant baculoviruses. Replacement of the CHIKV E2 B-domain with the corresponding region of the SAV E2

B-domain resulted in successful surface translocation and processing of the E2 protein. The chimeric polyprotein was expressed in *Sf* insect cells at 27°C and included in the vaccination trial, together with the SAV eVLP, and both CLP-B-domain fusions. However, the fish in the candidate vaccine test groups showed high mortality rates and at 3 wpc viremia was detected in almost all fish. From these results it was concluded that CLPs and chimeric eVLPs do not readily confer protection against SAV infection. A possible explanation is that the antigenic peptides in the vaccine preparations were not entirely presented (folded) in the correct conformation. The 17H23 mAb was previously shown to discriminate between correct and misfolded E2 glycoprotein (Chapter 4). The same antibody was able to detect the E2 protein in an immune fluorescence assay or dot-blot in all eVLP and CLP preparations derived from infected *Sf* cells, respectively. This indicates that protein folding in the vaccine preparations was at least partial correct (Fig. 1C, Chapter 3, Fig. 4C, and Chapter 4, Fig. 2D).

No significant amounts of CHIKV specific antibodies were detected in the post-challenge sera of fish immunized with chimeric VLPs. As the fish immune system relies more heavily on innate immunity than that of mammals (Collet, 2014), it is questionable whether antibodies are protective. In this study, we were unable to detect a specific antibody response against the candidate vaccines, and therefore we cannot conclude whether or not antibodies are correlated with protection.



Because SAV eVLPs have similar structure to inactivated virus, the main difference is the absence of inactivated genomic viral RNA in the eVLP. Hence, it can be hypothesized that the presence of viral RNA is essential for effective stimulation of the salmonid innate and adaptive immune system. Viral RNA is an important PAMP to stimulate innate immunity, and development of adaptive immune responses (Collet, 2014; Pietretti and Wiegertjes, 2014). In combination with the results shown in Chapter 4, we now hypothesize that future vaccines should contain viral RNA to activate both innate and adaptive immune pathways, and express at least both viral glycoproteins at low temperature. The results shed further light on the prerequisites for a potent immune response in salmon and may aid in future vaccine design against salmonid alphavirus infections.

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# Chapter 6

## Veterinary replicon vaccines

## ABSTRACT

Vaccination is essential in livestock farming and in companion animal ownership. Nucleic acid vaccines based on DNA or RNA provide an elegant alternative to classical veterinary vaccines that have performed suboptimal. Recent advancements in terms of rational design, safety and efficacy have strengthened the position of nucleic acid vaccines in veterinary vaccinology. The present review focuses on replicon vaccines designed for veterinary use. Replicon vaccines are self-amplifying viral RNA sequences that, in addition to the sequence encoding the antigen of interest, contain all elements necessary for RNA replication. Vaccination results in high levels of *in situ* antigen expression and induction of potent immune responses. Both positive and negative stranded viruses have been used as replicons, and they can be delivered as RNA, DNA, or as viral replicon particles (VRP). An introduction into the biology and the construction of different viral replicon vectors is given, and examples of veterinary replicon vaccine applications are discussed.

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## INTRODUCTION

In veterinary medicine, the main focus is on protection of the herd against pathogens. Immunization of sheep against sheep pox was mentioned to be in practice as early as the 16th century, and ever since veterinary vaccination strategies developed hand in hand with human vaccines. Ethical issues, herd health and economic benefits are important factors to be considered, and vaccination is now indispensable in livestock farming and in companion animal ownership (Lombard et al., 2007).

When a new virus or virus strain presents itself, the most straightforward vaccine approach is to propagate and subsequently inactivate the virus and formulate the viral antigen(s) with an appropriate adjuvant. This classical vaccination strategy may work efficiently, however, in some cases this type of vaccine is not efficacious, a susceptible production cell line cannot be selected, virus yields are low or the virus loses antigenic properties upon passaging in cell culture. Inactivated virus vaccines against porcine reproductive and respiratory syndrome (Zuckermann et al., 2007), and porcine epidemic diarrhea virus (Song et al., 2015) are examples of this. In addition, inactivated virus vaccines most often require adjuvants and booster vaccinations for seroconversion and induction of immune memory.

Live-attenuated viruses (LAV) are generally more potent vaccine alternatives to killed virus vaccines, as they are replication competent in their host and effectively induce innate and adaptive immune pathways without causing overt disease in the target animal (Meeusen et al., 2007). For instance, LAV vaccines have successfully been used to globally eradicate Rinderpest, a deadly disease in cattle and other ruminants (FAO/OIE, 2011; Normile, 2008). LAV can be generated by serial passaging of the virus in laboratory animals or on cell lines, which eventually may result in loss of pathogenicity, while the virion structure (antigenic determinants) is largely retained. Well-known examples are the globally used human oral polio vaccine (Garon et al., 2016), and the yellow fever vaccine (Beck and Barrett, 2015). However, LAV might cause problems in immunocompromised hosts and there may exist a chance of reversion to virulence despite extensive field testing. Striking examples are the LAV vaccination-related outbreaks of BTV in Africa (Coetzee et al., 2012) and the reversion to neurovirulence observed with the live-attenuated Sabin strains of oral poliovirus vaccine (Pliaka et al., 2012). However, due to increased understanding at the molecular level of the viral determinants of virulence and technical progress, reverse genetics systems have facilitated the development of LAV vaccines by rational design (e.g. by gene shuffling, gene deletions, or site-directed mutations) and have aided in increased LAV vaccine safety (Flanagan et al., 2001; Peters et al., 2007).

In situations in which traditional, killed-virus or LAV, vaccines have performed sub-optimally in terms of efficacy or safety, new strategies are sought, often using modern biotechnology approaches to produce viral subunits, virus-like particles (VLPs) or

nucleic acid vaccines. Subunit vaccines are safe but require adjuvants to induce a protective immune response. VLPs combine the efficacy of inactivated viruses with the production in facilities with lower biocontainment restrictions (Pijlman, 2015). VLPs are more potent immunogens as compared to subunits since they display epitopes in repeated regular patterns and have a particulate nature with sizes in the range of 10-103 nm (Metz et al., 2013). Nucleic acid vaccines based on DNA or RNA form an interesting alternative as they can be rapidly manufactured, are highly flexible and vaccination results in in situ antigen expression in its native configuration (Kutzler and Weiner, 2008; Vander Veen et al., 2012a).

Currently, four DNA vaccines are commercially available for veterinary use, two protect against viral disease in horses (West-Nile Innovator®, Fort Dodge) and salmonids (Apex-IHN®, Aqua Health Ltd), one prevents the development of carcinoma in dogs (Oncept®, Merial Health Ltd), and another one decreases perinatal piglet morbidity (LifeTide® SW5, VGX animal Health). DNA vaccination, leading to transient gene expression in the vaccinated animal, however, did not always proved successful in larger mammals (e.g. cattle, pigs) (Dufour, 2001), even though the use of genetic adjuvants (Bower et al., 2004; Salonius et al., 2007) and different delivery methods (e.g. by electroporation or gene-gun bombardment) greatly enhanced the efficacy (Kutzler and Weiner, 2008; Saade and Petrovsky, 2012).

To increase the potency by stimulating multiple immune pathways simultaneously, nucleic acid vaccines based on self-replicating viral RNAs (replicon vaccines) have been developed. An important feature that sets replicon vaccines apart from LAV or viral vectored vaccines is their inability to spread from cell to cell. Replicons do not code for viral structural proteins required for encapsidation, but code for a heterologous gene of interest instead. Thus, replicons are not infectious and are limited to a single round of replication. In its most simple form, a replicon is a cloned viral cDNA with the structural genes removed.

Replicon vaccines can be administered in different modalities: 1) as 'naked RNA', 2) as RNA launched from a DNA plasmid, and 3) as RNA packaged in viral replicon particles (VRP). Replicon vaccines can be administered in the target animal via different routes and are regarded "self-adjuvanting", i.e. replicon RNA replication triggers multiple immune pathways that are also activated during a natural virus infection. Replicon vaccines can be produced under good manufacturing practice (GMP) conditions and indeed, a few years ago the first veterinary VRP vaccine against swine influenza virus H3N2 produced by Harrisvaccines (now part of Merck Animal Health) obtained a full USDA license (Vander Veen et al., 2012b). Not much later, this company, which adopted the VRP technology developed at the biopharmaceutical company Alphavax, received three conditional licenses for VRP vaccines against porcine epidemic diarrhoea virus, rotavirus C and avian influenza H5N2, using the same SirraVax RNA Particle technology.

Current advances in next-generation sequence technologies and custom DNA synthesis have accelerated replicon development. Many replicon and VRP vaccines have been developed and tested in both human and veterinary trials. Recently, comprehensive reviews have been written on the use of veterinary DNA vaccines (Dhama et al., 2008; Redding and Weiner, 2009), RNA-based viral vectors (Khromykh, 2000; Mogler and Kamrud, 2015; Pijlman et al., 2006), and more specifically alphavirus-based viral vectors (Ljungberg and Liljeström, 2015; Lundstrom, 2014; Rayner et al., 2002; Vander Veen et al., 2012a). This review will focus on replicon vaccines designed for veterinary use. Chimeric viruses, and replicon vaccines that have been tested in animal models, but are intended for human use, are beyond the scope of this review. An introduction into the biology and the construction of different viral replicon vectors is given (Fig. 1), and examples of veterinary replicon vaccine applications are discussed.

## TYPES OF REPLICON VACCINES

Replicon vaccines are self-amplifying viral RNA sequences that, in addition to the sequence encoding the antigen of interest, contain all elements necessary for RNA replication (replicase genes and cis-acting elements). Vaccination with replicons results in viral RNA replication within the host cell to endogenously express the protein of interest in high amounts. This type of vaccination has an advantage over transient expression of the antigen, as antigen expression levels are generally higher and RNA replication will trigger a potent interferon response followed by additional T-cell mediated immune responses in the host (Abbas et al., 2014). Most replicons are based on positive-stranded RNA viruses, because replicon RNA produced from a viral cDNA clone is replication-competent. For example, the replicon RNA can be produced by, in vitro RNA transcription (typically driven by T7 or SP6 promoters) and can then be used as 'naked RNA' for vaccination. Alternatively, the in vitro transcribed replicon RNA can be co-transfected into a suitable cell line along with helper RNAs, which encode the viral structural proteins, for the formation of VRPs. Another option is to produce the replicon RNA in the target cell via RNA polymerase II-mediated transcription (usually driven by a human cytomegalovirus promoter). The DNA-launched replicons are then delivered to the target cell as plasmid DNA, or via a viral vector, e.g. vaccinia.

The best studied and most widely used replicon vaccines are based on alphaviruses (recently reviewed in Ljungberg and Liljeström, 2015; Lundstrom, 2014; Mogler and Kamrud, 2015; Rayner et al., 2002), followed by flaviviruses (Anraku et al., 2002; Pijlman et al., 2006; Varnavski and Khromykh, 1999), and picornaviruses (all three are also reviewed in Khromykh, 2000). Negative-stranded replicons have also been developed, and although these are more complicated to produce, they do have potential for use as vaccines. The sections below have been structured according to the different types of viral replicon vectors: alphaviruses, other positive stranded RNA viruses, and negative stranded RNA viruses.

## Alphavirus-based replicon vectors

Alphaviruses are enveloped viruses that have a positive, single-stranded RNA genome of ~12kb, which is 5' capped and has a 3' polyadenylation signal. An alphavirus replicon RNA is directly translated upon release in the cytoplasm to yield the non-structural polyprotein (nsP1-4), which is cleaved into the individual proteins by the nsP2 viral protease. Together, the nsPs form a replication complex that produces new full-length replicon RNA and a subgenomic messenger RNA (26S), and the latter translates into the viral structural proteins capsid (C), envelope (E) 3, E2, 6K and E1 (Fig. 1). The capsid protein is autocatalytically cleaved off from the nascent polyprotein and encapsidates new genomic RNA in the cytoplasm. In the ER, the glycoproteins precursor E2 (PE2) and E1 heterodimerize, and 6K is cleaved from the polyprotein by host signalases. Three heterodimers form a trimeric spike, which travel through the host cell secretory pathway to the cell surface. Before the spikes are exposed on the cell surface, host furin cleaves E3 from PE2, resulting in mature E2 (Kuhn, 2007).

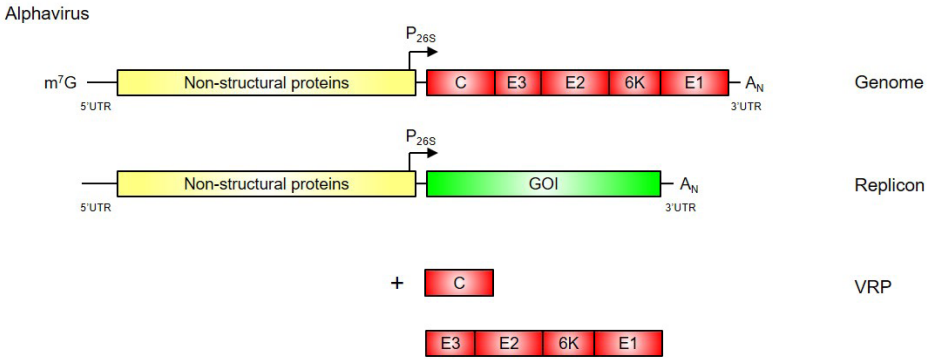



**Figure 1. Alphavirus genome and replicon.** The structural genes are replaced by the heterologous gene of interest. *In trans* complementation of the viral structural proteins via helper RNAs allows encapsidation of the replicon RNA into a viral replicon particle (VRP).

For replicon vaccine purposes, the alphavirus structural polyprotein is replaced by the gene(s) of interest. The absence of viral structural proteins results in the loss of viral progeny, while replication of the replicon RNA still occurs and the protein of interest is produced in high levels from the subgenomic promoter (Xiong et al., 1989)(Fig. 2B). Alphavirus replicon RNA can be delivered to the host as 'naked' RNA, or encoded on the sense-strand of a DNA plasmid, downstream from a suitable promoter (e.g. the human cytomegalovirus, CMV, promoter). RNA can relatively easy be produced *in vitro* under cell-free conditions, although storage conditions are not yet optimal (Jones et al., 2007). 'Naked' RNA delivery to target cells is not straightforward. RNA is readily degraded, and therefore caution needs to be taken during vaccine preparations. Also upon vaccination the RNA encounters some challenges. RNA is sensitive to oxidation and to multiple RNA-degrading enzymes, like RNAses and 5' and 3' exonucleases (Houseley and Tollervey,

2009). In addition, the RNA needs to enter the host cell, which is difficult due to its negative charge. Initially, RNA delivery was increased by use of in situ electroporation which increased the vaccine potency (Cu et al., 2013). Alternatively, to overcome most RNA-degrading problems, Mandl et al. package alphavirus viral replicon RNA in lipid nanoparticles. Their developed SAM technology (self-amplifying mRNA) keeps RNA stable and is rapidly adjustable to specific needs (Geall et al., 2012). Besides high immunogenicity, RNA has an additional advantage over DNA vaccines, as it does not first need to enter the cell nucleus for RNA replication to occur (Tavernier et al., 2011).

Vaccination using DNA-launched replicons is straightforward since the alphavirus RNA is encoded on the plasmid and is launched from a strong eukaryotic promoter. Insertion of a hepatitis delta virus ribozyme (HDVr) downstream of the polyA sequence at the 3'-end, although not essential, ensures an authentic viral 3' end and enhances replication. For salmonid alphavirus, it has been shown that addition of a hammerhead ribozyme at the 5' end of the replicon further enhances RNA replication, although the mechanism is not completely understood (Guo et al., 2015; Moriette et al., 2006). DNA vaccines can be produced in high amounts in bacterial cultures and purified DNA is stable for a long time at ambient temperatures. Geiss et al. (2007) generated a Sindbis virus replicon vector compatible with the Gateway expression system for ease of recombinant replicon production. DNA-launched alphavirus replicon vaccines enhanced humoral and cellular immunogenicity as compared to conventional DNA vaccines (Xiao et al., 2004; Yu et al., 2006). Especially for larger mammals, in which conventional DNA vaccines may not provide sufficient protection without the use of adjuvants, alphavirus replicon vaccines may provide a potent alternative. Besides their use in mammals, alphavirus replicon vaccines also find their application in aquaculture. A salmonid alphavirus replicon vector was shown to provide protection against infectious salmon anaemia virus (Wolf et al., 2013; Wolf et al., 2014), infectious pancreatic necrosis virus (Abdullah et al., 2015), and salmonid alphavirus (Hikke et al., 2014) (Table 1). The broad range of applications shows the high potential of replicon vaccines in the veterinary field. While DNA uptake into the cells was always considered a major hurdle in DNA (replicon) vaccination, improved delivery methods like electroporation, gene-gun injection, and co-delivery with cationic lipid complexes significantly have improved transfection efficacy (Dhama et al., 2008).

*In trans* delivery of the structural proteins by helper constructs allows encapsidation of the viral replicon RNA *in vitro*. The resulting VRPs simulate a natural virus infection upon vaccination, but without spreading the replicon RNA to neighbouring cells. Following the natural route of cell entry, the replicon RNA is released in the cytoplasm, where replication will occur and the heterologous gene is expressed in high amounts. Further spread is halted as the coding sequence for the structural proteins is absent (Fig. 1C). Use of a so-called "split-helper" structural construct to generate VRP *in vitro* increases the vaccine safety by minimalizing the chance for RNA recombination to generate

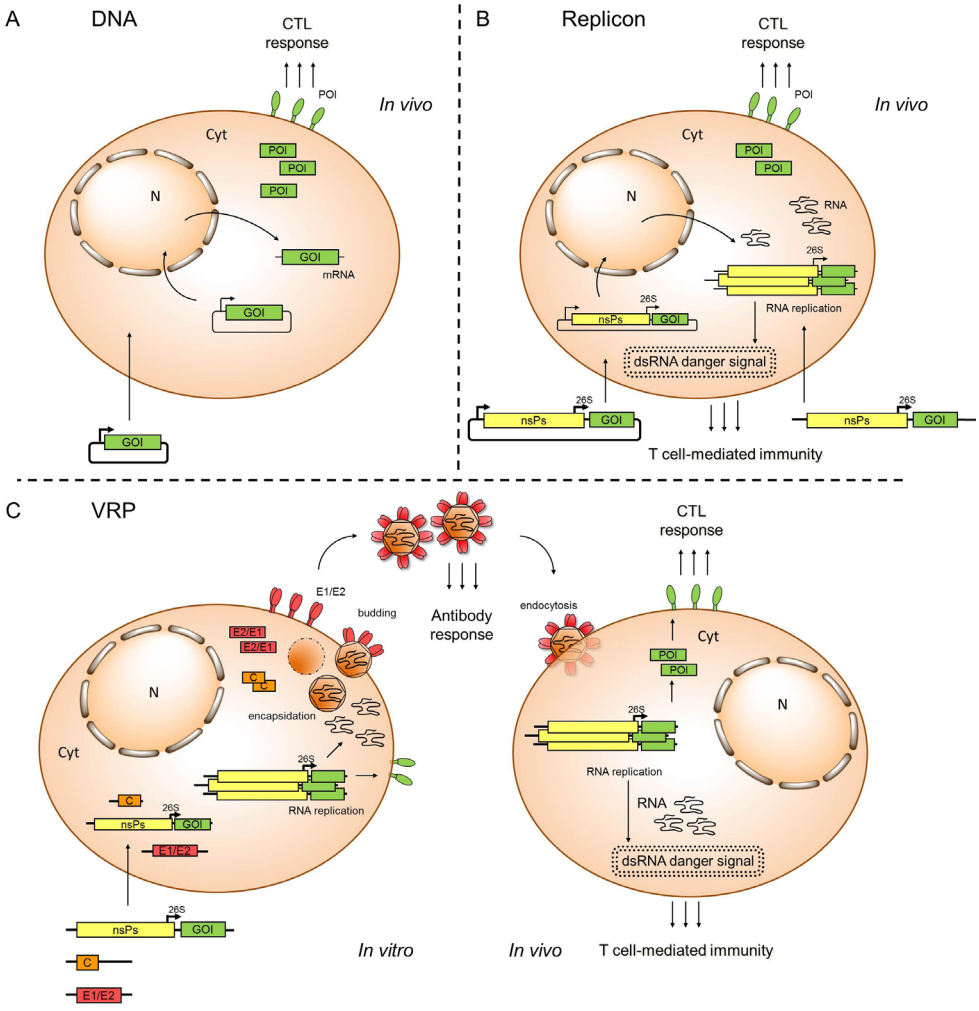



**Figure 2. Methods of heterologous gene expression.** A) Transient expression of the protein of interest from plasmid DNA. B) Expression of the protein of interest from a replicon. Alphavirus example: RNA is transcribed from a suitable promoter and encodes, besides the replication elements, the gene of interest. Subsequently, the RNA can self-replicate and the antigen of interest will be produced at high levels. C) Viral replicon particles (VRPs). Alphavirus example: RNA is transcribed from a suitable promoter and encodes, besides the replication elements, the gene of interest. Subsequently, the RNA replicates independently and the protein of interest will be produced. From a separate promoter the viral structural proteins are transcribed and subsequently translated. Encapsidation of the replicon RNA will occur and a viral replicon particle (VRP) will be secreted from the host cell. VRPs will enter a neighbouring cell and release the replicon RNA. The gene of interest will again be expressed, but VRPs cannot be formed (single round); GOI = gene of interest; POI = protein of interest; Cyt = cytoplasm; N = nucleus; CTL = cytotoxic T cell.

infectious virus (Frolov et al., 1997; Pushko et al., 1997; Smerdou and Liljeström, 1999). The first USDA licenced self-replicating vaccine against the highly pathogenic swine

influenza virus strain H3N2 is a VRP. This commercial vaccine, based on the patented SirraVax technology (Harrisvaccines), is based on the alphavirus genome of Venezuelan equine encephalitis virus (VEEV), and shows high potential for application to many other veterinary pathogens (Vander Veen et al., 2012b), including porcine reproductive and respiratory syndrome virus (PRRSV), porcine epidemic diarrhoea virus (PEDV), rotavirus C, canine influenza virus, and highly pathogenic avian influenza (HPAI) (FierceAnimalHealth, 2015; Mogler and Kamrud, 2015) (Table 1).

