

Graduate School for Cellular and Biomedical Sciences
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**Development of a novel marker vaccine
platform for protection against Bluetongue
Virus (BTV)**

PhD Thesis submitted by

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ABSTRACT

Bluetongue virus (BTV) is an economically important member of the genus Orbivirus and closely related to African horse sickness virus (AHSV) and Epizootic hemorrhagic disease virus (EHDV). Currently, 26 different serotypes of BTV are known. The virus is transmitted by blood-feeding *Culicoides* midges and causes disease (bluetongue [BT]) in ruminants. In 2006/2007, BTV serotype 8 (BTV-8) caused widespread outbreaks of BT amongst livestock in Europe, which were eventually controlled employing a conventionally inactivated BTV vaccine. However, this vaccine did not allow the discrimination of infected from vaccinated animals (DIVA) by the commonly used VP7 cELISA.

RNA replicon vectors based on propagation-incompetent recombinant vesicular stomatitis virus (VSV) represent a novel vaccine platform that combines the efficacy of live attenuated vaccines with the safety of inactivated vaccines. Our goal was to generate an RNA replicon vaccine for BTV-8, which is safe, efficacious, adaptable to emerging orbivirus infections, and compliant with the DIVA principle.

The VP2, VP5, VP3 and VP7 genes encoding the BTV-8 capsid proteins, as well as the non-structural proteins NS1 and NS3 were inserted into a VSV vector genome lacking the essential VSV glycoprotein (G) gene. Infectious virus replicon particles (VRP) were produced on a transgenic helper cell line providing the VSV G protein *in trans*. Expression of antigens *in vitro* was analysed by immunofluorescence using monoclonal and polyclonal antibodies.

In a pilot study, sheep were immunized with two different VRP-based vaccine candidates, one comprising the BTV-8 antigens VP2, VP5, VP3, VP7, NS1, and NS3, the other one containing antigens VP3, VP7, NS1, and NS3. Control animals

received VRPs containing an irrelevant antigen. Virus neutralizing antibodies and protection after BTV-8 challenge were evaluated and compared to animals immunized with the conventionally inactivated vaccine. Full protection was induced only when the two antigens VP2 and VP5 were included in the vaccine.

To further evaluate if VP2 alone, a combination of VP2 and VP5 or VP5 alone were necessary for complete protection, we performed a second animal trial. Interestingly, VP2 as well as the combination of VP2 and VP5 but not VP5 alone conferred full protection in terms of neutralizing antibodies, and protection from clinical signs and viremia after BTV-8 challenge.

These results show that the VSV replicon system represents a safe, efficacious and DIVA-compliant vaccine against BTV as well as a possible platform for protection against other Orbiviruses, such as AHSV and EHDV.

ABBREVIATIONS

BTV	Bluetongue virus
VSV	Vesicular stomatitis virus
VRP	Virus replicon particles
GFP or *	Green fluorescent protein
IFT	Immunofluorescent test/assay
MOI	Multiplicity of infection
NS	Non-structural protein
VP	Viral protein
G	Glycoprotein
Δ G	lacking Glycoprotein
AHSV	African horse sickness virus
EHDV	Epizootic hemorrhagic disease virus
RNA	Ribonucleic acid
kDA	kiloDalton
ELISA	Enzyme linked immunosorbent assay
dsRNA	double-stranded ribonucleic acid
kb	kilobases