The above-described VRPs have a single-round infection phenotype, but propagating replicon particles (PRP) have also been developed. Semliki Forest virus (SFV) RNA was designed to express the Simian immunodeficiency virus (SIV) Env and Gag proteins from the subgenomic SFV promoter. In addition, the vesicular stomatitis virus (VSV) G protein sequence was inserted behind a duplicate subgenomic promoter. Replication resulted in expression of both the VSV-G protein and the antigens of interest, followed by budding of VSV-G coated vesicles from the host cell. These vesicles had non-specifically encapsidated viral RNA, and could be passaged multiple times, thereby closely resembling an actual virus (Rolls et al., 1994). Even though the RNA is encapsidated in a heterologous envelope, this type of vaccine is comparable to the iDNA vaccines, in which a DNA plasmid encodes for the complete viral RNA/DNA. An iDNA vaccine, launching a LAV Venezuelan equine encephalitis virus (TC-83) provided protection in mice and is awaiting vaccine potency confirmation in equine species (Berge et al., 1961; Tretyakova et al., 2013).

Viral vector-delivery of alphavirus replicon vaccines may further enhance the immunogenic properties of the replicon or facilitate delivery. Wang et al. (2007) found that vaccination using recombinant baculovirus, designed to protect against porcine reproductive and respiratory virus (PRRSV) by transient expression of antigens from a CMV promoter, resulted in enhanced immunogenicity when compared to a conventional DNA plasmid expression of the same antigens in mice. This showed the possibility to use recombinant baculoviruses as a vehicle for gene delivery and generation of an antigen-specific immune response (Wang et al., 2007). After that, the same group used a similar recombinant baculovirus to launch a SFV replicon, which expressed the same PRRSV antigens from the SFV subgenomic promoter. The immunogenicity was compared to that obtained from the earlier tested recombinant baculovirus. They found that the recombinant BV containing the replicon resulted in elevated PRRSV antigen expression in vitro and an increased Th1 response in vaccinated mice, in comparison to the baculovirus that transiently expressed PRRSV antigens (Wu et al., 2013). Similarly, a recombinant adenovirus was used to deliver a SFV replicon that expressed the classical swine fever E2 protein. This resulted in complete protection in pigs, whereas vaccination with a recombinant adenovirus vector expressing the same E2 protein, or a DNA-launched SFV replicon expressing E2, resulted only in partial protection (Sun et al., 2011; Sun et al., 2010) (Table 1).

## Other positive stranded RNA viruses

Besides alphaviruses, other positive-stranded RNA viruses have also been used in the production of viral replicon vaccines for veterinary use. These encompass members of the *Flaviviridae*, *Picornaviridae* and *Arteriviridae*.

*Flaviviridae*

The flavivirus genome consists of positive-stranded RNA of ~11 kb, which is, like in alphaviruses, encapsidated by a nucleocapsid protein and an envelope. Unlike alphaviruses, the viral RNA encodes a single polyprotein, which can directly be translated due to a 5' cap and specific RNA structures at both the 5'- and 3' end. The 5' portion of the RNA encodes the structural proteins (C, prM, E), followed by the non-structural proteins. The resulting polyprotein spans the ER membrane multiple times and is processed by both viral and host proteases to release the individual proteins (Lindenbach et al., 2007). Flavivirus virions assemble in the ER lumen, and mature in the slightly acidic environment of the late host secretory pathway, by furin cleavage of prM into pr and M, before exocytosis from the cell surface (Mukhopadhyay et al., 2005).

Flavivirus reverse genetic systems are well established (reviewed by Aubry et al., 2015) and are often used for the production of chimeric viruses, by replacement of the structural proteins PrM and E by equivalent genes from related flaviviruses (recently reviewed by Mogler and Kamrud, 2015). However, as chimeric viruses are outside the scope of this review, we will solely focus on what is known about flavivirus replicon vaccines for veterinary applications.

The best studied flavivirus replicons are based on West Nile virus (WNV) lineage 1 using the naturally attenuated strain Kunjin virus (Khromykh and Westaway, 1997; Pijlman et al., 2006), of which replication was shown to result in non-cytopathic, long-lasting heterologous antigen expression and potent immune responses (Anraku et al., 2002). Insertion of a Hepatitis delta virus ribozyme at the 3' end significantly increased viral replication (Varnavski et al., 2000) and this WNV replicon shows great potency in its application as a viral vector. Delivery of the WNV structural proteins *in trans* in a tetracyclin-inducible 'packaging cell line' results in high levels of VRP production (Harvey et al., 2004; Khromykh et al., 1999; Liu et al., 2002) (Fig. 3A). VRPs can also be produced *in vitro* by a dual replicon system, in which flavivirus replicon RNA was packaged by WNV structural proteins produced from a replicating SFV replicon (Khromykh et al., 1998). Recently, WNV-based VRP were shown to provide protection against Ebola virus in non-human primates (Pyankov et al., 2015). Flavivirus replicons have also been developed for yellow fever virus (YFV) (Jones et al., 2005; Lindenbach and Rice, 1997; Shustov et al., 2007), highly pathogenic WNW (Scholle et al., 2004; Suzuki et al., 2009), tick-borne encephalitis virus (TBEV) (Gehrke et al., 2003; Yoshii et al., 2005; Yoshii and Holbrook, 2009) and DENV (Lai et al., 2008). Besides VRP production, it is important

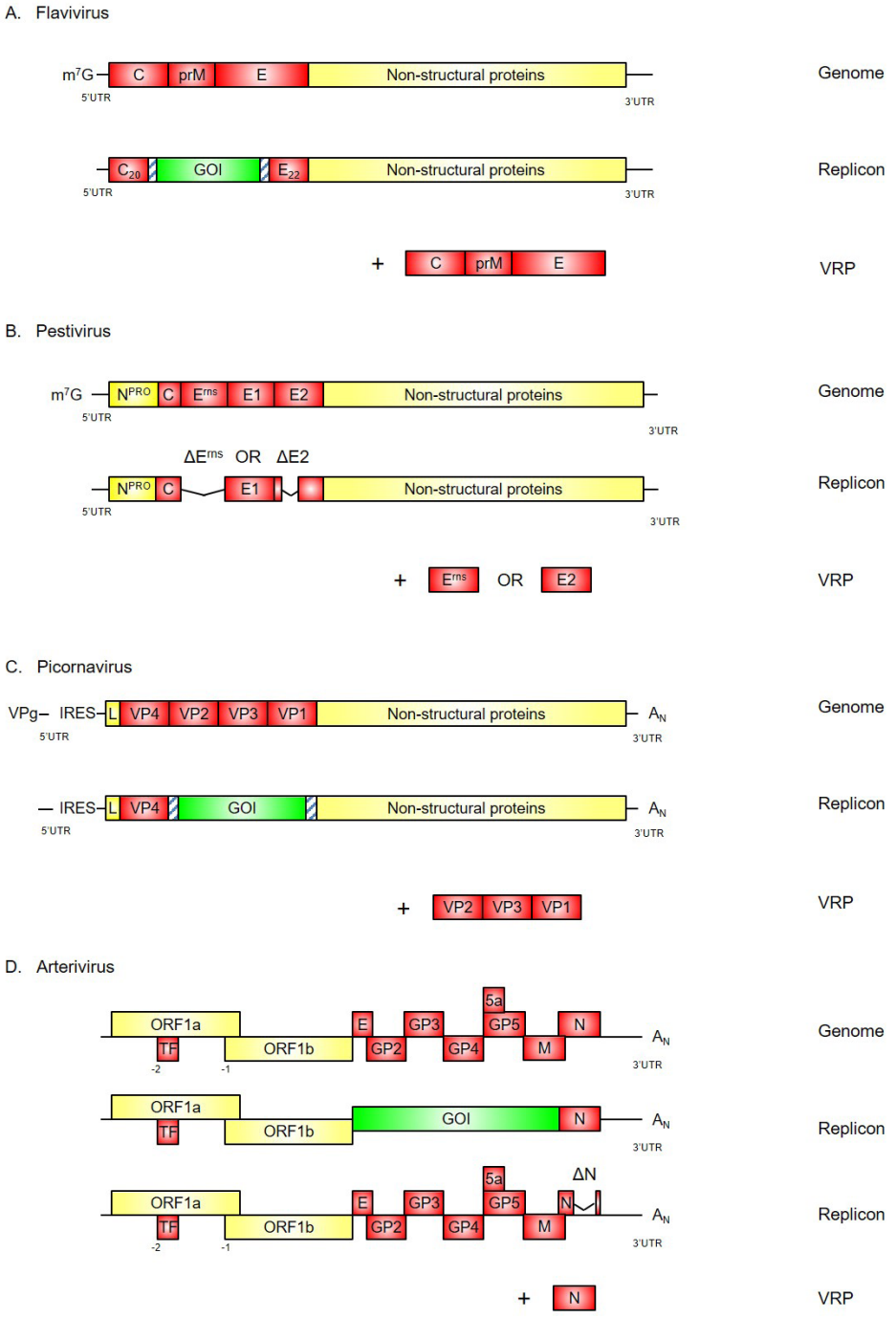



**Figure 3. Schematic overview of the genome organization and replicon construction of positive stranded RNA viruses.** (A) flaviviruses, (B) pestiviruses, (C) picornaviruses and (D) arteriviruses. Complementation of the viral RNA with the lacking structural proteins *in trans* results in the generation of viral replicon particles (VRP).

to note that the ability of flaviviruses to generate prME subviral particles (SVP) can be used to further boost the efficacy of flavivirus replicon vaccines. Gene-gun vaccination with *in vitro* generated TBEV RNA, containing a deletion in the capsid gene, resulted in *in vivo* RNA replication and non-infectious SVP budding from the cell, and led to protective immunity in mice (Aberle et al., 2005; Kofler et al., 2004; Mandl, 2004). However, to the best of our knowledge, veterinary applications for flavivirus replicon vaccines have only been explored for use in horses. A DNA plasmid was developed to launch a WNV replicon, and from a duplicated CMV promoter the capsid protein was expressed *in trans*. Gene-gun bombardment delivery of the DNA plasmid resulted in VRP production *ex vivo*, which were able to spread the replicon RNA for a single additional round to neighbouring cells. Replication of replicon RNA in these cells led to secreted SVPs, but absence of the capsid protein ensured dead-end replication. The secreted SVPs gave an additional 'kick' to the replicon vaccine and evoked improved protection as compared to a conventional DNA vaccine in mice and yielded neutralizing antibodies in horses (Chang et al., 2008). This is an elegant example of how rational design can help generate a self-limiting and highly potent replicon vaccine for veterinary use.

The genus *Pestivirus* contains important infectious agents of livestock like classical swine fever virus (CSFV) and bovine viral diarrhoea virus (BVDV) and is therefore widely studied. The enveloped virion contains a RNA genome of ~12kb that encodes one long, single polyprotein. Both viral and host proteases process the polyprotein into 12 mature proteins; 4 structural proteins (C, E2, Erns, and E1) and 8 non-structural proteins (Tautz et al., 2015). Replicon vaccines based on alphavirus genomes have been developed to protect against economically important pestiviruses (Loy et al., 2013; Reddy et al., 1999; Sun et al., 2010), however, also replicons have been made based on the pestivirus itself. Pestivirus replicons have been developed for BVDV (Horscroft et al., 2005), and CSFV. Such BVDV and CSFV replicon vaccines are obtained by deletion mutagenesis of one or more of the structural proteins (Fig. 3B) and are mostly used for protection against the homologous virus. Viral replicon expression in cell lines that constitutively express the complementary structural protein, results in the production of VRPs of either BVDV (Reimann et al., 2007) or CSFV (Frey et al., 2006; Maurer et al., 2005; Van Gennip et al., 2002; Widjojoatmodjo et al., 2000), that are subsequently used for vaccination. In addition, a CSFV replicon was used in a VRP vaccine against Japanese encephalitis (JE). In this study the E2 protein of CSFV was replaced by a truncated form of the JE E2 protein. Replication of this chimeric replicon in a helper cell line constitutively expressing the CSFV E2 protein, resulted in the secretion of VRP that elicited a protective immune response against JE in pigs (Yang et al., 2012).

*Picornaviridae*

Picornaviruses have a positive sense RNA of ~7.5 kb, which contains a 5' internal ribosomal entry site and a polyadenylation signal at the 3' end. The RNA encodes a

single polyprotein that translates into four structural proteins (VP1-4) and the non-structural proteins, and is processed into the mature proteins by viral proteases (Jiang et al., 2014). The family of *Picornaviridae* encompasses human and veterinary important pathogens such as poliovirus, rhinovirus, hepatitis A virus and foot-and-mouth disease virus (FMDV). Even though the genome organization is similar among these viruses, sequence diversity is high (Jia et al., 1998). Picornavirus replicons were first generated from poliovirus (Kaplan and Racaniello, 1988) and have subsequently been developed for amongst others, FMDV (Fig. 3C). The replicons are generated by an in-frame deletion of – part of – the capsid polyprotein, and are encapsidated by expression of a hetero- or homologous capsid protein *in trans* (Jia et al., 1998). Recently, a FMDV replicon was generated that contained all non-structural proteins, expressed a reporter gene and still replicated efficiently (Tulloch et al., 2014). To the best of our knowledge, however, no FMDV replicon vaccines have been reported yet, which was possibly related to the decision to cease routine vaccination against FMDV throughout the EU in 1991. Re-emergence of FMDV outbreaks in Europe and presence of multiple global endemic area's specifies the need for a FMDV vaccine (Brito et al., 2015; Sobrino and Domingo, 2001). Inactivated virus vaccines against FMDV have to be applied every 4-6 months to maintain immunity and hinder global trade, as vaccinated animals cannot be distinguished from affected animals (Sobrino and Domingo, 2001). In addition, because there is a wide sequence variety in field strains and new isolates continue to arise, next-generation FMDV replicon vaccines that are more easily adapted to new strains may have future potential, as they may be quicker to generate.



*Arteriviridae*

Porcine reproductive and respiratory syndrome virus (PPRSV) is a member of the family *Arteriviridae*, which only includes three other viruses; equine arteritis virus, simian haemorrhagic fever virus and lactate dehydrogenase-elevating virus. The genome consists of a single stranded positive sense RNA of ~15 kb, which encodes ten open reading frames. Out of the seven structural proteins, the N protein is most abundant and specific antibodies arise early during infection. Therefore these specific antibodies are often used for diagnostics. Most neutralizing antibodies are generated against the GP5 protein (Loving et al., 2015).

Being an important infectious agent for livestock itself, PRRSV is also used in the generation of vaccines against heterologous viruses. A PRRSV DNA-launched replicon was generated that expressed GFP. This replicon elicited increased IFN-γ responses in mice when compared to a DNA plasmid transiently expressing GFP (Huang et al., 2009). A similar replicon was used to launch expression of the FMDV antigen VP12A3C, which was shown to produce the antigen *in vitro,* but this replicon awaits further *in vivo* analysis (Jeeva et al., 2014). A DNA-launched replicon vaccine was also developed with the aim to vaccinate swine against the homologous virus. A partial deletion in ORF2

rendered the virus non-infectious and partial protection was obtained in a vaccination trial using pigs (Pujhari et al., 2013). PRRSV replicon RNA can also be packaged in a packaging cell line continuously expressing the virus capsid protein N. However, so far, the VRP titers obtained were not very high (Song et al., 2011). More research is needed to optimize and test the efficacy of PRRSV replicon applications.

### Negative stranded RNA viruses

Generation of self-replicating vaccines for negative stranded RNA viruses is not as straightforward as for positive stranded RNA viruses, as their genome is antisense and non-infectious. However, reverse genetic systems have been developed for multiple negative-sense RNA viruses and are now also applied in vaccine development. Bunyaviruses are one example of negative-sense RNA viruses to cause severe disease in both animals and humans. An important member of the genus *Phlebovirus* is Rift Valley fever virus (RVFV), which causes severe disease in ruminants. Besides disease in adult ruminants, mortality in foetuses can reach 100% (Kortekaas et al., 2011). Bunyaviruses have a 3-segmented genome, consisting of a large (L), medium (M) and small (S) segment. The L-segment encodes the viral RNA polymerase, the M-segment encodes two structural proteins (Gn, Gc) and two non-structural proteins (NSm1 and NSm2), and the ambi-sense S-segment encodes the structural protein N and the non-structural protein NSs (Acheson, 2011) (Fig. 4A). The L- and S-segment are prerequisites for viral replication and can be maintained in a stable replicon cell line. Transfection of this cell line with a helper plasmid encoding the structural glycoproteins, resulted in the secretion of VRPs, which are, due to a lack of an encapsidated M segment, incapable of further spread. These VRPs induced protective immunity when used as a vaccine against the homologous virus in mice and sheep (Kortekaas et al., 2011; Oreshkova et al., 2013). This system was further optimized by the use of Newcastle disease virus (NDV) to deliver the glycoproteins to the stable replicon cell lines, rather than by transfection (Wichgers Schreur et al., 2014). A similar RVFV VRP was developed, which besides the lack of structural glycoproteins, also lacked both the virulent NSs and NSm. The authors showed that viral replication was essential for induction of protective immunity, and also this VRP provided protection against RVFV challenge in mice (Dodd et al., 2012).

Replicons have also been developed for non-segmented negative stranded RNA viruses, in particular for paramyxoviruses and rhabdoviruses. The genome organization of both virus families is very similar and codes for 5-10 genes, and the viral RNA is not capped or polyadenylated (Acheson, 2011). The proteins N, L and P are indispensable for replication, however deletions within the structural proteins have been made without abrogation of viral RNA replication. Paramyxoviruses comprise among others the veterinary important viruses NDV and Peste des petites ruminants virus. These viruses are mostly used for the generation of chimeric and/or pseudotyped viruses and therefore beyond the scope of this review (reviewed in Bukreyev et al., 2006; Mogler

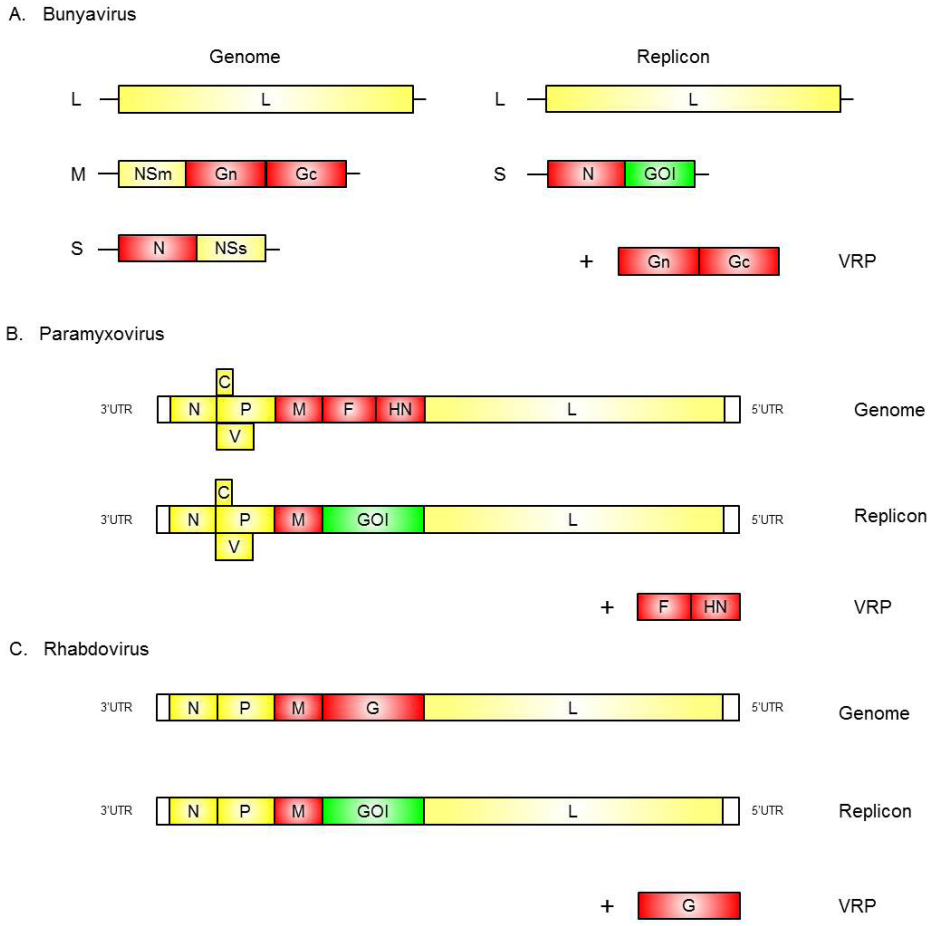



**Figure 4. Schematic overview of the genome organization and replicon construction of the negative stranded RNA viruses.** (A) bunyavirus and (B) paramyxoviruses and (C) rhabdoviruses. Complementation of the viral RNA with the lacking structural proteins *in trans* results in the generation of viral replicon particles (VRP).

and Kamrud, 2015). Paramyxovirus replicons can be made by replacement of either or both the F and HN protein with a heterologous GOI, and VRPs are secreted following complementation by the lacking structural protein(s) (Fig. 4B). Sendai virus (SeV), also known as murine parainfluenzavirus type 1, is a well-known paramyxovirus, on the basis of which a replicon has been made (Bernloehr et al., 2004; Iida and Inoue, 2013). To the best of our knowledge, however, no veterinary applications for this replicon have been established. Rhabdovirus replicons and VRPs based on vesicular stomatitis virus (VSV), an infectious agent of livestock, have been made by replacement of the glycoprotein G with a heterologous GOI (Fig. 4C). Replacement of the G protein with the HA protein of avian influenza and subsequent complementation with the G protein generated VSV VRPs which gave protection against lethal virus challenge in chickens

(Halbherr et al., 2013; Kalhoro et al., 2009; Schwartz et al., 2007). Similarly, VSV VRPs, adapted to express the bluetongue virus VP2 protein, resulted in protection against viral challenge in sheep (Kochinger et al., 2014).

## CONCLUDING REMARKS

Viral replicons have great potential to be used in next generation vaccines, mainly due to their ability to replicate RNA *in vivo* and the concomitant high antigen expression levels, which together induce a potent immune response. Progress in sequencing and DNA synthesis technologies allows for precise and rapid generation of antigen coding sequences. Therefore, replicon vaccines can be produced in a time-span of a few weeks after a new virus strain has emerged. DNA-launched replicons are easily produced and are stable under ambient temperatures for long periods of time. In addition, improved DNA delivery methods have contributed to enhanced efficacy. The use of replicons in an RNA form circumvents the need for delivery and transcription initiation in the cell nucleus. Finally, VRP vaccines, which are encapsidated forms of the replicon RNA, bypass the difficult uptake of negatively charged nucleic acid material into the cells.

As with any new technology, there are still some important hurdles to overcome. Within Europe, the question remains whether animals held for consumption vaccinated with DNA vaccines (including the DNA-launched replicon vaccines as described in this review) are considered genetically modified or not. However, at present four DNA vaccines have obtained licenses outside Europe. In addition, the first VRP vaccine has also been registered for use in the field. Replicons provide an elegant alternative to classical veterinary vaccines that perform suboptimal. Further advancements in terms of safety and efficacy may strengthen its position in veterinary vaccinology. Combined, replicons represent a next-generation class of veterinary vaccines with great prophylactic potential.

## ACKNOWLEDGEMENTS

We acknowledge Just M. Vlak for his suggestions and critical reading of the manuscript.

**Table 1. Alphavirus-based, veterinary replicon vaccine candidates.**

| Agent/disease | Antigen | Use for | Tested in | Vector | DNA/RNA | Method | Ref |
|---|---|---|---|---|---|---|---|
| Infectious salmon anaemia virus | HE | Salmon | Salmon | Salmonid alphavirus | RepDNA | i.m. | (Wolf et al., 2013; Wolf et al., 2014) |
| Infectious pancreatic necrosis virus | Structural polyprotein | Salmon | Salmon | Salmonid alphavirus | RepDNA | i.m. | (Abdullah et al., 2015) |
| Salmonid alphavirus | Envelope genes | Salmon | Salmon | Salmonid alphavirus | RepDNA | i.m. | (Hikke et al., 2014) |
| Bovine viral diarrhea virus | NS3 | Cattle | Mice | Semliki Forest virus | RepDNA | i.m. | (Reddy et al., 1999) |
| Classical swine fever virus | CS-E2 | Swine | Pigs | Semliki Forest virus (adenovirus vectored) | RepDNA | i.m. | (Sun et al., 2011; Sun et al., 2010) |
| Classical swine fever virus | E2 | Swine | Pigs | Semliki forest virus | RepDNA | i.m. | (Li et al., 2007) |
| Infectious bursal disease virus | VP2 | Chicken | Chicken | Semliki Forest virus | RepDNA | i.m. | (Phenix et al., 2001) |
| Louping ill virus | prME | Sheep | Mice, sheep | Semliki Forest virus | RepRNA | i.m. | (Fleeton et al., 2001; Fleeton et al., 2000; Morris-Downes et al., 2001) |
| Semian immunodeficiency virus | GP160, env, gag-pol, nef, rev, tat | Primates | Macaques | Semliki Forest virus | VRP | i.m. | (Mossman et al., 1996; Nilsson et al., 2001) |

*The RNA is packaged in a heterologous VLP

i.d. = intradermal; i.m. = intramuscular; i.n. = intranasal; i.o. = in ovo; PRP = propagating infectious particle; RepDNA = DNA-launched replicon; s.c. = subcutaneous; VRP = viral replicon particle

**Table 1, continued. Alphavirus-based, veterinary replicon vaccine candidates.**

| Agent/disease | Antigen | Use for | Tested in | Vector | DNA/RNA | Method | Ref |
|---|---|---|---|---|---|---|---|
| Semian immunodeficiency virus | Env, Gag | Primates | Macaques | Semliki Forest virus | PRP* | i.m. + i.n. | (Rolls et al., 1994; Schell et al., 2015; Schell et al., 2011) |
| Brucella abortus | IF3 | Cattle | Mice | Semliki Forest virus | VRP | i.p. | (Cabrera et al., 2009) |
| Pseudorabies virus | gC | Swine | Mice | Semliki Forest virus | RepDNA | i.m. | (Xiao et al., 2004) |
| Foot and mouth disease virus | VP1-VP4 (P1) | Ruminants | Mice | Semliki Forest virus | RepDNA | i.m. | (Yu et al., 2006) |
| Foot and mouth disease virus | VP1 | Ruminants | Guinea pigs | Sindbis virus | DNArep | i.m. | (Dar et al., 2012) |
| Rabies virus | G | Dog | Mice | Sindbis virus | RepDNA | i.m. | (Saxena et al., 2008) |
| Pseudorabies virus | gB, gC, gD | Swine | Pigs | Sindbis virus | RepDNA | i.m. | (Dufour and De Boisséson, 2003) |
| Rift Valley fever | GnGc, NSm | Ruminants | Mice | Sindbis virus | VRP | Gene-gun | (Bhardwaj et al., 2010; Heise et al., 2009) |
| Rift Valley fever | Gn | Ruminants | Mice | Sindbis virus, VEE virus | DNArep, VRP | s.c. | (Gorchakov et al., 2007) |
| Porcine influenza virus | HA | Swine | Mice, Swine | Venezuelan equine encephalitis virus , (SirraVax$^{sm}$) | VRP | i.m. | (Vander Veen et al., 2012b) |
| Porcine reproductive and respiratory syndrome virus | n.a. | Swine | Mice, swine | Venezuelan equine encephalitis virus (SirraVax$^{sm}$) | VRP | n.a. | n.a. |

*The RNA is packaged in a heterologous VLP

i.d. = intradermal; i.m. = intramuscular; i.n. = intranasal; i.o. = in ovo; PRP = propagating infectious particle; RepDNA = DNA-launched replicon; s.c. = subcutaneous; VRP = viral replicon particle

| Agent/disease | Antigen | Use for | Tested in | Vector | DNA/RNA | Method | Ref |
|---|---|---|---|---|---|---|---|
| Porcine epidemic diarrhea virus | n.a. | Swine | n.a. | Venezuelan equine encephalitis virus (SirraVax$^{sm}$) | VRP | i.m. | n.a. |
| Rotavirus C | n.a. | Swine | n.a. | Venezuelan equine encephalitis virus (SirraVax$^{sm}$) | VRP | n.a. | n.a. |
| Canine influenza virus | n.a. | Dogs | n.a. | Venezuelan equine encephalitis virus (SirraVax$^{sm}$) | VRP | n.a. | n.a. |
| Avian influenza | n.a. | Poultry | n.a. | Venezuelan equine encephalitis virus (SirraVax$^{sm}$) | VRP | n.a. | n.a. |
| Equine arteritis virus | GL and M | Equine | Mice, horses | Venezuelan equine encephalitis virus | VRP | s.c. | (Balasuriya et al., 2002; Balasuriya et al., 2000) |
| Bovine viral diarrhea virus | E2 | Cattle | Calves | Venezuelan equine encephalitis virus | VRP | i.m. | (Loy et al., 2013) |
| Avian influenza virus | HA | Poultry | Chicken | Venezuelan equine encephalitis virus | VRP | i.o. and/ or s.c. | (Schultz-Cherry et al., 2000) |
| Venezuelan equine encephalitis virus | Structural proteins | Equine | Mice | Venezuelan equine encephalitis virus | iDNA | EP | (Tretyakova et al., 2013) |
| Foot and mouth disease virus | poINF, boIFN | Ruminants | Mice | Venezuelan equine encephalitis virus | RepDNA | s.c | (Diaz-San Segundo et al., 2013) |

*The RNA is packaged in a heterologous VLP

i.d. = intradermal; i.m. = intramuscular; i.n. = intranasal; i.o. = in ovo; PRP = propagating infectious particle; RepDNA = DNA-launched replicon; s.c. = subcutaneous; VRP = viral replicon particle

**Table 2. Positive RNA virus-based, veterinary replicon vaccine candidates.**

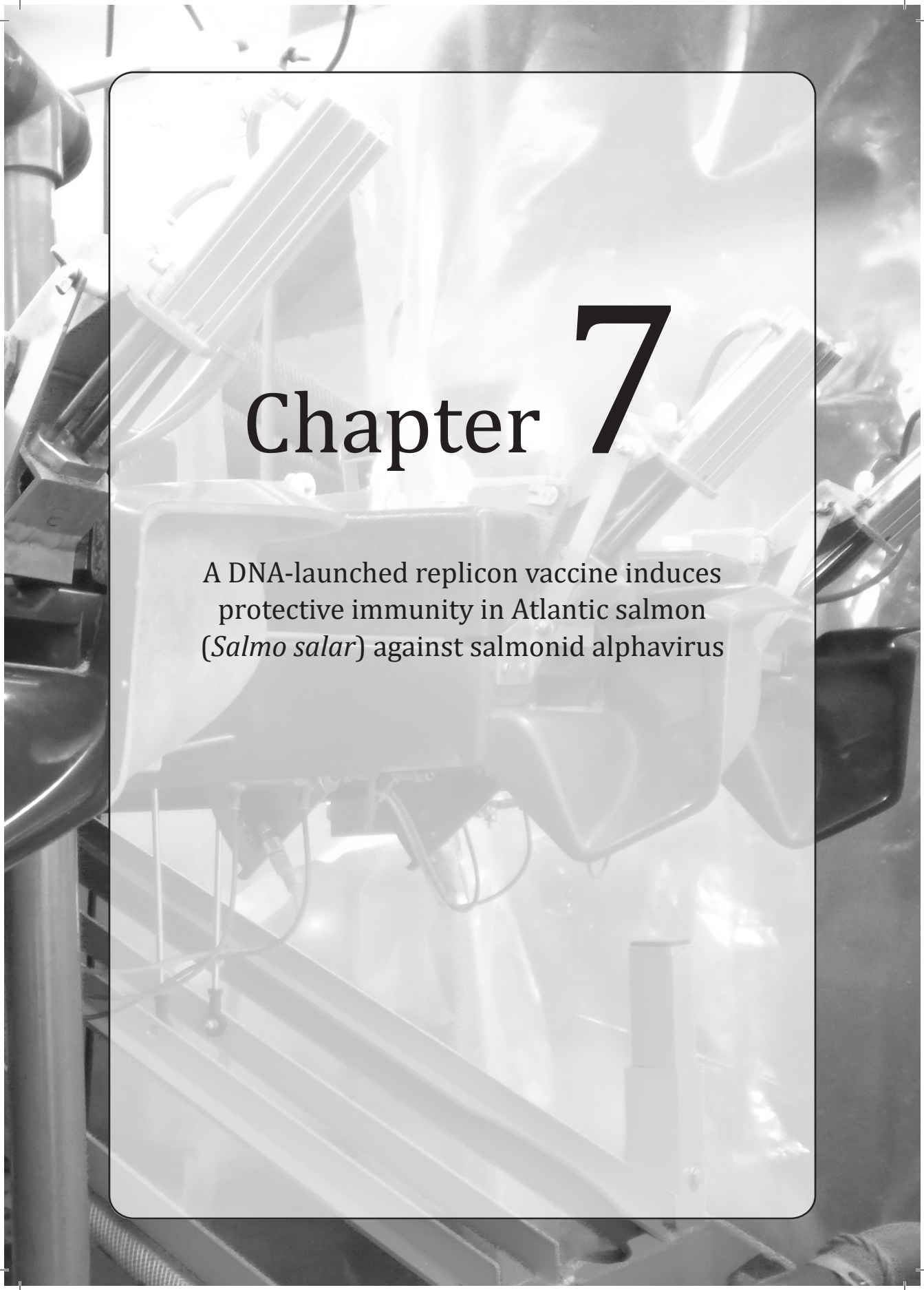
| Agent/disease | Antigen | use for | Tested in | Vector | DNA/RNA | Method | Ref |
|---|---|---|---|---|---|---|---|
| West Nile virus | prM and E | Horses | Mice, horses | West Nile virus | DNA-VRP | Gene gun | (Chang et al., 2008) |
| Japanese encephalitis | E2 | Swine | pigs | classical swine fever virus | VRP | i.d., oronasal | (Yang et al., 2012) |
| classical swine fever virus | Ems (ΔE2) | Swine | Pigs | classical swine fever virus | VRP | oronasal | (Maurer et al., 2005) |
| classical swine fever virus | E2 (ΔErns) | Swine | Pigs | classical swine fever virus | VRP | i.n., i.d. and i.v. | (Widjojoatmodjo et al., 2000) |
| classical swine fever virus | Erns (ΔE2) | Swine | Pigs | classical swine fever virus | VRP | i.d. | (Van Gennip et al., 2002) |
| classical swine fever virus | E2 (ΔErns) | Swine | Pigs | classical swine fever virus | VRP | i.d. injection | (Frey et al., 2006) |
| Bovine viral diarrhea virus | E2 and Erns (ΔC) | Bovine | calves | bovine viral diarrhea virus | VRP | i.m. or i.m. and i.n. | (Reimann et al., 2007) |
| Foot and mouth disease virus | VP12A3C | Ruminants | n.a. | Porcine reproductive and respiratory syndrome virus | DNA, VRP | n.a. | (Jeeva et al., 2014) |
| Porcine reproductive and respiratory syndrome virus | Structural proteins | Swine | n.a. | Porcine reproductive and respiratory syndrome virus | VRP | n.a. | (Song et al., 2011) |
| Porcine reproductive and respiratory syndrome virus | Structural proteins | Swine | pigs | Porcine reproductive and respiratory syndrome virus | DNArep | i.m. | (Pujhari et al., 2013) |

DNArep = DNA-launched replicon; i.d. = intradermal; i.m. = intramuscular; i.n. = intranasal; s.c. = subcutaneous; VRP = viral replicon particle

**Table 3. Negative RNA virus-based, veterinary replicon vaccine candidates.**

| Agent/disease | Antigen | use for | Tested in | Vector | DNA/RNA | Method | Ref |
|---|---|---|---|---|---|---|---|
| Rift Valley fever | Gn, Gc | Ruminants | Mice | Rift Valley fever virus | VRP | s.c. | (Dodd et al., 2012) |
| Rift Valley fever | Gn, Gc | Ruminants | Mice, sheep | Rift Valley fever virus | VRP | i.m. | (Kortekaas et al., 2011; Oreshkova et al., 2013) |
| Avian influenza | HA | Poultry | Mice, chickens | Vesicular stomatitis virus | VRP | i.m. | (Halbherr et al., 2013; Kalhoro et al., 2009; Schwartz et al., 2007) |
| Bluetongue virus | VP2 | Ruminants | Sheep | Vesicular stomatitis virus | VRP | i.m. | (Kochinger et al., 2014) |

i.d. = intradermal; i.m. = intramuscular; i.n. = intranasal; PRP = propagating viral particle; s.c. = subcutaneous; VRP = viral replicon particle



# Chapter 7

## A DNA-launched replicon vaccine induces protective immunity in Atlantic salmon (*Salmo salar*) against salmonid alphavirus

## ABSTRACT

Salmonid alphavirus (SAV; also known as Salmon pancreas disease virus; family *Togaviridae*) causes pancreas disease and sleeping disease in Atlantic salmon and rainbow trout, and poses a major burden to the aquaculture industry. Previously, we demonstrated that membrane trafficking and surface expression of SAV glycoprotein E2 occurs only in the presence of E1. In addition, we showed that vaccination of Atlantic salmon with virus-like particle and core-like particle vaccines did not readily result in protection upon SAV challenge. It was hypothesized that presence of viral RNA in a SAV vaccine may be needed to generate a protective immune response. Self-replicating RNA (replicon) vaccines have shown great potential as vaccines in different animals. Therefore, in this study, DNA-launched SAV replicon vaccines were tested in a vaccination-challenge model in Atlantic salmon. A SAV replicon vaccine encoding E2 elicited protective immunity only when E1 was co-expressed. This proved the biological significance of E1-dependent surface translocation of glycoprotein E2. By expression of the SAV capsid protein *in trans*, it was hypothesized that viral replicon particles (VRP) were formed *in vivo*, which might further elevate the immune response in comparison to the replicon vaccine. However, a second animal trial showed that the inclusion of capsid did not yet improve vaccine efficacy. These trials also showed that a DNA vaccine transiently expressing the SAV structural proteins provided superior protection over both replicon vaccines (with and without capsid). These studies further define the prerequisites for induction of a potent immune response in Atlantic salmon by DNA-launched (replicon) vaccination against SAV, identify two promising vaccine candidates, and give insight on future directions for SAV vaccine development.