INTRODUCTION BLUETONGUE

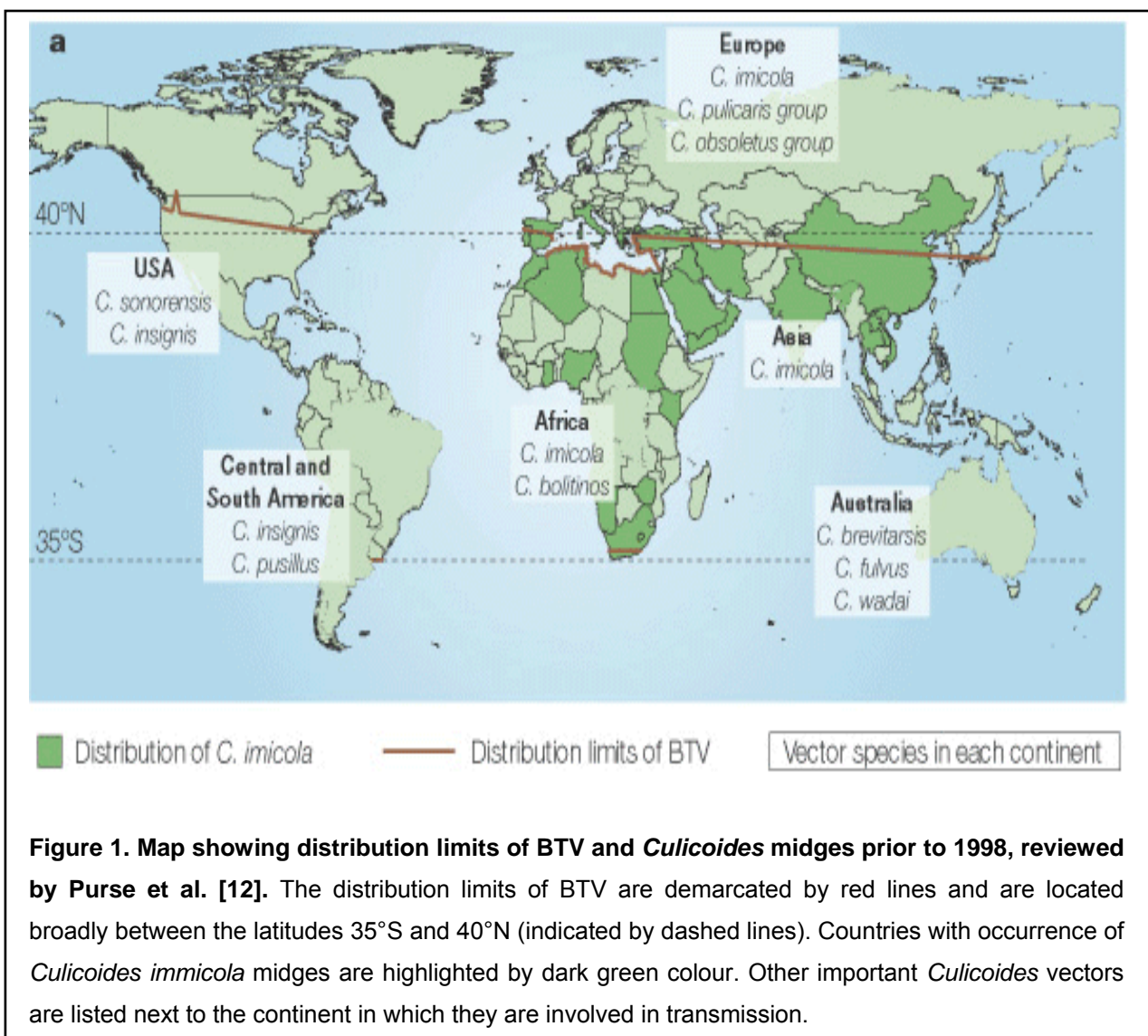
Bluetongue virus (BTV) is an *Orbivirus* of the *Reoviridae* family, and the causative agent of a haemorrhagic disease (bluetongue [BT]) in ruminants, mainly sheep [1]. There are currently 26 recognised serotypes of BTV, which are transmitted by blood-feeding *Culicoides* midges and sometimes vertically in animals [2, 3]. BTV is closely related to African horse sickness virus (AHSV).

History and Epidemiology

Bluetongue was first described in Merino sheep in the late 19th century in South Africa and got its name due to severe cyanosis of the tongue of some severely affected sheep [4]. Later in the 1990s, it was determined that BT was a disease of ruminants occurring worldwide from latitudes of approximately 40 degrees North to 35 degrees South [5]. Furthermore, the disease was responsible for considerable economic losses especially at the incursional margins of the global range of BTV infection [6] (**Figure 1**). Although BTV had transiently made brief incursions into Southern Europe, these did not persist in the continent [5]. However, between 1998 and 2001, eight BTV strains of five different serotypes (BTV-1, 2, 4, 8, 9 and 16) emerged in several countries around the northern rim of the Mediterranean Basin where they had previously not been recorded, likely because of climatic changes which allowed overwintering of the virus in *Culicoides* vector midges [7].