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## 1. INTRODUCTION

Salmonid alphavirus (SAV) is the etiological agent of pancreas disease (PD) and sleeping disease (SD) in Atlantic salmon (*Salmon salar* L.) and rainbow trout (*Oncorhynchus mykiss* W.). SAV, also known as Salmon pancreas disease virus (SPDV; ICTV 9th report, 2011), is a unique member of the genus *Alphavirus* (family *Togaviridae*) as it lacks an identified invertebrate vector, its viral replication is abrogated at temperatures above 18°C, and it contains on average larger individual proteins (Graham et al., 2008; McLoughlin and Graham, 2007). Still, the infectious cycle of SAV is expected to consist of general alphavirus 'life' cycle elements (Villoing et al., 2000; Weston et al., 2002; Weston et al., 1999). The viral genome consists of a single, positive stranded RNA, which can directly be translated into the non-structural proteins (nsPs) 1-4 necessary for viral replication. A subgenomic mRNA is produced that encodes the viral structural proteins capsid (C), envelope (E)3, E2, 6K and E1. Upon translation, the C protein is autocatalytically cleaved off from the nascent polyprotein. The remaining envelope polyprotein (precursor E2 (PE2), 6K and E1) is embedded into the endoplasmic reticulum (ER), where host signalases segregate the PE2, 6K and E1 proteins. The trimeric alphavirus spikes consisting of three PE2-E1 heterodimers are then formed and travel to the trans-Golgi network (TGN). Here PE2 is processed by the host enzyme furin, which separates E3 from E2 and renders the spike sensitive for low-pH induced activation. Finally, the glycoprotein spikes appear on the cell surface and by association with the viral nucleocapsid the enveloped virions then leave the cell by budding (Garoff et al., 2004; Kielian, 2010; Kuhn, 2007). The exact mechanism by which nucleocapsid formation and subsequent virion budding in alphaviruses occurs is unclear, however it has been shown that delivery of pre-formed nucleocapsids into the cytoplasm still leads to virion budding from the cell (Cheng and Mukhopadhyay, 2011; Snyder et al., 2011).

SAV C core-like particles (CLP) (Chapter 3), SAV virus-like particles (eVLP) (Metz et al., 2011a), and chimeric CHIKV/SAV$^{Bdom}$ eVLP were previously tested in a vaccination/cohabitation challenge trial. It was concluded that eVLP and CLP vaccination of Atlantic salmon did not readily confer protection against SAV challenge (Chapter 5). It was hypothesized that presence of viral RNA in the vaccine may be needed to generate a protective immune response. Self-replicating RNA (replicon) molecules have shown great potential as vaccines in different animal models (Chapter 6), and might also be a potent vaccine candidate for use in Atlantic salmon against SAV. In addition, in Chapter 4, we showed that the E2 glycoprotein needs co-expression of E1 for proper translocation and presentation at the cell surface of insect cells. In the first trial, we describe vaccination with DNA-launched SAV replicon of Atlantic salmon, in which the PE2 subunit or the envelope cassette (Env, E3E26KE1) was translated from the alphavirus 26S subgenomic mRNA. The results from this trial confirmed the biological significance of the E1-dependent mechanism of E2 presentation at the cell its surface (Chapter 4), as it appeared to be essential for the generation of protective immunity against SAV

in a vaccination/cohabitation challenge trial. To further increase vaccine potency, the SAV capsid protein was expressed *in trans* from the same plasmid DNA to produce viral replicon particles (VRPs) *in vivo*. Both DNA-launched replicon vaccines were compared to a classical DNA vaccine, which transiently expressed the SAV structural polyprotein, in a second vaccination-challenge trial in Atlantic salmon.

## 2. MATERIALS AND METHODS

### 2.1 Plasmid construction

The SAV envelope cassette (Env) and PE2 subunit, and capsid protein were cloned into the pcDNA-DEST40 (Invitrogen) for constitutive expression in vertebrate cells (Fig. 1A) or cloning purposes, respectively. SAV replicons for vaccination have been described (Fig. 2A) (Wolf et al., 2013). For the immunization constructs of the first animal trial, PE2 or Env genes were amplified by RT-PCR from purified viral RNA (QIAamp Viral RNA Mini Kit, Qiagen; Valencia, CA) of SAVH20/03 strain propagated in CHSE-214 cells and were cloned into the replicon vector by AgeI/AscI to generate pSAV/PE2 and pSAV/Env. For the second animal trial, the structural proteins in pFISH (Chapter 4) were replaced by the Env genes via AsiSI/FseI digestion and ligation. Subsequently, the GFP sequence and second subgenomic promoter were removed by restriction/re-ligation

**Table 1. Primers used for generation of the constructs.**

| Name Primer | Primer sequence (5'→ 3')* |
|---|---|
| *Construction of Gateway compatible constructs* | |
| SAV3_E3_F | ATTB1-GGATCCACC**ATG**ACACGTGCTCCAGCCCTCC |
| SAV3_E2_R | ATTB2-GGATCC**TGA**CGCACGAGCCCCAGGTATG |
| SAV3_E1_R | ATTB2-GGATCCTGAGCTCTTGACTATCCGGATTCT |
| SAV3_Capsid_F | ATTB1-GGATCCACC**ATG**TTTCCCATGCAATTCACAAA |
| SAV3_Capsid_R | ATTB2-GGATCC**CTA**ATGGTGATGGTGGTGATGCCAAGGTATGGCCTCGCTG |
| ATTB1 | GGGGACAAGTTTGTACAAAAAAGCAGGCTTA |
| ATTB2 | GGGGACCACTTTGTACAAGAAAGCTGGGTA |
| *Constructs for immunization* | |
| SAV_E3_AgeI_F | GCGACCGGTGCCACC**ATG**GGAACACGTGCTCCAGCCCTCCT |
| SAV_E2_AscI_R | CCGGCGCGCC**TTA**CGCACGAGCCCCAGGTATG |
| SAV_E1_AscI_R | CCGGCGCGCC**TTA**GCTCTTGACTATCCGG |
| SAV_E3_AsiSI_F | TAGCGATCGCACC**ATG**ACACGTGCTCCAGCCCTCC |
| SAV_E1_FseI_R | TAGGCCGGCC**CTA**GCTCTTGACTATCCGGATTC |
| CMV_NotI_F | TTGCGGCCGCGTTGACATTGATTATTGACTAG |
| BGH pA_NotI_R | TTGCGGCCGGCCATAGAGCCCACCGCATC |

*Restriction sites are underlined, and start/stop codons are printed in bold.

with the compatible restriction enzymes PacI and AsiSI to generate pSAV/Env. pcDNA-DEST40-Capsid served as a PCR template for the linear DNA fragment CMV-Capsid-BGH pA, flanked by NotI restriction sites. This sequence was cloned into the unique NotI restriction site, in reverse orientation, at the 5' end of the CMV promoter in pSAV/Env, resulting in pVRP. Restriction enzymes were from New England Biolabs (Ipswich, MA), and primers are listed in Table 1.

### 2.2 Cells and viruses

Chinook salmon embryo CHSE-214 cells were cultured as described (Metz et al., 2011a). CHSE-214 cells were transfected at 12°C for 4 h using FuGene (Promega) according to manufacturer's protocol, then placed at 12°C or 20°C. Transfections with pcDNA-DEST40 plasmids and DNA replicons were incubated at 15°C.

### 2.3 Immunofluorescence assay

Cells were fixed with 4% paraformaldehyde in PBS for 10 min at RT and optionally permeabilized with ice-cold acetone/methanol for 10 min. Immunofluorescence analysis with mAb α-E2 (17H23 (Moriette et al., 2005), 1:1000) was conducted as described (Chapter 2).

### 2.4 Immunization, sampling and challenge

**Animal trial #1**. Plasmid DNA was purified using a NucleoBond® Xtra EF plasmid purification kit (Macherey-Nagel; Düren, Germany) and quantified spectrophotometrically (Nanodrop [ND-1000], Thermo Scientific; Wilmington, DE). For vaccine preparation, replicons were diluted to 0.2 µg/µl in sterile PBS. As a positive control for protection against SAV challenge, a group vaccinated with inactivated, whole virus SAV-antigen was included. SAV challenge experiment was conducted at the VESO Vikan aquatic research facility, Vikan, Norway. The trial was performed as a cohabitation challenge, using unvaccinated Atlantic salmon (*Salmo salar*) presmolts, i.e. not adapted to seawater, with a mean weight of 35 g each. The fish were confirmed free of known salmon pathogens at the onset of the experiment. The fish were kept in tanks supplied with filtered and UV-radiated fresh water, 12°C, fed according to appetite using commercial fish feed, and anesthetized by bath immersion (2–5 min) in benzocaine chloride (0.5 g/10 L water) before handling. The fish were randomly divided into five groups of 52 fish each (Table 2), marked by fin clipping and immunized by intramuscular (i.m.) injection of 50 µl (10 µg) replicon construct. At 6 weeks post vaccination (wpv), shedder fish (20% of the total number of fish), which had been infected i.p. with SAV isolate PD03-13p2 on the same day as the 'replicon vaccination', were placed into the tank for cohabitation. Blood samples were taken at 3 weeks post challenge (wpc) from anaesthetized fish (20 fish/group) and sera were tested for presence of SAV by RT-PCR. Hearts were sampled at 5 wpc (15 fish/group/sampling) and fixed in formalin and

processed for histological evaluation. The relative percent survival {RPS = (1-cumulative mortality vaccinated group/cumulative mortality non-vaccinated group) × 100} was calculated. Fish were killed using concentrated benzocaine chloride (1 g/5 L water) for 5 min.

**Animal trial #2.** The animal experiment was conducted at the Industrielaboriet (ILAB) facilities, Bergen, Norway. Replicon DNA was purified using a Maxiprep EF plasmid purification kit (Qiagen), quantified spectrophotometrically, and diluted with sterile PBS to 0.2 µg/µl in sterile PBS. As a positive control for protection against SAV challenge, a group vaccinated with DNA plasmid, constitutively expressing SAV structural proteins from a CMV promoter, was included. A group mock-vaccinated with a saline solution was included as a negative control. Unvaccinated Atlantic salmon presmolts with a mean weight of 35 g were kept in UV-radiated freshwater at 12°C (500 L tank) and fed according to appetite using commercial fish feed. Before vaccination, the fish were starved for 36 h, anesthetized by bath immersion in Finquel (0.1 mg/mL for 2–3 min) and divided in 5 groups of 42 fish. The fish were marked by fin clipping and injected with 50 µl (10 µg) (replicon) DNA construct. At 6 wpv, the vaccine efficacy was tested in a cohabitation challenge experiment, by releasing SAV infected shedders (20% of the total number of fish) amongst the vaccinated fish (500 L tank), which had been i.p. injected with SAV isolate PD03-13p2. At 3 wpc, serum samples of 20 fish per group were collected and analyzed for the presence of nsP1 transcripts by quantitative real-time PCR (Applied Biosystems). The relative percentage protection at 3 wpc was calculated (RPP= [1-(% PCR positive fish in vaccinated group/% PCR positive fish in unvaccinated group)] x 100). At 5 wpc, hearts were sampled and processed for histological analysis (20 fish/group), after which the trial was terminated.

Both animal trials had been approved by the Norwegian Animal Research Authority.

## 3. RESULTS AND DISCUSSION

### 3.1 Surface expression of SAV glycoprotein E2 in Salmonid cells depends on E1

Previously, it had been determined in *Spodoptera frugiperda* insect cells that surface translocation of SAV glycoprotein E2 was dependent on co-expression of E1 (Chapter 4). To make sure this interdependence is also true in vertebrate cells, prior to the vaccination studies an experiment was conducted to see whether E1-dependent surface expression of E2 was also seen in Salmonid CHSE-214 cells. CHSE-214 cells were transfected with plasmids constitutively expressing either the PE2 subunit (CMV-PE2) or the envelope cassette (CMV-Env) (Fig. 1A) at 15°C. In cells transfected with CMV-Env but not CMV-PE2, E2 was detected at the cell surface at 4 dpt (Fig. 1B, left). The same cells were then permeabilized and subjected to intracellular E2 staining (Fig. 1B, right), resulting in detection of E2 for both constructs. Thus, also in fish cells, SAV E2 is

dependent on glycoprotein E1 for translocation to the cell surface.

### 3.2 Replicon DNA vaccine efficacy relies on expression of both E2 and E1 glycoproteins

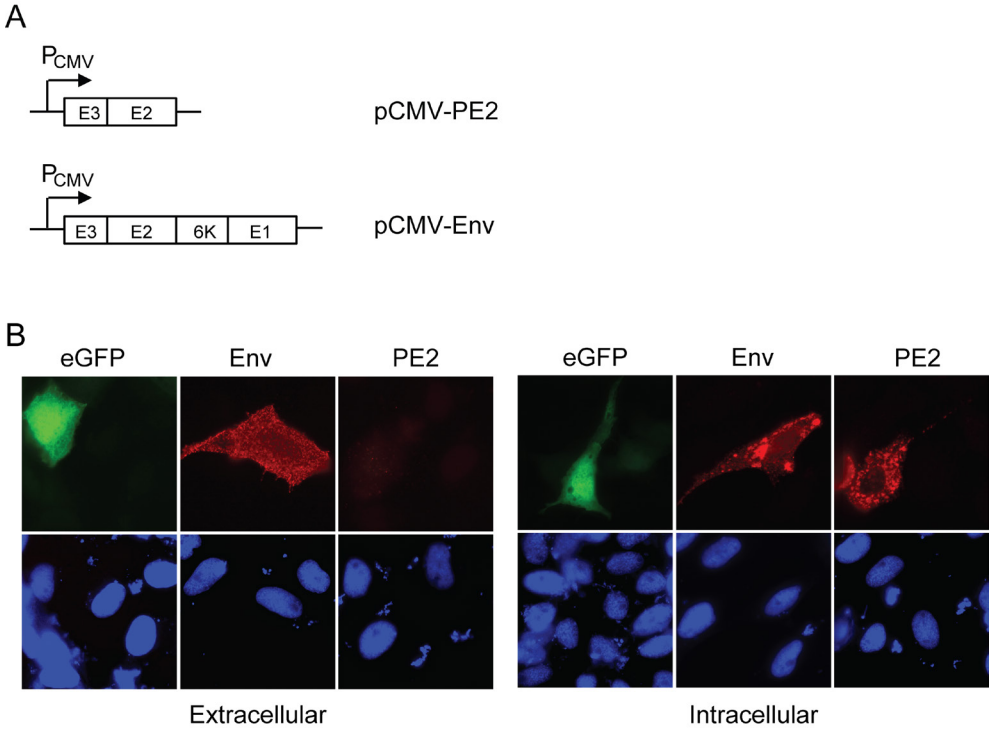


**Figure 1. Surface expression of SAV glycoprotein E2 in Salmonid cells depends on E1.** (A) Schematic presentation of the CMV constructs used in the immunofluorescence assay. (B) SAV glycoprotein surface translocation in CHSE-214 cells. Surface (left) and intracellular (right) detection of glycoprotein E2 (red). Cell nuclei are shown by counterstaining cells with Hoechst DNA dye (blue).



To determine whether surface exposure of SAV glycoprotein E2 is important for the generation of a protective immune response against SAV challenge, replicons pSAV/PE2 and pSAV/Env (Fig. 2A) were used for DNA vaccination of Atlantic salmon. Expression of the E2 glycoprotein by these replicons was confirmed by an immunofluorescence staining of the surface of transfected CHSE-214 cells (Fig. 2B). Atlantic salmons were challenged with SAV by cohabitation challenge 6 wpv. The PBS control group demonstrated successful SAV cohabitation challenge with a cumulative mortality of 50% at the end of the vaccination-challenge experiment (Fig. 2C). Dead fish showed classical SAV-induced histopatological changes in the heart (Table 2, last column). Following SAV challenge, fish immunized with the pSAV/Env replicon showed a mortality rate identical to the group immunized with inactivated, whole virus SAV-antigen (positive control), with a relative percentage survival (RPS) endpoint of 75% for both groups, while the pSAV/PE2 and pSAV/eGFP injected groups were not protected and showed

a $RPS_{endpoint}$ of 0% and -6.3%, respectively (Table 2). In contrast, all fish in the pSAV/PE2 and negative control pSAV/eGFP groups that were tested by RT-PCR of serum and histology of heart, were viremic for SAV at 3 wpc and showed typical SAV-induced histopathological changes in the heart at 5 wpc. Only minor proportions of the fish in the groups pSAV/Env and inactivated antigen were positive in these tests (Table 2).

Thus, pSAV/Env vaccination induced effective protection against SAV challenge, whereas no protection was observed with the pSAV/PE2 replicon. The difference between these two DNA vaccines is that in the case of pSAV/Env the combination of glycoproteins E1/PE2 are properly expressed and exposed. The first possible explanation for this difference in observed protection might be that E1 is essential for induction of protective immunity, possibly by additional antibodies raised against E1. However, from literature it is known that the most immunogenic alphavirus epitopes are located on the E2 protein and not on E1 (Grosfeld et al., 1989; Hunt et al., 2010; Vrati et al., 1988). In our experiments we show that in the absence of E1, E2 does not translocate to the cell surface in both insect (Chapter 4, Fig. 2) and salmon (Fig. 1B) cells. Hence, a more likely explanation is that the presence of E1 aids in the correct folding of E2, which allows translocation of the PE2/E1 heterodimer to the cell surface and correct presentation to the immune system. Although a construct only expressing E1 was not tested as an independent group in this animal trial, we expect that both viral glycoproteins are required at the cell surface for the generation of an immune response able to protect against SAV challenge. Therefore, for SAV vaccine design, we conclude that E2 should ideally be expressed in the context

**Table 2. Vaccine trial #1 experimental design and outcome.**

| Group | Dose and route | Cumulative mortality (%) | $RPS_{endpoint}$ (%) | Prevalence of SAV viremia, 3 wpc | RPP (%) | SAV typical cardiac changes, 5 wpc |
|---|---|---|---|---|---|---|
| pSAV/eGFP | 10 µg/50 µl, i.m. | 53.1 | -6 | 20/20 | 0 | 14/14 |
| pSAV/PE2 | 10 µg/50 µl, i.m. | 50.0 | 0 | 20/20 | 0 | 15/15 |
| pSAV/Env | 10 µg/50 µl, i.m. | 12.5 | 75 | 9/20 | 55 | 7/15 |
| Inactivated antigen | 0.1 ml, i.p. | 12.5 | 75 | 6/20 | 70 | 7/15 |
| PBS | 50 µl, i.p. | 50.0 | 0 | 20/20 | 0 | 15/15 |

$RPS_{endpoint}$ = relative percentage survival = {(1-cumulative mortality vaccinated group/cumulative mortality non-vaccinated group) × 100}.
RPP = relative percentage protection = {1-(% PCR positive fish in vaccinated group/% PCR positive fish in unvaccinated group) × 100}.
i.m. = intramuscular, i.p. = intraperitoneal, wpc = weeks post infection

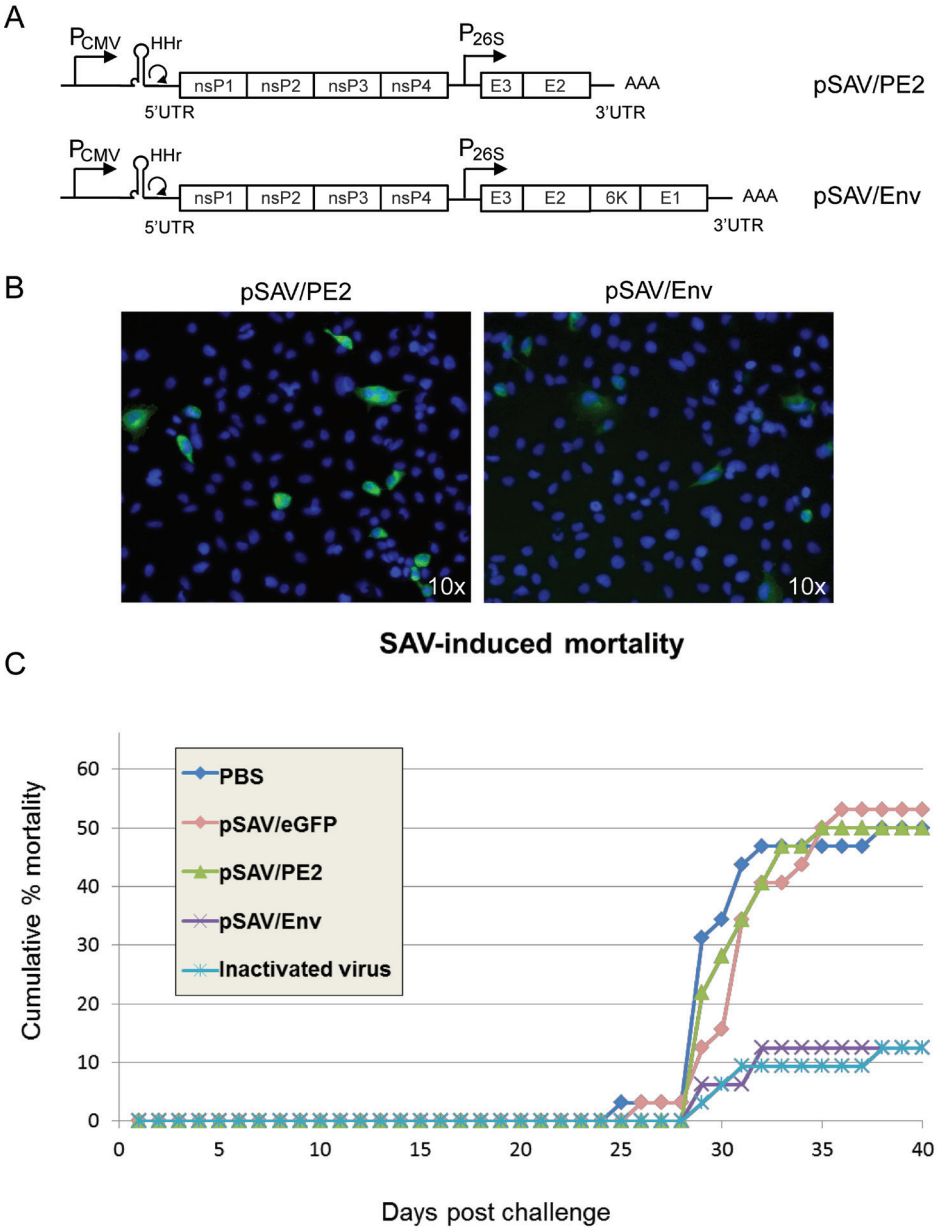


**Figure 2. Vaccination-challenge trial with DNA replicons expressing SAV structural proteins.** (A) Schematic representation of the replicons used for vaccination. (B) Transfection of CHSE-214 cells with the SAV-replicons pSAV/PE2 and pSAV/Env show expression of the E2 glycoprotein, following incubation for 4 days at 15°C in CHSE-214 cells. Pictures were taken at 10 × magnification. (C) Mortality curves of immunized Atlantic salmon exposed to cohabitant SAV challenge 6 weeks post vaccination. Cumulative mortalities groups immunized intramuscularly with SAV-based replicons expressing the PE2 and Env and control groups PBS and mock-replicon expressing the eGFP, a group immunized intraperitoneally with inactivated SAV vaccine. The experiment was terminated at day 40 post challenge, when the mock-vaccinated control group plateaued at a cumulative mortality of 50%.

of a larger polyprotein (e.g. E3E26KE1) to ensure proper processing and presentation to the salmon's immune system.

### 3.3 Classical DNA vaccination provides superior protection over replicon DNA vaccination

DNA vaccination with a SAV replicon encoding Env was successful and therefore, to further improve vaccine efficacy, the SAV capsid sequence was cloned in the same DNA plasmid. This was done to deliver the capsid protein *in trans*, which could promote spread of the replicon to neighbouring cells. Vaccination with this DNA plasmid was expected to result in the formation of VRP *in vivo*. Both the replicon (pSAV/Env) and VRP (pVRP) DNA plasmids were used to transfect CHSE-214 cells and used in an immunofluorescence assay to confirm E1 and E2 antigen expression (Fig. 3A). As a negative control for E1 and E2 mAb binding, pMAXeGFP was included. The negative control did not show antigen expression as expected (Fig. 3B, 1st panel), whereas both DNA replicon plasmids did show antigen expression (Fig. 3B, 2nd and 3rd panel).

These plasmids were then used in vaccination trial #2. As a positive control, a DNA

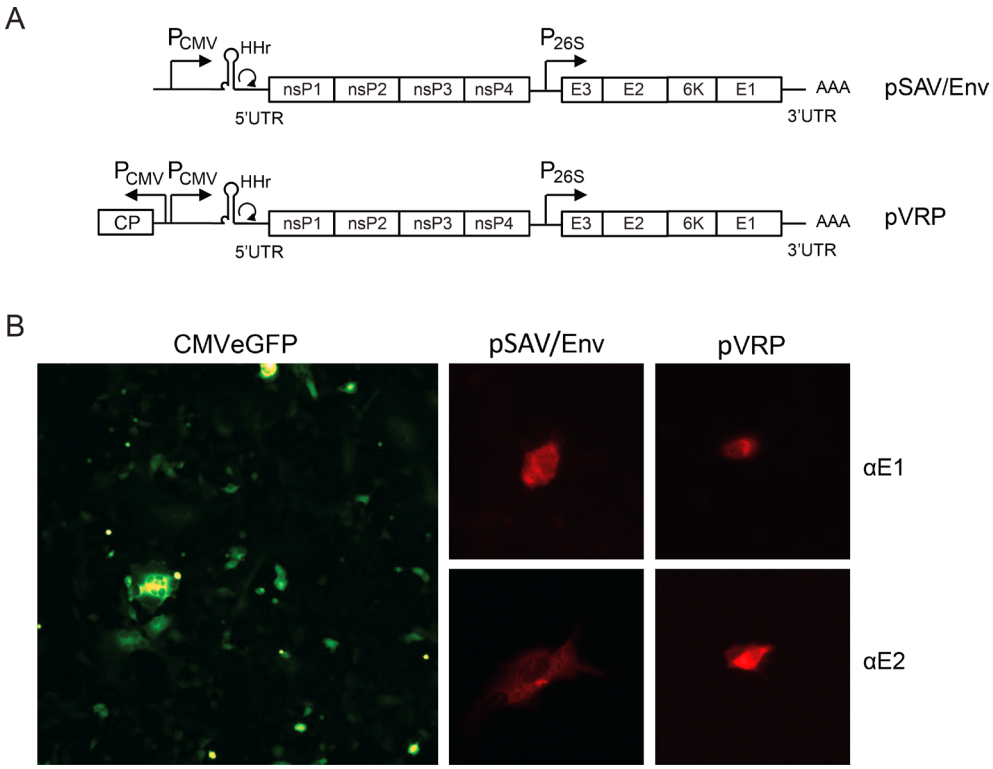


**Figure 3. *In vitro* validation of DNA replicons expressing SAV structural proteins.** (A) Schematic representation of the DNA replicons used for vaccination. (B) Transfection of CHSE-214 cells with the SAV-replicons pSAV/Env and pVRP show expression of the E1 and E2 glycoprotein, following incubation at 15°C in CHSE-214 cells. Pictures were taken at 20 × magnification.

plasmid expressing all SAV structural proteins was included (Simard and Horne, 2014). As a negative control, one group was injected with a saline solution (Table 3). At 6 wpv, all groups were exposed to SAV by cohabitation challenge. In contrast to vaccine trial #1, in vaccine trial #2, none of the fish groups showed mortality following SAV challenge, including the negative control groups, pSAV/eGFP and saline. Despite the lack of mortality, RT-PCR analysis did show high SAV prevalence in sera collected from the negative control groups as expected. Vaccination with the previously tested vaccine pSAV/Env again resulted in significant protection (RPP=41%, Fisher's exact test, $p<0.05$) and confirmed the vaccine potential of this DNA-launched replicon vaccine. However, the vaccine candidate pVRP showed SAV viremia in 70% of the tested fish, which did not significantly differ from the negative saline control group (85% SAV positive). This result was unexpected, because the plasmid pVRP contains an identical pSAV/Env replicon, with the only addition being CMV-controlled expression of the capsid protein *in trans.*

**Table 3. Vaccine trial (2) experimental design and outcome.**

| Group | Dose and route | Prevalence of SAV viremia 3wpc | RPP (%) |
|---|---|---|---|
| pSAV/eGFP | 10 µg/50 µl, i.m. | 16/20 | 5.9 |
| pSAV/Env | 10 µg/50 µl, i.m. | 10/20 | 41.2 |
| pS-VRP | 10 µg/50 µl, i.m. | 14/20 | 17.6 |
| pSAV SP | 10 µg/50 µl, i.m. | 2/20 | 88.2 |
| PBS | 50 µl, i.m. | 17/20 | 0 |

RPP = relative percentage protection = {1-(% PCR positive fish in vaccinated group/% PCR positive fish in unvaccinated group) × 100}. wpc = weeks post infection). i.m. = intramuscular

Previous research had shown that VRP vaccine candidates provided better protection than replicon DNA vaccines and some VRP vaccinations resulted in complete protective immunity (Chapter 6). The capsid protein might have influenced the negative outcome, as it, similar to some related alphaviruses, may inhibit cell proliferation (Karlsen et al., 2010), However, since capsid is also expressed in the positive control group pSAV SP, this cytopathic effect is unlikely to be the cause of decreased vaccine efficacy. Second, the presence of two CMV promoters adjacent to each other might have led to interference of both promoters, thereby leading to decreased transcription of both the

capsid protein and viral replicon RNA. However, this interference is more often seen in unidirectional promoters, rather than in bidirectional (Curtin et al., 2008). Therefore, the pVRP plasmid strategy needs further optimization. Possible improvements might include enhancement of capsid expression via a self-replicating RNA (Bredenbeek et al., 1993), and the generation of VRP *in vitro* rather than generation *in vivo*. Vaccination with *in vitro* generated VRP may lead to direct release of viral replicon RNA in the cytoplasm, avoiding initial transcription start in the cell nucleus. Finally, to enhance antigen expression from the DNA replicon constructs, the 5' end of the capsid protein gene might be inserted upstream of the envelope cassette to boost expression levels (Guo et al., 2015; Sjoberg et al., 1994).

Surprisingly, where classical DNA vaccination often only provides partial protection, especially in larger terrestrial mammals, and replicon/VRP vaccination shows better potential (Chapter 6), in the current trial the positive control plasmid pSAV SP showed superior protection (RPP=88%) over pSAV/Env (RPP=41%) (Table 3, last column). Possibly, the combination of bacteria-derived unmethylated CpG dinucleotide motifs, and subsequent fast and high amounts of viral antigen production, are more efficient than the stimulation by viral RNA replication in induction of immunity. However, this cannot be said with certainty, as immunogenic profiles in the vaccinated fish were not examined.

DNA vaccination of salmon has shown mixed results for both transient and replicon vaccines. Similar to our vaccine trial #2, a SAV replicon expressing the full structural polyprotein of infectious pancreatic necrosis virus (IPNV), showed modest protection (Abdullah et al., 2015), while a DNA vaccine expressing the same structural proteins showed better results (Mikalsen et al., 2004). Vaccination using the same SAV replicon, expressing the HE protein from infectious salmon anaemia virus (ISAV) showed protection levels similar to vaccination with inactivated virus, as was seen in the initial vaccine trial described here (trial #1). DNA vaccination with a plasmid expressing the same ISAV HE protein, however, only resulted in modest protection. These results show that vaccination of salmonids against viral infections is not straightforward and no general statements about comparative efficacy of DNA versus replicon vaccines can be made at this point.

## 4. CONCLUSIONS

In this study we showed that SAV glycoprotein E2 surface translocation in CHSE-214 salmon cells only occurs in the presence of E1. The importance of E1-dependent cell surface translocation was shown by a DNA-launched replicon vaccine expressing both glycoproteins, which induced a protective immune response against SAV challenge, similar to a SAV-inactivated antigen vaccine. DNA-launched expression of VRP did not yet improve DNA replicon vaccine efficacy, and a classical DNA vaccine transiently

expressing the SAV structural proteins was found to provide superior protection over both DNA-launched replicon vaccines. These studies further define the prerequisites for induction of a potent immune response in Atlantic salmon by DNA-launched (replicon) vaccination against SAV, identify two promising DNA (replicon) vaccine candidates, and may have implications for future vaccine design and production.

## ACKNOWLEDGEMENTS

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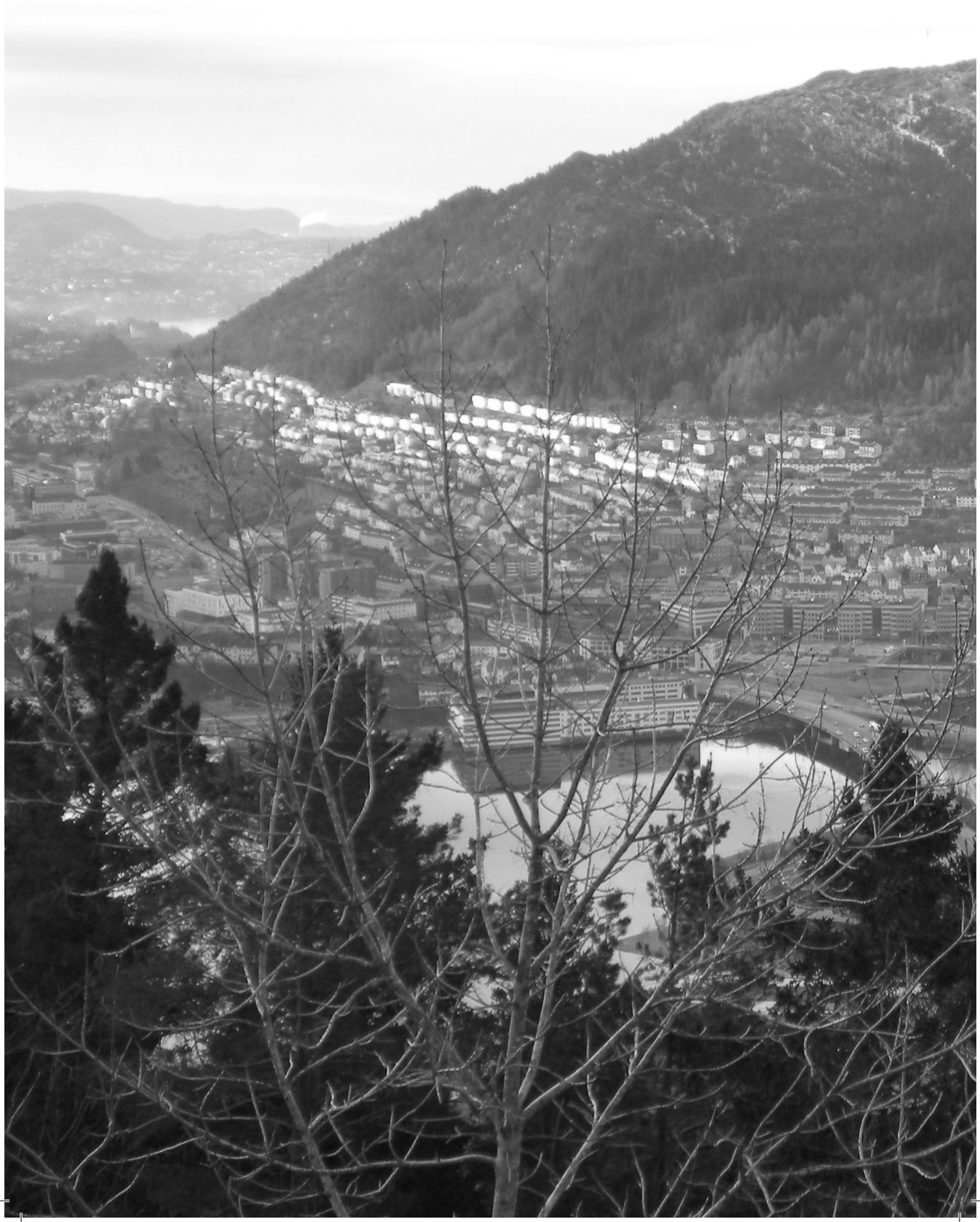

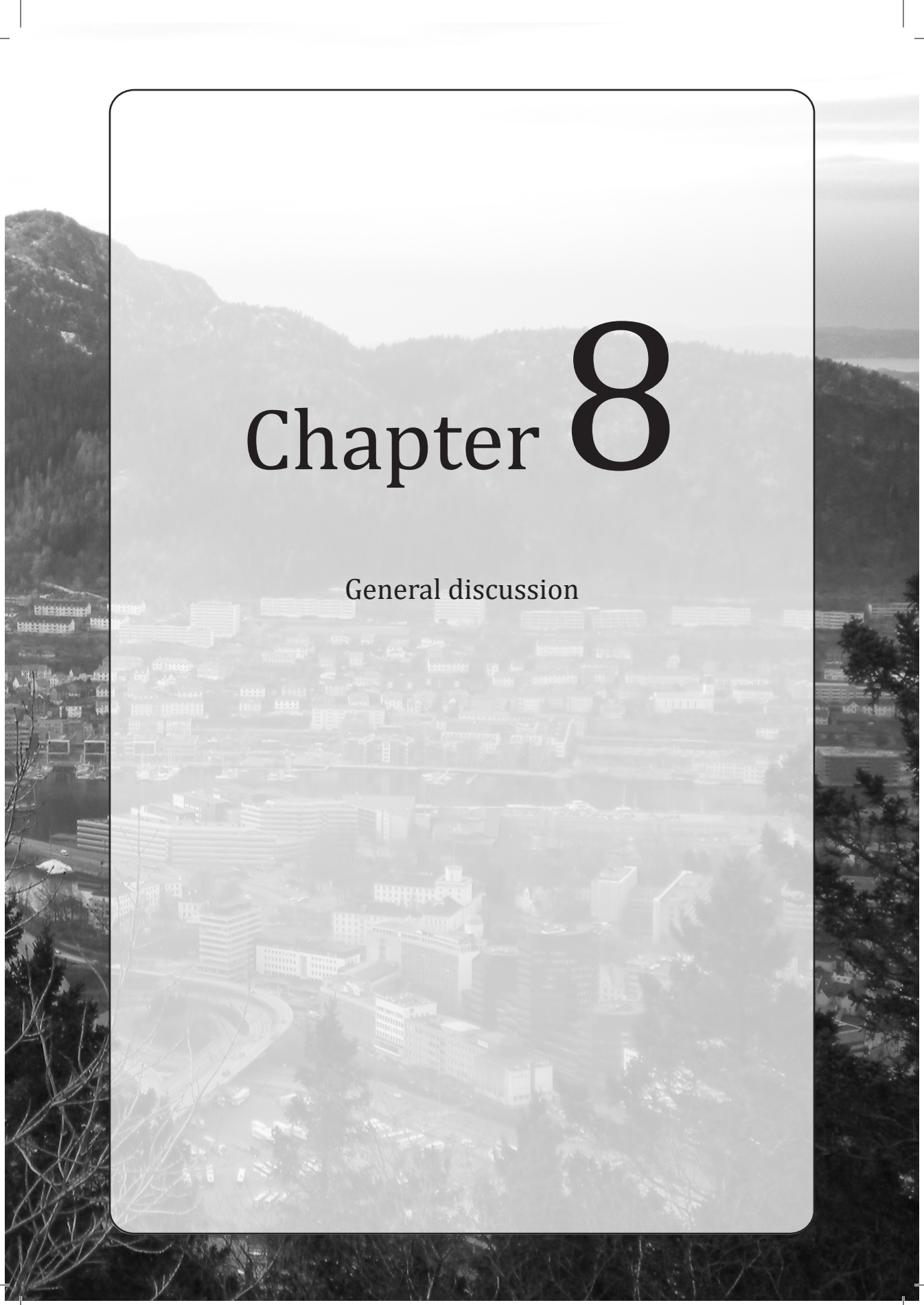

# Chapter 8

General discussion

# 1. INTRODUCTION

The growing demand for seafood makes aquaculture indispensable to provide sufficient supplies for future generations. The two most extensively cultured marine- and fresh water species, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), respectively, are of major economic importance. The viral agent salmonid alphavirus (SAV), poses a major burden on aquafarming of these species, and therefore, these fish are currently vaccinated with an inactivated virus vaccine to reduce the negative effects of natural SAV infection. The vaccine, albeit relatively efficacious and cross protective against multiple SAV subtypes, leaves room for improvement. Therefore, the work described in this thesis aimed to increase the general knowledge on SAV and current vaccine technologies, and to use this knowledge in the design of next-generation vaccines against SAV. In this synthesis chapter, the main results will be interconnected and discussed, and future perspectives are given.