A novel strain of BTV serotype 8 (BTV-8), which had never previously been detected in Europe, emerged in 2006 as an epidemic wave in Western and Northern Europe [4, 8, 9]. This outbreak had a significant economic impact, not only because of morbidity and mortality caused by the disease in sheep and cattle, but also because

of restrictions imposed on livestock movement and trade [10]. In August 2006, BTV-8 was detected in sheep in the Netherlands, followed by Germany, Belgium and France. In 2007, the outbreak was confirmed in Switzerland, the United Kingdom, Luxembourg, Denmark and the Czech Republic. In 2008, Spain, Italy, Greece and Austria also detected BTV-8 infection in cattle and sheep. It remains uncertain precisely how BTV-8 arrived in Northern Europe, but the absence of outbreaks in southern parts of the continent points towards a direct entry mechanism rather than a linear extension from its ancestor, potentially a Western lineage virus from sub-Saharan Africa [11].



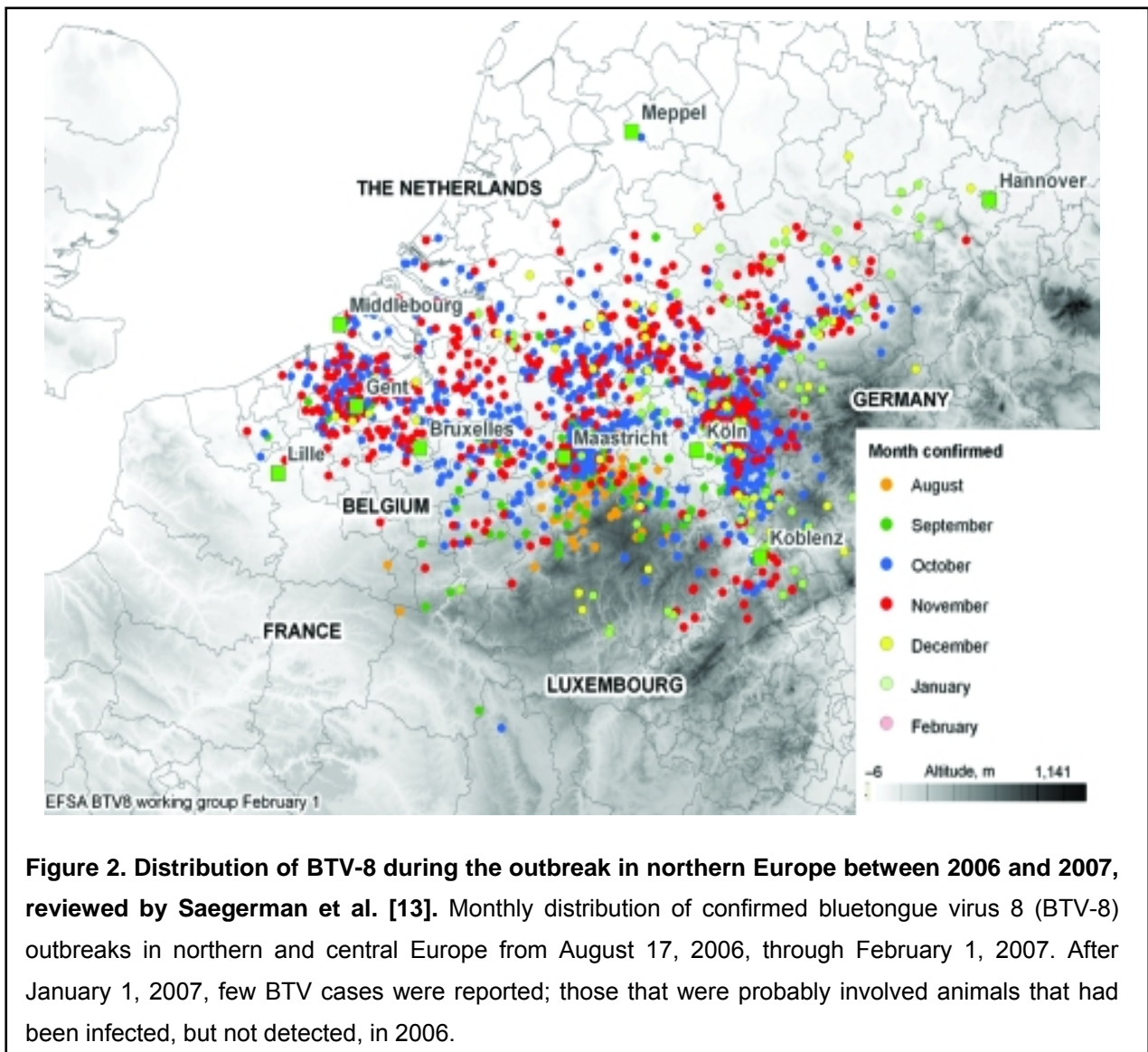


Figure 2. Distribution of BTV-8 during the outbreak in northern Europe between 2006 and 2007, reviewed by Saegerman et al. [13]. Monthly distribution of confirmed bluetongue virus 8 (BTV-8) outbreaks in northern and central Europe from August 17, 2006, through February 1, 2007. After January 1, 2007, few BTV cases were reported; those that were probably involved animals that had been infected, but not detected, in 2006.