# 2. SAV: AN ATYPICAL ALPHAVIRUS?

## 2.1 SAV host range

To develop new vaccines, it is important to understand the intrinsic properties of the virus and its interaction with the host. SAV was described earlier as an 'atypical' alphavirus (Villoing et al., 2000). Despite a similar genome organization and the same conserved functional regions as the terrestrial alphaviruses, there are some major differences. One of the most prominent differences between SAV and other alphaviruses is the lack of an invertebrate vector for virus transmission. Apart from Eilat virus, the only invertebrate-restricted alphavirus known to date (Nasar et al., 2012), all others are transmitted via invertebrate vectors, typically mosquitoes (Strauss and Strauss, 1994). SAV, however, can be horizontally transmitted, as shown by cohabitation-challenge experiments (Graham et al., 2011). Recently, it was shown for the first time, that also terrestrial alphaviruses may be horizontally transmitted, as infectious CHIKV was present in the saliva of mice, monkeys and humans following CHIKV infection (Gardner et al., 2015) and this may contribute to virus transmission (Rolph et al., 2015).

*In vitro* experiments showed that SAV can readily infect mosquito cells, without alterations (mutations) to the viral genome. Furthermore, the mosquito cell-derived SAV was able to re-infect salmonid cells (Chapter 2). This may suggest that SAV is an arbovirus, although a competent (invertebrate) vector in the aquatic ecosystem has not yet been identified. In addition to mosquito cells, SAV RNA replication was also observed in invertebrate *Sf* cell lines and in mammalian baby hamster kidney (BHK) cells (Olsen et al., 2013), which demonstrates that its RNA replication machinery is functional in cell lines derived from completely different hosts. Glycoprotein folding in insect cells is very similar to that of mammalian cells and therefore insect cells are widely used in vaccine

production. However, glycosylation in insect cells lacks sialylation, the final addition of sialic acid to the glycan (Marchal et al., 2001). Lack of sialic acid on mosquito-derived virions however, does not seem to affect the infectivity and pathogenicity of terrestrial alphaviruses (Stollar et al., 1976). Also the fish rhabdoviruses IHNV and VHSV, which have an envelope with glycoproteins, could propagate on mosquito and *Sf*9 cells, respectively, and virus grown on these cells remained pathogenic to trout (Lorenzen and Olesen, 1995). These observations, respectively, show that SAV is actually not so dissimilar to its terrestrial relatives with respect to host range, and this strengthens the idea that invertebrate cell lines may be used as an alternative virus/vaccine production cell line. The low vaccine efficacy of nanoparticle vaccines produced in insect cells observed in Chapter 5, is therefore more likely to be a result of fish immune responses against the specific type of vaccine, rather than the cell type in which the vaccines were produced.

### 2.2 Phylogenetic placement of SAV

Phylogenetic sequence analysis of SAV and other alphaviruses places SAV in a highly divergent clade, branching directly at the base of a rooted phylogenetic tree (Powers et al., 2001). Hypotheses are, that alphaviruses have a marine (Forrester et al., 2012) or an invertebrate origin (Ventoso, 2012), and the research described in this thesis provides further support to both these hypotheses. Apart from studying alphavirus evolution based upon genome sequence homologies, intrinsic and extrinsic properties of the virus may also be considered to interpret alphavirus evolution. For example, the N-terminal region of the viral capsid protein is responsible for the inhibition of cellular proliferation following SAV infection (Karlsen et al., 2010). Transcriptional shut-off by New World and Old World alphaviruses is allocated to the capsid protein and the non-structural protein nsP2, respectively, which makes SAV more similar to New World alphaviruses in this aspect (Garmashova et al., 2007; Powers et al., 2001). Furthermore, SAV infections are associated with the inability of fish to maintain their position in the water, which is attributed to lesions in red muscle tissue. However, since New World and Old World alphaviruses are in general associated with encephalitic and arthritic disease, respectively, it would be interesting to see if SAV infection, besides the known aetiology, also affects salmonid joints. If so, this would place SAV more closely to the arthritic alphaviruses, such as CHIKV. Finally, SAV lacks the commonly present leaky stop codon between nsP3 and nsP4 (Hodneland et al., 2005). The absence of this stop codon was also found in several (quasi species of) alphaviruses and was suggested to be related to the efficiency of the virus to replicate in either their mosquito or mammalian host. Presence of both quasi species in a virus isolate, suggests that this is an adaptation of the virus to the current host (Hwang Kim et al., 2004; Myles et al., 2006). Presence of the stop-codon was related to increased O'Nyong-Nyong virus (ONNV) infectivity of mosquitoes (Myles et al., 2006) and multiple passages of an ONNV strain on mammalian

cells resulted in replacement of the stop codon by an arginine (Lanciotti et al., 1998). It would be interesting to see what consequences the insertion of this opal stop codon into the SAV sequence would have, and it may even increase replication efficacy (speed) in mosquito cells.

### 2.3 Glycoprotein trafficking as function of temperature and E2-E1 interaction

Before the onset of this research, it was known that SAV could not efficiently be propagated at temperatures above 15°C. This growth constraint was not caused by instability of the virion, as virions were still infectious following incubation at 37°C (Villoing et al., 2000). It was hypothesized that the cause for low-temperature dependency must be sought intracellularly. The low-temperature dependent replication is not defined by the susceptible cell line, as replication of SAV on mosquito cells was also only observed at low temperature (15°C) and not at 18°C (Chapter 2). By the use of a synthetically designed SAV (pFISH), in Chapter 4, we indeed confirmed that SAV RNA is able to replicate at 20°C in CHSE-214 cells, but that viral spread at this temperature is halted (Chapter 4, Fig. 1). This suggested that the temperature restriction is most likely associated with the SAV structural proteins. Further analysis of the individual SAV structural subunits and envelope cassette showed that at higher temperatures, the PE2 protein did not appear at the cell surface, but is retained in the ER, most likely due to misfolding of the PE2 protein. Even at the permissive temperature of 15°C, in the absence of glycoprotein E1, PE2 remains in the ER. Because it is generally accepted that alphavirus E1 and E2 travel together to the cell surface as heterodimers (Fig. 1), where E2 protects E1 from premature fusion (Kielian, 2010), these results are not completely unexpected. However, the ability of glycoprotein subunits of different alphaviruses to translocate to

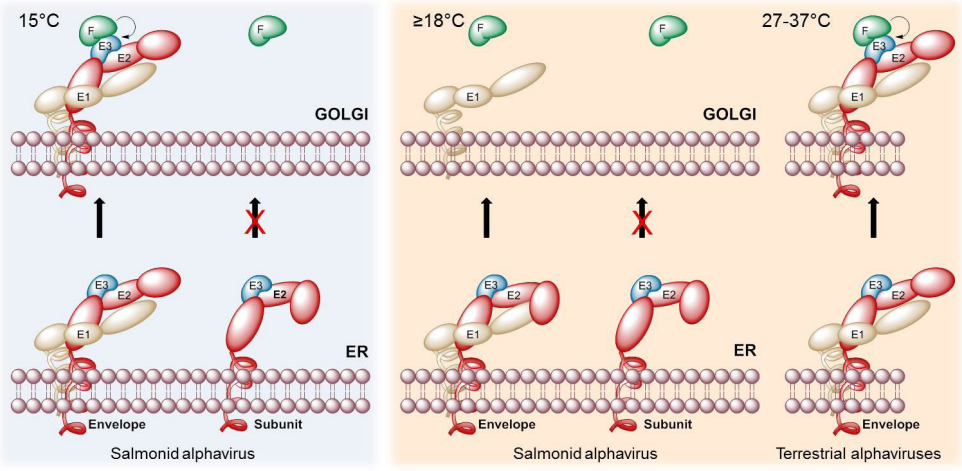


**Figure 1. Schematic presentation of the glycoprotein trafficking profile of salmonid alphavirus and the terrestrial alphaviruses.**

the cell surface greatly varies and does not follow a common rule. For example, CHIKV subunits were both able to travel to the cell surface independently resulting in fusogenic E1, when expressed in *Sf*21 insect cells (Metz et al., 2011b). On the other hand, the Semliki Forest virus (SFV) PE2 subunit could travel to the cell surface alone, although less efficiently, in the absence of SFV E1, yet the SFV E1 protein could not be detected when expressed alone (Barth and Garoff, 1997). Studies with Sindbis virus (SINV) show variable results. Some reports state that expression of E1 alone is insufficient for surface relocation of glycoprotein E1 to the cell surface (Carleton et al., 1997; Frolov and Schlesinger, 1994), whereas others showed that a signal in the carboxy-terminal of 6K is required for correct translocation of E1 into the ER lumen and relocation of E1 to the cell surface, a process which was independent of E2 (Migliaccio et al., 1989). Quite similarly to the latter report, in our experiments the SAV E1 glycoprotein was still able to travel to the cell surface independent of heterodimerization with E2 (Chapter 4, Fig. 2D).

These apparent discrepancies have not yet been resolved, but differences in the ability of alphavirus glycoproteins to appear at the cell surface might result from the differences in pH thresholds for E1 fusion. As the *trans*-Golgi network has a slightly acidic pH (~6.0) (Martín et al., 2009), some E1 proteins might, in the absence of protection by E2, start fusion before they appear at the cell surface. This is supported by the observation of an increase in intracellular vacuoles upon expression of SINV E1, thus in the absence of E2 (Sanz et al., 2003), and the ability of solely the E1 ectodomain to oligomerize and associate with artificial liposomes upon low-pH induction (Klimjack et al., 1994). The fact that the fusion threshold of SINV was determined to be around pH 6.0 (Smit et al., 1999), but more specifically pH 5.6 for the TOTO 1102 SINV strain (Glomb-Reinmund and Kielian, 1998) used by Sanz et al., (2003) might explain the differences in the efficiency of SINV E1 surface localization. Thus, low-pH fusion threshold may correlate with surface localization of E1 in absence of E2. Indeed, the pH threshold of SFV was found to be 6.2 (Glomb-Reinmund and Kielian, 1998; Qin et al., 2009), possibly explaining why E1 did not appear at the cell surface, as fusion might have occurred earlier in the secretory pathway. The pH threshold for SAV E1 fusion is expected to be below 6.0 (5.0~5.4) (Villoing et al., 2000), which might explain why these E1 proteins can freely travel to the cell surface without undergoing premature fusion.

Effective glycoprotein trafficking is of major importance for the generation of new (infectious) virus particles. The ability of arthropod-borne viruses to cope with different temperatures within both their vertebrate and invertebrate host, is therefore a fascinating feature, as the permissive temperature range of SAV is clearly a lot smaller. Knowledge on the ability or inability of individual glycoproteins to correctly fold and pass the secretory pathway finds its application in the generation of new vaccines, where correct conformation and secretory pathway trafficking may be essential for antigen purification, or may determine the vaccine efficacy.

## 3. FISH IMMUNITY AND VACCINES

### 3.1 The immune system of fish

The immune system in teleost fish is somewhat different from mammals, although many types of mammalian immune responses find their homologues in teleost fish (Jørgensen, 2014). Like in mammals, antigen-presenting cells (APCs) recognize pathogen associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) on their cell surface (Munang'andu and Evensen, 2015). Besides direct elimination of antigens, fish APCs are capable of antigen processing and presentation, leading to potent adaptive immune responses (Jørgensen, 2014). It is interesting to note that immunoglobulin (Ig) types have been identified in zebrafish (IgZ), carp (IgZ), fugu (IgT), Atlantic salmon (IgT) and rainbow trout (IgT) that are not present in mammals (Danilova et al., 2005; Hansen et al., 2005; Savan et al., 2005; Zhang et al., 2011). Salmonid IgT was shown to be specialized in mucosal immunity (Tadiso et al., 2011; Zhang et al., 2011).

Vaccination depends on the generation of an immune response that leads to establishment of memory cells that are effective in elimination of a foreign pathogen upon infection. The ability of vaccines to provoke protective immunity highly depends on the type of vaccine. The vaccine modality determines which immune pathways are triggered, and are roughly divided into an endogenous and an exogenous antigen delivery pathway (recently reviewed by (Munang'andu and Evensen, 2015). In our studies, nanoparticles (eVLPs, CLPs; exogenous) and DNA-launched replicon vaccines (endogenous) are compared to whole inactivated virus (exogenous) and classical DNA vaccines (endogenous), and all are expected to induce a different array of immune responses.

### 3.2 Natural SAV infections and live attenuated virus vaccines

Pathogenic SAV live virus and live-attenuated vaccines are both able to enter the host cell and start their viral replication cycle. Following viral replication, RIG-I like receptors (RLR) in the cytoplasm sense viral RNA and are activated. RLRs have been identified in rainbow trout and displayed antiviral functions (Chang et al., 2011). Subsequent cytokine signalling results in induction of an antiviral response, which is generally associated with interferon (IFN) production. Early involvement of IFN stimulated genes and adaptive immune responses was noted following infection of Atlantic salmon with SAV (Gahlawat et al., 2009; Herath et al., 2012; Xu et al., 2010) and ISAV (Røkenes et al., 2007), and specific protection is mediated by neutralizing antibodies (Desvignes et al., 2002; McLoughlin et al., 1996). The large contribution of neutralizing antibodies in clearance of infection was shown by transfer of serum retrieved from SAV-recovered salmon to naïve salmon, which were then protected against SAV infection (Houghton and Ellis, 1996). Salmon that have recovered from SAV infections are subsequently protected against secondary SAV infections. A live-attenuated virus vaccine is expected

to provoke similar immune responses, and has been developed against SAV infections by Moriette et al. (2006). However, even though live attenuated vaccines are very effective in generating a potent immune response and they can be administered via immersion, safety issues (i.e. potential reversion to virulence) for the environment will probably prevent this vaccine type to become widely used.

### 3.3 Inactivated virus vaccines

The current SAV vaccine consists of chemically inactivated virus particles. Since these particles do not fuse with the host cell and do not replicate, the vaccine is expected to follow the exogenous antigen delivery route. Herein, exogenous antigens are taken up by APC into endosomes, processed and presented on MHC class II molecules, which are then recognized by naïve T cells. Subsequently, a humoral antibody response is triggered, wherein B cells are stimulated to produce and secrete antibodies and generate memory B cells (Abbas et al., 2007; Haugland et al., 2005; Munang'andu and Evensen, 2015). However, an inactivated virus vaccine also contains viral ssRNA, which may serve as a PAMP for recognition by PRR in the cell (Pietretti and Wiegertjes, 2014). 'Sensing' viral RNA may therefore lead to an MHC class I mediated antiviral response by cytotoxic T cells (CTLs) (Forlenza et al., 2008) in addition to the MHC class II primed antibody response. Inactivated virus vaccines have shown to be effective in generation of significant protection against multiple fish viruses (reviewed by Munang'andu and Evensen, 2015).

### 3.4 Subunit and VLP vaccines

From the nanoparticle vaccination trial described in Chapter 5, it followed that vaccination of Atlantic salmon with CLP and eVLP vaccines did not confer protection against SAV challenge. Surprisingly, in a study by another group, bacterial derived E2 and E1 subunits did provide significant protection against SAV infection (Xu et al., 2012), which is not in line with the outcome of our vaccination trial. Possibly the delivery of the bacterial subunits in inclusion bodies aided in improved uptake by antigen presenting cells, although eVLP and CLP conformation should have similar positive uptake effects (Noad and Roy, 2003). Subunit and VLP(-like) vaccines, like the CLP and eVLP vaccines developed in our studies (Chapters 3 and 5), are expected to follow the exogenous antigen delivery route and largely depend on the generation of a humoral immune response for protective immunity. Similar to inactivated virus, they are taken up by APC, presented on MHC class II molecules and thereby induce amongst others an humoral immune response (Munang'andu and Evensen, 2015). In the study by Xu et al. (2012), fish were i.p. vaccinated with the individual subunits according to a prime/booster regime, where at 6 weeks post vaccination (wpv), a booster vaccine was administered, followed by SAV challenge at 3 weeks post-boost vaccination. In our studies, no booster vaccination was given and fish were challenged at 6 wpv. Also, insect-derived glycoproteins are

likely more similar to those expressed in fish cells than bacteria-derived subunits. In this comparison, however, it may be that activation of a different array of PRRs upon sensing of bacterial cell components is more important than the folding or glycosylation profile of the antigens for induction of protective immunity. Viral antigens produced in bacteria might still contain some remaining bacterial constituents (e.g. LPS), which stimulate the production of pro-inflammatory cytokines. Similar to our studies, VLPs of piscine myocarditis virus (PMCV) and infectious pancreatic necrosis virus (IPNV) were produced by the baculovirus insect cell expression system and both vaccines failed to confer protection against SAV challenge in Atlantic salmon (Nilson et al., 2014; Shivappa et al., 2005). However, a subunit vaccine of the IPNV VP2 protein, added to a multivalent vaccine containing bacterial components, showed a decrease in viral titers post-challenge (Frost and Ness, 1997). Also, adjuvanted vaccination with whole insect cells, infected with recombinant baculovirus expressing the VHSV G protein resulted in modest protection against VHSV (Lecocq-Xhonneux et al., 1994) and baculovirus/insect cell-produced VLPs have been produced for viral nervous necrosis virus (VNNV) and found to induce protective immunity against its pathologic agent in the phylogenetically distant European sea bass, *Dicentrarchus labrax* (Thiéry et al., 2006). Thus, the protective outcome of subunit vaccines seems to differ per virus and/or fish species. Our vaccination studies with eVLPs and CLPs are in line with the overall outcome, that exogenous delivered subunit and VLP vaccines are in general less potent than those that in addition to MHC class II, also stimulate MHC class I mediated immunity (Munang'andu and Evensen, 2015). Whole inactivated virus vaccines in general have good vaccine efficacy and if the structure is compared with an eVLP vaccine, this suggests a role for the presence of viral RNA in synergistic induction of protective immunity.

### 3.5 Nucleic acid vaccines

DNA vaccination with a plasmid encoding the complete SAV structural polyprotein (pSAV SP) results in efficient protection upon SAV challenge. In the study by Xu et al. (2012), DNA vaccination with either SAV E2 or E1 subunits did not lead to protection. These authors hypothesized that the absence of the capsid protein might be the reason for absence of immunity. The E2 and E1 glycoproteins were expressed from the DNA construct without E3 and 6K, their respective ER localization signals, which might explain the lack of protective immune responses (Xu et al., 2012). However, data from Chapter 4 and 7 indicate that expression of both glycoproteins is necessary for induction of protective immunity, so it is expected that even correct orientation in the ER membrane will not rescue protective abilities of a DNA vaccine expressing either the E1 or E2 subunit, but that expression of minimal the complete envelope cassette is necessary. This is also in agreement with the inability of DNA vaccine expression of IHNV VP2 to confer protection, whereas DNA-based expression of the complete segment A of IHNV did provide protection (Mikalsen et al., 2004). However, even if the requirements

for correct antigen exposure are unravelled, still no general statements can be made about the efficacy DNA vaccination of salmonids. A commercialized DNA vaccine protective against the rhabdovirus infectious haematopoietic necrosis virus (IHNV) has successfully been used in Canada since 2005 (Apex-IHN, Aqua Health Ltd) (Garver et al., 2005) and a DNA vaccine against a related rhabodovirus, viral haemorrhagic septicaemia virus (VHSV) (Lorenzen et al., 2009; Lorenzen et al., 1998), also showed to be very effective. However, DNA vaccination against another rhabdovirus, spring viremia of carp virus (SVCV), showed mixed results (Emmenegger and Kurath, 2008; Kanellos et al., 2006).

DNA and DNA-launched replicon vaccines deliver the viral antigen via the endogenous antigen delivery route (Munang'andu and Evensen, 2015). This is expected to result in MHC class I regulated cell mediated immunity, primarily by CTL responses. Both plasmids are made in bacteria, and therefore also contain non-methylated CpG motifs (XCGY, where X is any base but C and Y is any base but G). In vertebrates, DNA is routinely methylated at the 5' end position of the cytosines (Krieg, 2002) and absence of this methylation possibly activates an endosomal PRR, TLR-9 (Pietretti and Wiegertjes, 2014), which in turn leads to fast induction of IFNs (Abbas et al., 2014). Indeed, CpG DNA has been shown to induce an antiviral response and to bind TLR-9 in Atlantic salmon (Iliev et al., 2013; Jørgensen et al., 2003). Also, DNA vaccines have been shown to attract both B and T cells to the site of antigen expression, and evoke both humoral and cellular mediated immune responses (Castro et al., 2014). Thus, besides endogenous production of viral antigen, induction of TLR-9 may be of importance for vaccine efficacy. In order to investigate this experimentally, pSAV SP and pSAV/Env DNA plasmids can be treated with CpG methylase to examine whether CpG methylation leads to loss of protective immunity. This may indicate that there is an essential role for activation of TLR-9, or that solely endogenous expression of the viral antigen is sufficient for the establishment of a protective immune response (Klinman et al., 1997).

In Chapter 6 it was concluded that DNA-launched replicons are likely to be more potent vaccines than transient DNA vaccines, as they, besides bacterial dinucleotide CpG motifs and endogenous viral antigen expression, also induce active RNA replication, and thereby trigger additional immune responses like activation of RLRs. It was therefore unexpected that pSAV SP was more potent in induction of immunity than the replicon vaccine and VRP vaccine. However, no general statements about comparative efficacy of DNA vaccines and replicons in salmonids could be made (Chapter 7). It would be interesting to see if co-injection of pSAV SP and pSAV/Env DNA plasmids would provide superior protection over vaccination with pSAV SP DNA plasmid alone. We could conclude though that, at least for endogenous antigen expression (e.g. DNA and replicon vaccination), co-expression of both glycoproteins and cell surface display is needed for induction of protective immunity. This is supported by the study of Acosta et al. (2006), in which transfection of a rainbow trout gonad cell line with a plasmid

encoding the VHVG G protein, and subsequent intracellular arrest of the G protein, led to a decrease in the antiviral response, as shown by a decrease in type I IFN expression in neighbouring cells.

Long-term antigen expression has been observed post-DNA vaccination, yet studies in mice showed that 95-99% of the plasmid DNA is already degraded within the first 90 minutes (Tonheim et al., 2008). In addition, due to the negative charge of the DNA, it is more difficult for larger plasmids to enter host cells and the cell nucleus (Hølvold et al., 2014). Direct delivery of the replicon in the form of ssRNA into the cytoplasm, by e.g. delivery of 'naked' RNA, would circumvent the need for a nuclear transcription start and directly stimulate an antiviral state by induction of RLR in the cytoplasm, followed by a CTL response caused by intracellular antigen expression (Chang et al., 2011). *In vitro* generated VRP would in addition provoke APC to phagocytose viral antigen and present those on their MHC class II. Also, these would bypass the difficult initial uptake of negatively charged DNA plasmids into cells (Tonheim et al., 2008).

### 3.6 Ideal vaccine components

The above described research suggests that multiple and synergistic responses may be key for the establishment of protective immunity in salmonids. Exogenous delivered vaccines can be subunit vaccines, produced by bacteria or with an adjuvant inducing similar immune responses, or inactivated whole virus, live attenuated, or VRP vaccine, containing an 'RNA danger signal'. Endogenous expressed viral antigens need correct cell surface expression for induction of immunity and depending on the virus, endogenous expression of the major antigen on its own is sufficient, or it might need co-expression with its 'protein partners'. In addition it needs a second trigger like unmethylated CpG motifs or RNA replication. In short, to induce protective immunity, a trigger from 'inside, and from outside' the cell is needed, and synergistic responses will finally lead to both induction of MHC class I and class II regulated immune responses.

## 4. OTHER FACTORS THAT INFLUENCE VACCINE POTENCY

### 4.1 Fish age

Besides the stimulation of correct immune responses in the fish, several other factors influence the vaccination outcome. The age of the fish has been shown to play a role in vaccine efficacy, because while innate immunity is effective immediately after hatching, adaptive immunity needs time to mature (Castro et al., 2015). Rainbow trout and Atlantic salmon have been shown to be fully immunocompetent at 2-3 months and 5 grams of age, respectively (Lillehaug, 2014).

### 4.2 Environmental factors

The temperature of the water has been shown to influence the nature of immune

responses following infection and/or vaccination (Bly and Clem, 1992). IPNV infection in rainbow trout at 16°C resulted in a significant increase of IFNα secretion compared to those fish kept at 8°C and 12°C early post-infection, and clinical disease only developed in fish kept at 12°C (Arguedas Cortés et al., 2015). DNA vaccination against VHSV infection induced better long-lasting, non-specific immunity at 5°C than at 10°C and 15°C, while humoral immune responses were most prominent at 15°C (Lorenzen et al., 2009). Preferably vaccination is performed at a temperature at which the most long-lasting protective immunity is provoked, and sudden changes in temperature should be avoided, as this might compromise immunological responses (Roberts and Rodger, 2012).

The fish being kept at open water results in more factors influencing vaccine efficacy. Presence of multiple aquafarms in the same area influences the likelihood of a farm to become infected. Presence of parasitic sea lice has been shown to make salmonids more susceptible to viral infection and the density of salmonid farms plays a role in the occurrence of sea lice infestations (Jansen et al., 2012). Also, if a farmer decides to culture non-vaccinated salmon, the infection pressure increases for the surrounding farms. A higher infection pressure means that the vaccine used by the farmer should be more effective, i.e. must have a higher RPS value.

### 4.3 Vaccination method

Last but not least, the injection route has been shown to have major influence on the vaccine efficacy. For instance, intramuscular injection of a DNA-launched replicon vaccine protective against ISAV resulted in high protection, whereas intraperitoneal injection did not (Wolf et al., 2014). Whole inactivated virus vaccines are mostly intraperitoneally injected (Dhar et al., 2014). However, whether intramuscular or intraperitoneal injected, both manual and mechanic injection of fish is very labour intensive, and not possible for small fish. Therefore, ideally fish vaccines are administered via immersion or via the feed. Immersion vaccination has been shown effective against multiple pathogens, like NNV (Kai and Chi, 2008), *Yersinia ruckeri* (Chettri et al., 2013) and SAV (Moriette et al., 2006). Vaccination via oral intake has been shown effective for several antigens (Adelmann et al., 2008; Allnutt et al., 2007; Chen et al., 2015; Ghosh et al., 2015; Tobar et al., 2011) and DNA vaccines (summarized by Mutoloki et al., 2015). An oral vaccine, comprising an alginate microsphere encapsulated DNA plasmid expressing the VP2 protein of IPNV, was shown to be protective in rainbow trout (Ballesteros et al., 2015b; de las Heras et al., 2010). However, the same approach worked less efficient when a DNA vaccine expressing the IHNV G protein was orally administered. For induction of significant protection, 20 times more plasmid DNA was needed in comparison to intramuscularly injection vaccination (Ballesteros et al., 2015a). Oral vaccination against IPNV in rainbow trout has also been shown feasible when a DNA plasmid encoding VP2 was added to commercial food pellets (Ballesteros et al., 2014). Apparently, for DNA-

based VP2 vaccination against IPNV, oral intake may have some advantages in induction of immunity over intramuscular injection (Mikalsen et al., 2004). Uptake via food, however, cannot be controlled per individual fish. For Atlantic salmon, antigen uptake and distribution following oral intake has also been reported for IPNV inactivated virus antigen (Chen et al., 2015) and was shown to increase immune responses when used as booster vaccination (Chen et al., 2014). An oral vaccine for use in Atlantic salmon against IPNV is commercially available (Merck, AquaVac® IPN Oral). However, very few 'mucosal' vaccines are commercially available, most likely attributed to their variable efficacies (summarized by Rombout and Kiron, 2014). To the best of our knowledge, no oral (DNA) vaccine protective against SAV, however, has been tested in Atlantic salmon. Finally, as mentioned in Chapter 6, viral vectors may improve delivery of antigens by either antigen incorporation in the viral structure or encoded within the genomic material. For instance, recombinant baculovirus (Xue et al., 2013) and the frog virus *Rana grylio* virus (He et al., 2012) have been examined for potential use as viral vectors in fish.

## 5. FUTURE PERSPECTIVES AND CONCLUDING REMARKS

In fish vaccinology, nothing seems to be straightforward. Immune responses to an infection or a vaccination in fish vary amongst fish species, with fish age, with environmental conditions and are highly dependent on a correct interplay of all these

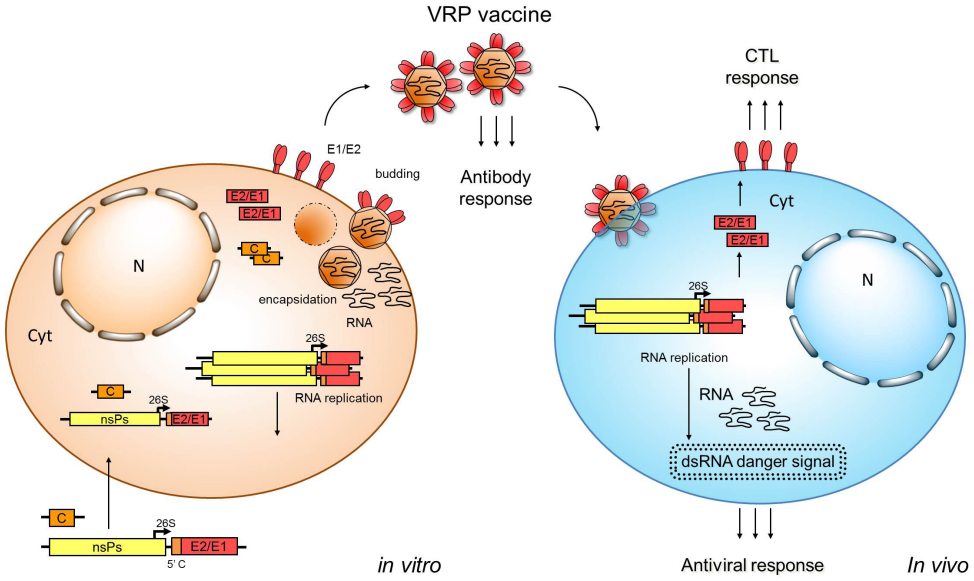


**Figure 2. Schematic overview of an example of a SAV VRP vaccine.** VRP are generated in vitro by separate introduction of the replicon construct (expressing the complete envelope cassette) and complementary structural proteins in a suitable cell system, preferably at ambient temperature. VRP can be isolated from the medium fraction and used for immersion and injection vaccination trials.

factors. Next-generation vaccines like subunits, nanoparticles, DNA vaccines and DNA-launched replicons against viral diseases work, albeit with variable efficacies. Next-generation SAV vaccines should provoke both cellular and humoral immune responses, preferably early post vaccination in concurrency with innate immunity. The developed DNA-launched replicons developed in this thesis would need further optimization in terms of antigen expression levels. Ideally, the replicon should be delivered by an in vitro generated VRP, as it is most similar to 'live' virus and would circumvent the drawbacks of plasmid vaccination (Fig. 2). However, efficient generation of VRP vaccines for salmonid alphavirus in cell culture has not yet been demonstrated. Thus, even though VRP can be administered via bath-immersion, the large quantities of particles needed for this vaccination method may be a limitation. A prime-boost regime, in which the mucosal immunity of young salmon is primed via bath immersion with a nanoparticle vaccine (e.g. VRP), or via oral vaccination with encapsulated plasmid DNA (expressing the viral antigen), may result in complete protection when a boost is given with the current inactivated virus vaccine. As salmonids recovered from SAV infection are no longer prone to get viral disease upon a second encounter, continuation of studies on immune responses following SAV infection may enhance our understanding on which immune responses to provoke upon vaccination.

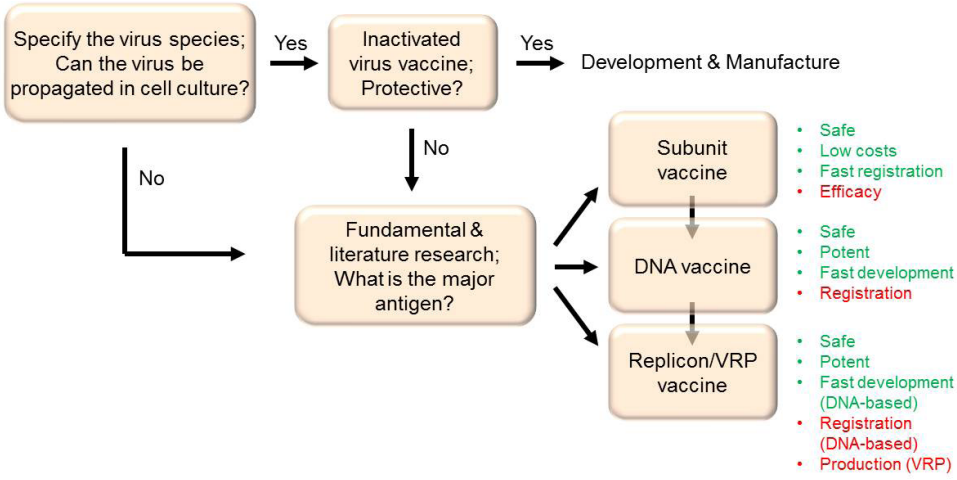


**Figure 3. Schematic flow diagram for the development of a new fish vaccine.** Advantages and disadvantages are indicated in green and red text colouring, respectively.

For most fish viruses, or for vaccination against pathogens in general, the obvious approach of vaccination with inactivated virus has been tested. However, the virus may not always be able to replicate in cell culture at sufficient levels, protection obtained may not be complete, and the application method may not be ideal. If the initial approach of vaccination with inactivated virus does not provide sufficient protection, fundamental research and literature study on the virus species may identify the major viral antigen(s).

This knowledge can be used to (concurrently) produce and test a subunit vaccine, DNA vaccine and replicon/VRP vaccine, each of which have advantages and disadvantages (Fig. 3). Further improvements can be made by prime-boost vaccination regimes, and by the use of adjuvants to extent the time of antigen release, and/or to induce synergistic MHC class I and MHC class II responses.

The work described in this thesis aimed to generate alternative vaccines to the current inactivated virus vaccine, protective against SAV infections. Important characteristics of this unique aquatic virus were further explored, which aided in the design of multiple 'next-generation' vaccine prototypes, and which were tested in SAV's natural host, Atlantic salmon. Besides confirmation of the high vaccine efficacy of a classical DNA vaccine, DNA-launched replicon vaccination has shown potential for further development. The research described in this thesis contributes to the development of next-generation vaccines in the challenging area of fish vaccinology.

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# Summary
# Samenvatting

## SUMMARY

The consumption of sea-food has increased over the last decades and is expected to do so in the upcoming years. For example, to maintain a healthy life-style, the Netherlands Nutrition Centre now recommends eating fish twice a week (Voedingscentrum, schijf van vijf). To prevent sea-food wild-catch to outcompete the carrying capacity of aquatic ecosystems, aquaculture has become indispensable to meet the future demands for aquaculture products. Despite good hygiene, a lot of pathogens still threaten the sea-food industry. Vaccination against pathogens is generally accepted and has substantially aided in the decrease of antibiotic use in aquaculture. The salmonid species Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* W.) are the most cultured fish species in cold-water marine and fresh water aquaculture, respectively (FEAP, 2014). One pathogen that causes a huge burden on aquaculture of both Atlantic salmon and rainbow trout is salmonid alphavirus (SAV). This virus is in many aspects different from its terrestrial family members and the viral 'life' cycle is not yet completely understood. Current vaccination against SAV is performed with an inactivated virus vaccine, which is efficacious and cross-protective against various SAV subtypes. The vaccine does not provide 100% protection and is cultured on a fish cell line at low temperature, which makes vaccine production time consuming. In addition, the vaccine needs to be intraperitoneally injected, which is labour intensive and cannot be carried out until the fish reach a specific size. Salmon undergo a 'smoltification' process, at which they are transferred from fresh-water to marine water habitats. This leaves a small time window for farmers to vaccinate their fish, and the young salmon are unprotected until they reach the desired size for vaccination. The work described in this thesis, aimed to increase the general knowledge on SAV and current vaccine technologies, and to use this knowledge in the design for next-generation vaccines.

SAV is a member of the genus *Alphavirus* (family *Togaviridae*), of which the viruses are generally transmitted between vertebrate hosts via blood-sucking arthropod vectors, typically mosquitoes. SAV is unique in this respect since it can be transmitted directly from fish to fish and has no known invertebrate vector. Interestingly, we showed that SAV is able to complete a full infectious cycle within arthropod cells derived from the Asian tiger mosquito *Aedes albopictus*. Progeny virus was produced in C6/36 and U4.4 cells in a temperature-dependent manner (at 15°C but not at 18°C), and could be serially passaged and remained infectious to salmonid CHSE-214 cells, without amino acid modifications. This suggested that SAV is not necessarily a vertebrate-restricted alphavirus and may have the potential to replicate in invertebrates, including an elusive aquatic vector. The study further showed the ability of SAV to be propagated to high titer in mosquito cells, which could possibly provide an alternative SAV production system for vaccine applications.

The current inactivated virus vaccine against SAV, and therefore also a similar vaccine

produced on a different cell line, is a classical first generation vaccine, and is produced at low temperature. A possible alternative to this inactivated virus vaccine would be a nanoparticle vaccine. Before the onset of this study, an enveloped virus-like particle (eVLP) prototype vaccine was developed, by expressing SAV structural proteins using recombinant baculoviral vectors in insect cells. These cells grow over a wide temperature range (12-30 °C). SAV eVLPs could only be obtained when the incubation temperature was lowered to below 18°C. However, the protein expression levels at low temperatures are lower then at 27°C. An eVLP structure with core, lipid membrane, surface glycoproteins may be more similar to the wild-type virus, but is more complex than that of non-enveloped, protein-only VLPs, which are structurally and morphologically 'simple', and therefore easier to produce. In order to develop an alternative to the eVLPs, we engineered recombinant baculovirus vectors to produce high levels of alphavirus core-like particles (CLPs) in insect cells by expression of the alphavirus capsid protein at 27°C. The CLPs localized in dense nuclear bodies within the infected cell nucleus and therefore could be purified through a rapid and scalable protocol involving cell lysis, sonication and low-speed centrifugation steps. Furthermore, an immunogenic epitope from the SAV E2 glycoprotein, the E2 B-domain, could be successfully fused to the N-terminus of the capsid protein without disrupting the CLP self-assembling properties. This approach can be in principle expanded to multiple alphaviruses, and it is proposed that immunogenic epitope-tagged alphavirus CLPs produced in insect cells represent a simple and perhaps more stable alternative to alphavirus eVLPs.

SAV infection *in vivo* is temperature-restricted and progeny virus is only produced at low temperatures (10-15°C). To understand why SAV replication is restricted to low temperatures and to circumvent SAV eVLP production at low temperature, the molecular basis of low-temperature dependent SAV virion replication was studied. Using engineered SAV replicons we showed that at least viral RNA replication is not temperature-restricted, which suggested that the viral structural proteins are involved in low-temperature dependency. The processing and trafficking of SAV glycoproteins E1 and E2 as a function of temperature were investigated in insect cells via baculovirus vectors. We demonstrated that protein trafficking and surface expression of E2 occurs only at low temperature and only in the presence of E1. The study identified E2 as the critical determinant of SAV low-temperature dependent virion formation.

As said, SAV eVLPs can only be formed at low temperature (<18°C), whereas eVLPs of a related alphavirus, chikungunya virus (CHIKV), can be produced at 27°C. Using different combinations of chimeric viral polyproteins, we showed that the immunogenic SAV E2 B-domain is not responsible for the temperature-sensitivity of SAV assembly. Subsequently, this knowledge was used in the design of an additional prototype nanoparticle vaccine. A chimeric CHIKV/SAV eVLP vaccine was produced at 27°C, in which the CHIKV E2 B-domain was exchanged with the corresponding SAV E2 B-domain within the structural protein cassette of CHIKV. All nanoparticle vaccine candidates,

SAV eVLP, SAV peptide-fused CLP and CHIKV/SAV chimeric eVLP, were tested in a vaccination/cohabitation challenge trial of Atlantic salmon. High mortality occurred upon viral challenge, except in the group of fish vaccinated with SAV inactivated antigen, and PCR analysis confirmed high viremia in all other test groups. It was concluded that eVLP or CLP vaccination does not readily result in the establishment of a protective immune response in salmon against SAV challenge and that further improvements on this concept need to be made.

The major difference between the non-protective eVLP and protective whole inactivated virus vaccines was the absence/presence of viral RNA. Therefore, it was hypothesized that presence of (replicating) viral RNA in a SAV vaccine may be needed to generate a protective immune response. Self-replicating RNA (replicon) vaccines have shown great potential as vaccines in different animals. Therefore, we developed different types of DNA-launched SAV replicon vaccines, which were tested in cell culture and in a vaccination-challenge model in Atlantic salmon. A SAV replicon vaccine encoding E2 elicited protective immunity only when E1 was co-expressed. This showed that expression of both glycoproteins is essential in the establishment of protection. By expression of the SAV capsid protein *in trans*, it was hypothesized that viral replicon particles (VRP) were formed *in vivo*, which would cause an additional single round of infection and might further elevate the immune response in comparison to the replicon vaccine. A second animal trial indicated that the inclusion of capsid did not yet improve vaccine efficacy. These trials showed that a DNA vaccine transiently expressing the SAV structural proteins provided superior protection over both replicon vaccines (with and without capsid).

To conclude, the work described in this thesis aimed to generate alternative, next-generation vaccines to the current inactivated virus vaccine, protective against SAV infections. Some characteristics, such as the cause of temperature-dependency of SAV replication, of this unique aquatic virus were further explored. The production and *in vivo* testing of multiple next-generation vaccines further defined the prerequisites for induction of a potent immune response in Atlantic salmon. A prototype DNA-launched replicon vaccine has shown potential for further development. The research described in this thesis contributes to the development of next-generation vaccines in the challenging area of fish vaccinology.

## SAMENVATTING

Al vele jaren stijgt de wereldwijde visconsumptie en men verwacht dat deze trend zal doorzetten. In Nederland wordt aangeraden om tweemaal per week vis te eten (Voedingscentrum, Schijf van Vijf). We kunnen echter maar een bepaalde hoeveelheid vis uit zeeën en meren betrekken zonder dat dit een negatieve invloed heeft op de aquatische ecosystemen. Daarom is visteelt tegenwoordig onmisbaar. Ondanks de goede hygiëne wordt de aquacultuur bedreigd door vele pathogenen. Vaccinatie van economisch belangrijke vissen tegen deze pathogenen is momenteel gebruikelijk en heeft een grote bijdrage geleverd aan de vermindering van het antibioticagebruik. De Atlantische zalm en de regenboogforel zijn respectievelijk de meest gekweekte zout- en zoetwatervissen in Europa. Een pathogeen dat veel problemen bij beide soorten in de kweek veroorzaakt is het zalmalphavirus, beter bekend als salmonid alphavirus (SAV). Dit virus is in vele opzichten anders dan zijn familieleden die problemen veroorzaken in landdieren en men begrijpt de virale levenscyclus nog niet volledig. Het huidige vaccin tegen SAV is een geïnactiveerd virusvaccin, dat zeer effectief is en werkzaam tegen de verschillende SAV-subtypes. Echter, het vaccin biedt geen complete bescherming en wordt geproduceerd bij een lage temperatuur (10-15°C), wat de productietijd aanzienlijk langer maakt ten opzichte van vele andere vaccins. Daarnaast wordt het vaccin toegediend middels een intraperitoneale injectie, wat erg arbeidsintensief is en pas uitgevoerd kan worden als de vis een bepaalde grootte heeft bereikt. Dit betekent ook dat de vissen tot die tijd onbeschermd zijn. Het werk dat is beschreven in dit proefschrift had enerzijds als doel om de kennis over SAV te vergroten en anderzijds om deze kennis te gebruiken voor het ontwerpen en het produceren van een volgende generatie vaccins tegen SAV.

SAV behoort tot het genus Alphavirus, familie Togaviridae, waarvan de virussen over het algemeen worden overgedragen door ongewervelde vectoren, voornamelijk steekmuggen. SAV is hierin uniek, aangezien dit virus ook direct overgedragen kan worden van vis naar vis en er tot nog toe geen ongewervelde vector bekend is. Een interessante vondst was dat SAV kon repliceren in verscheidene muggencellijnen van de Aziatische tijgermug Aedes albopictus. Deze cellen produceerden nieuwe virusdeeltjes, die vervolgens enkele malen van cultuur naar cultuur konden worden overgezet zonder dat het virus de infectiositeit voor zalmcellen verloor. Dit kon plaatsvinden zonder veranderingen in de basenvolgorde van het virus, maar wel enkel bij lage temperatuur. Dit resultaat suggereerde dat SAV-replicatie misschien toch niet beperkt is tot virusvermenigvuldiging in gewervelde gastheren, maar wellicht een nog onbekende ongewervelde aquatische vector heeft. Het is dus mogelijk is om SAV tot hoge titers te laten groeien in een muggencellijn. Deze cellijn is daarom een mogelijk alternatief voor de huidige zalmcellijn om het vaccinvirus te vermeerderen.

Het huidige vaccin tegen SAV, en daarmee ook een soortgelijk vaccin gegroeid via een

alternatieve cellijn, is een klassiek eerstegeneratievaccin, gemaakt bij lage temperatuur. Een alternatief voor dit geïnactiveerd-virusvaccin, zou een vaccin zijn in de vorm van een nanodeeltje. Voordat de huidige studie begon was een nieuw type vaccin ontwikkeld op basis van een virusachtig deeltje (virus-like particle, VLP). Dit werd gedaan door de viruseiwitten van de buitenkant van SAV, tot expressie te brengen in insectencellen met behulp van het baculovirus-insectencelexpressiesysteem. Deze insectencellen kunnen op vele temperaturen groeien, maar veelal wordt dit expressiesysteem gebruikt bij $27^0$C. SAV VLP's werden met behulp van dit expressiesysteem enkel gevormd bij lage temperatuur. Echter, bij lage temperatuur is ook de eiwitopbrengst minder en daarom was de opbrengst van dit VLP-vaccin niet zo hoog. Het VLP-vaccin bestaat uit de volledige eiwitmantel van het virus, zijnde het nucleocapside, zijnde het lipidemembraan verkregen van de gastheer en de oppervlakte-eiwitten. Een VLP lijkt vanaf de buitenkant precies op het echte SAV-virus, maar is ingewikkelder om te maken dan een vaccin dat enkel uit het nucleocapside bestaat. Als een alternatief voor de SAV VLP's maakten wij een nucleocapsidevaccin op $27^0$C, door het capside eiwit met behulp van een aangepaste baculovirusvector in insectencellen te produceren. Deze eiwitten gingen uit zichzelf in de juiste vorm aan elkaar zitten en vormden daardoor nucleocapsiden in de celkern van de insectencellen. Deze nucleocapsiden konden met een simpel protocol van cellysis, sonicatie en centrifugatie worden opgezuiverd. Daarnaast konden we een sterk antigeen, het B-domein van het E2 oppervlakte-eiwit van SAV, aan dit nucleocapside hangen, zonder dat de vorm hierdoor werd aangetast. Hierdoor kon ook dit deeltje als nanodeeltjesvaccin gebruikt worden. Deze methode kan toegepast worden bij alle alphavirussen en dit vormt wellicht een stabieler alternatief voor de SAV VLP. Daarnaast heeft het als voordeel dat het geproduceerd kan worden bij $27^0$C.

SAV-infecties bij vissen worden gelimiteerd door de temperatuur en nieuwe virusdeeltjes kunnen enkel gevormd worden bij lage temperatuur (10-$15^0$C). Om te begrijpen waarom SAV-replicatie enkel op lage temperatuur plaatsvindt, en om zo mogelijk vaccinproductie bij lage temperatuur te kunnen omzeilen, werd de moleculaire verklaring voor de lage-temperatuurafhankelijke productie bestudeerd. Door middel van een synthetisch SAV-replicon, werd aangetoond dat de lage-temperatuurafhankelijkheid in ieder geval niet veroorzaakt wordt door de RNA-replicatie, want deze vindt bij hogere temperaturen nog steeds plaats. Dit suggereerde dat de structurele eiwitten de oorzaak zijn. Om dit te bevestigen werd het transport van de structurele eiwitten E1 en E2 van binnen in de cel naar het oppervlak bestudeerd aan de hand van baculovirusexpressie in insectencellen. We hebben hiermee laten zien dat het E2-eiwit alleen naar het celoppervlak reist wanneer het samengaat met het E1-eiwit, en alleen bij lage temperatuur. Dit leidde tot de conclusie dat het E2-eiwit verantwoordelijk is voor de lage-temperatuurafhankelijke replicatie van SAV.

Zoals gezegd konden SAV VLP's alleen geproduceerd worden bij lage temperatuur, terwijl VLP's van een ander alphavirus, chikungunya virus (CHIKV), wel bij $27^0$C gemaakt

kunnen worden. Gebruikmakend van verschillende combinaties van structurele eiwitten van deze twee virussen, hebben we geprobeerd om de regio in E2, welke verantwoordelijk is voor de lage-temperatuursfhankelijke replicatie, verder in kaart te brengen. Aangetoond kon worden dat het B-domein van E2 niet temperatuurafhankelijk is. Deze kennis werd vervolgens gebruikt om een chimere VLP te produceren bij $27^0$C, bestaande uit de structurele eiwitten van CHIKV, maar waarin het antigene B-domein van CHIKV omgewisseld was met het B-domein van SAV. Vervolgens zijn alle vaccin kandidaten gebaseerd op nanodeeltjes, zijnde SAV VLP's, SAV-nucleocapsiden met B-domeinfusie en de CHIKV/SAV VLP's, getest door middel van een vaccinatieproef bij Atlantische zalmen. Hoge mortaliteit vond plaats na blootstelling aan SAV, behalve in de groep zalmen die was gevaccineerd met het bestaande vaccin. We konden hieruit concluderen dat VLP of nucleocapsidevaccinatie geen bescherming biedt tegen blootstelling aan SAV.

Het grootste verschil tussen de VLP die geen bescherming biedt en het geïnactiveerde virusvaccin dat wel bescherming biedt, is de respectievelijke af- en aanwezigheid van viraal RNA. Daarom werd gedacht dat wellicht de aanwezigheid van (zelf-replicerend) viraal RNA in een SAV-vaccin nodig is. Zelf-replicerende RNA-vaccins, ook wel replicons genaamd, zijn veelbelovend als vaccins voor verschillende diersoorten. Daarom werden verschillende typen replicon-vaccins ontwikkeld, die vanaf een DNA-plasmide tot expressie werden gebracht. Deze replicons werden getest in cellijnen en bij Atlantische zalm. Het bleek dat het replicon-vaccin slechts bescherming bood wanneer het E2-eiwit samen met E1 tot expressie werd gebracht. Dit liet zien dat gelijktijdige expressie van deze eiwitten essentieel is voor het opwekken van immuniteit. Door ook het capside-eiwit in trans tot expressie te brengen werd verwacht dat virale replicondeeltjes gevormd zouden worden. Deze deeltjes zouden een additionele ronde van replicatie in de gastheer bewerkstelligen waardoor de immuunreactie mogelijk nog verder aangewakkerd zou worden. Een tweede dierstudie liet echter zien dat dit nog niet het geval is. Ook liet deze studie zien dat een DNA-vaccin, waarmee enkel de oppervlakte-eiwitten tot expressie werden gebracht, betere bescherming bood dan het replicon vaccin.

Kortom, het werk dat in dit proefschrift is beschreven had als doel om alternatieve vaccins voor het huidige SAV-vaccin te ontwikkelen. Enkele viruseigenschappen, zoals de oorzaak van de lage-temperatuurafhankelijke replicatie van dit unieke aquatische virus zijn verder onderzocht. Door de productie en het testen van de verschillende repliconvaccins wordt duidelijk wat de vereisten zijn voor een effectieve immuunreactie in zalm. Daarnaast is een prototype repliconvaccin ontworpen, dat aanknopingspunten biedt voor verdere ontwikkeling. Het onderzoek, beschreven in dit proefschrift, draagt hopelijk bij aan de ontwikkeling van een volgende generatie vaccins in het uitdagende veld van de visvaccinologie.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AA | amino acid |
| APC | antigen presenting cell |
| BHK | baby hamster kidney |
| Bdom | B-domain |
| BVDV | bovine viral diarrhoea virus |
| C | capsid |
| CP | capsid protein |
| CHIKV | chikungunya virus |
| CHSE | Chinook salmon embryo |
| CSFV | classical swine fever virus |
| CLP | core-like particle |
| CMV | cytomegalovirus |
| CTL | cytotoxic T cell |
| dpt | days post-transfection |
| DENV | dengue virus |
| dsRNA | double stranded RNA |
| ER | endoplasmatisch reticulum |
| E | envelope |
| Env | envelope proteins |
| eVLP | enveloped virus-like particle |
| FMDV | foot-and-mouth disease virus |
| FISH | full infectious SAV with Hammerhead ribozyme |
| F | furin |
| GOI | gene of interest |
| HPAI | highly pathogenic influenza virus |
| Ig | immunoglobulin |
| IHNV | infectious heamophoetic necrosis virus |
| IPNV | infectious pancreatic necrosis virus |
| ISAV | infectious salmon anemia virus |
| IFN | interferon |
| JE | Japanese encephalitis virus |
| LAV | live attenuated virus |
| MHC | major histocompatibility complex |
| nsP | nonstructural protein |
| N | nucleus |
| PD | pancreas disease |

| | |
|---|---|
| PAMP | pathogen associated molecular pattern |
| PRR | pattern recognition receptor |
| PBS-T | PBS-tween20 |
| PEDV | porcine epidemic diarrhoea virus |
| PRRSV | porcine reproductive and respiratory syndrome virus |
| PE2 | precursor E2 |
| POI | protein of interest |
| RPP | relative percentage protection |
| RPS | relative percentage survival |
| RVFV | Rift Valley fever virus |
| RLR | RIG-I like receptor |
| RT | room temperature |
| SAV | salmonid alphavirus |
| SIV | semian influenza virus |
| SFV | Semliki Forest virus |
| SINV | sindbis virus |
| ssRNA | single stranded RNA |
| SD | sleeping disease |
| Sf9 | *Spodoptera frugiperda* 9 |
| SVCV | spring viremia of carp virus |
| SP | structural proteins |
| SPDV | salmon pancreas disease virus |
| SVP | subviral particles |
| TBEV | tick-borne encephalitis virus |
| TCID | tissue culture infectious dose |
| TLR | toll-like receptor |
| TGN | trans-golgi network |
| UTR | untranslated region |
| VEEV | Venezualan equine encephalitis virus |
| VSV | vesicular stomatitis virus |
| VHSV | viral haemmorhagic disease virus |
| VRP | viral replicon particle |
| VLP | virus-like particle |
| wpv | weeks post-vaccination |
| WNV | West Nile virus |
| WB | western blot |
| YFV | yellow fever virus |

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Besides my project at the Laboratory of Virology, I was a member of the PhD council of the PE&RC graduate school and a member of the Wageningen PhD council. I learned a lot from these meetings and I met a lot of new people. Thank you to all who have joined me in these councils. Claudius en Lennart, een speciaal bedankje voor jullie!

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## ABOUT THE AUTHOR

Maria (Mia) Cornelia Hikke was born on September 5th, 1987 in Leiderdorp. After attending primary school in Ter Aar and high school in Alphen aan den Rijn, she moved to Wageningen, to start her BSc studies in Biotechnology at Wageningen University. For her BSc thesis, she worked at the Scottish Fish Immunology Research Centre in Aberdeen, Scotland. After her BSc graduation, she started her MSc studies in Medical Biotechnology at Wageningen University. Her MSc study was finalized by an MSc thesis on chikungunya virus at the Laboratory of Virology at Wageningen University, and an internship in virology at the University of Otago in Dunedin, New Zealand. After she obtained her MSc degree, she started her first job as junior researcher at the Laboratory of Virology of Wageningen University, under supervision of Gorben P. Pijlman. Here, she worked on a feasability study for next-generation salmonid alphavirus vaccine development. After one year, this contract was elongated by a four year PhD contract, during which she continued her studies on salmonid alphavirus. Currently she is employed by MSD Animal Health, where she works as a project leader at the department of Bioprocess Technology and Support - Viral.

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Hikke MC and Pijlman GP. Veterinary replicon vaccines. 2016; Article in submission.

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Salmonid alphavirus glycoprotein E2 requires low temperature and E1 for virion formation and induction of protective immunity. Mia Hikke, Stine Braaen, Stephane Villoing, Kjartan Hodneland, Corinne Geertsema, Lisa Verhagen, Petter Frost, Just Vlak, Espen Rimstad, Gorben Pijlman. Presented at the 9th International Symposium on Viruses of the Lower Vertebrates, Malaga, Spain, October 1-4, 2014

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## PE&RC TRAINING AND EDUCATION STATEMENT


With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Veterinary replicon vaccines

Writing of project proposal (4.5 ECTS)

- Salmonid alphavirus 3rd generation vaccine development

Post-graduate courses (3 ECTS)

- Isotope / radiation course (2012)
- Electron microscopy course (2014)

Laboratory training and working visits (3 ECTS)

- Work visit: animal trial-SAV vaccine testing; Intervet Norbio AS (2014)
- Work visit: lab experiments; Intervet Norbio AS (2015)

Invited review of (unpublished) journal manuscript (2 ECTS)

- Virus Research: the role of Salmonid alphavirus E2 protein in viral fitness (2014)
- Journal of General Virology: glycoprotein E of the Japanese Encephalitis Virus forms virus-like particles and induces Syncytia when expressed by a baculovirus (2014)

Competence strengthening / skills courses (6.4 ECTS)

- Mini symposium: how to write a world class paper; WUR Library + Elsevier Science (2013)
- PhD Council; PE&RC (2013-2015)
- Scientific writing; Wageningen in'to Languages (2015)

PE&RC Annual meetings, seminars and the PE&RC weekend (3.3 ECTS)

- PE&RC Weekend (2012)
- PE&RC Day (2012-2015)
- Wageningen PhD symposium (2013, 2015)
- PE&RC Last year weekend (2015)

Discussion groups / local seminars / other scientific meetings (5.1 ECTS)

- Meetings MSD animal health (2012-2015)
- Dutch Annual Virology symposium (2012-2015)

- Vaccine symposium; Infection and Immunity Centre, Utrecht (2012-2015)
- WEES Seminars (2012-2015)

International symposia, workshops and conferences (11 ECTS)
- American Society of Virology annual meeting; Penn State University, USA (2013)
- Fish immunology workshop; Wageningen University, NL (2014)
- American Society of Virology annual meeting; Colorado State University, USA (2014)
- International Symposium on viruses of lower vertebrates; Malaga, SP (2014)
- Veterinary Virology conference; Montpellier, FR (2015)

Lecturing / supervision of practical's / tutorials (26.7 ECTS)
- Practical's immunotechnology (2012-2014)
- Introduction to BBT (2012-2015)
- PGO Molecular virology (2012-2015)
- Lecture during fundamental and applied virology (2015)

Supervision of MSc students
- Temperature dependent processing of the E2 glycoprotein (Lisa Verhagen)
- SAV CLP As protein carriers for an immunogenic epitope (Vincen Wu)
- Chimeric Salmonid alphavirus virus –like particles (Andi Asnayanti)
- Alphavirus replicon particles (Zyfra Day)
- Increased replication of Salmonid alphavirus (Jelle Sterk)

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