Bluetongue disease

BTV is transmitted to livestock by blood-feeding *Culicoides* midges, in Europe mainly by *C. imicola*, *C. pulicaris* and *C. obsoletus*. In cattle, goats, and wild ruminants, BTV infection is typically asymptomatic despite prolonged viremia. These host species represent a potential reservoir for unnoticed dissemination of BTV in ruminant populations. In sheep, however, BTV infection often results in an acute disease with associated high morbidity and mortality, depending on the virulence of the virus and

the sheep breed affected [14]. Following an initial replication in the lymph nodes, BTV targets endothelium and mononuclear phagocytes [15]. After an incubation period of 2 to 15 days, clinical signs in the acute phase of disease comprise high fever, salivation, depression, nasal discharge, hyperaemia and congestion of the muzzle, lips, face, eyelids and ears leading to tissue oedema (**Figure 3 A**). Ulcerations and necrosis of the mucosae of the mouth are often observed, the tongue may become hyperaemic and oedematous. Lameness due to coronitis, pneumonia or lung oedema can be observed in severe cases. In cases with fatal outcome, sheep usually die after 8 to 10 days.

Certain strains of BTV, notably the northern European strain of BTV-8, can cross the placental barrier, leading to infection of the developing fetus [3]. Hence, abortions and malformations of offspring are frequently associated with infection of pregnant animals with certain strains of the virus [16-18].

Frequently observed pathological findings comprise hemorrhages at the base of the pulmonary artery (A. pulmonalis), the papillary muscles of the left ventricle of the heart, enlarged haemorrhagic lymph nodes, erosions on ruminal pillars, and ulcers of the mucosal lining throughout the upper gastrointestinal tract (**Figure 3 B-F**).

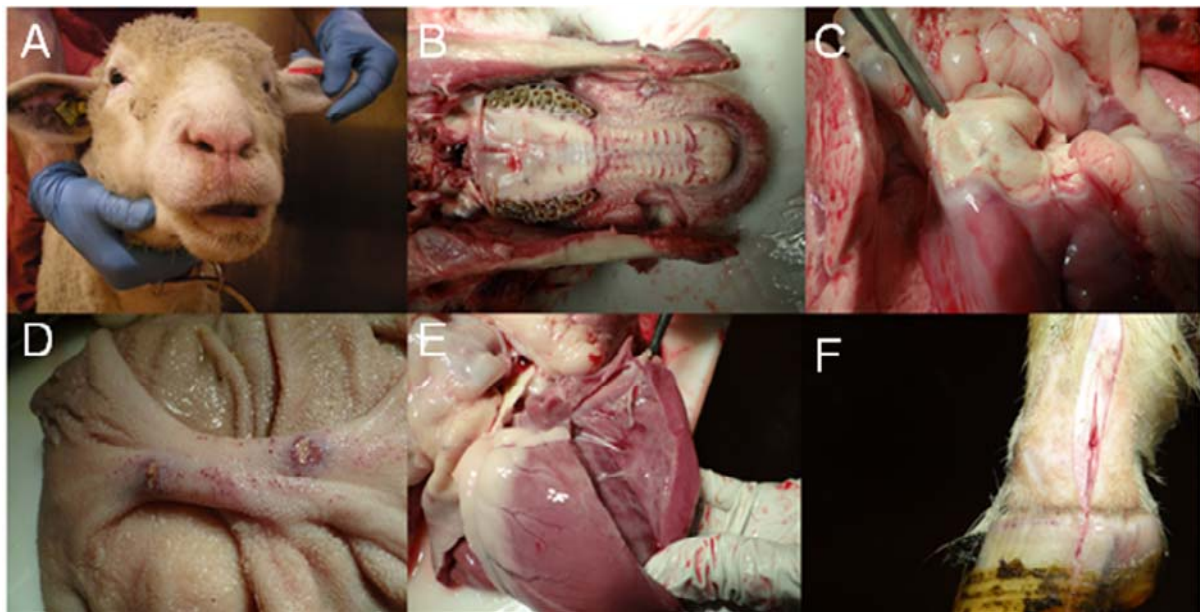
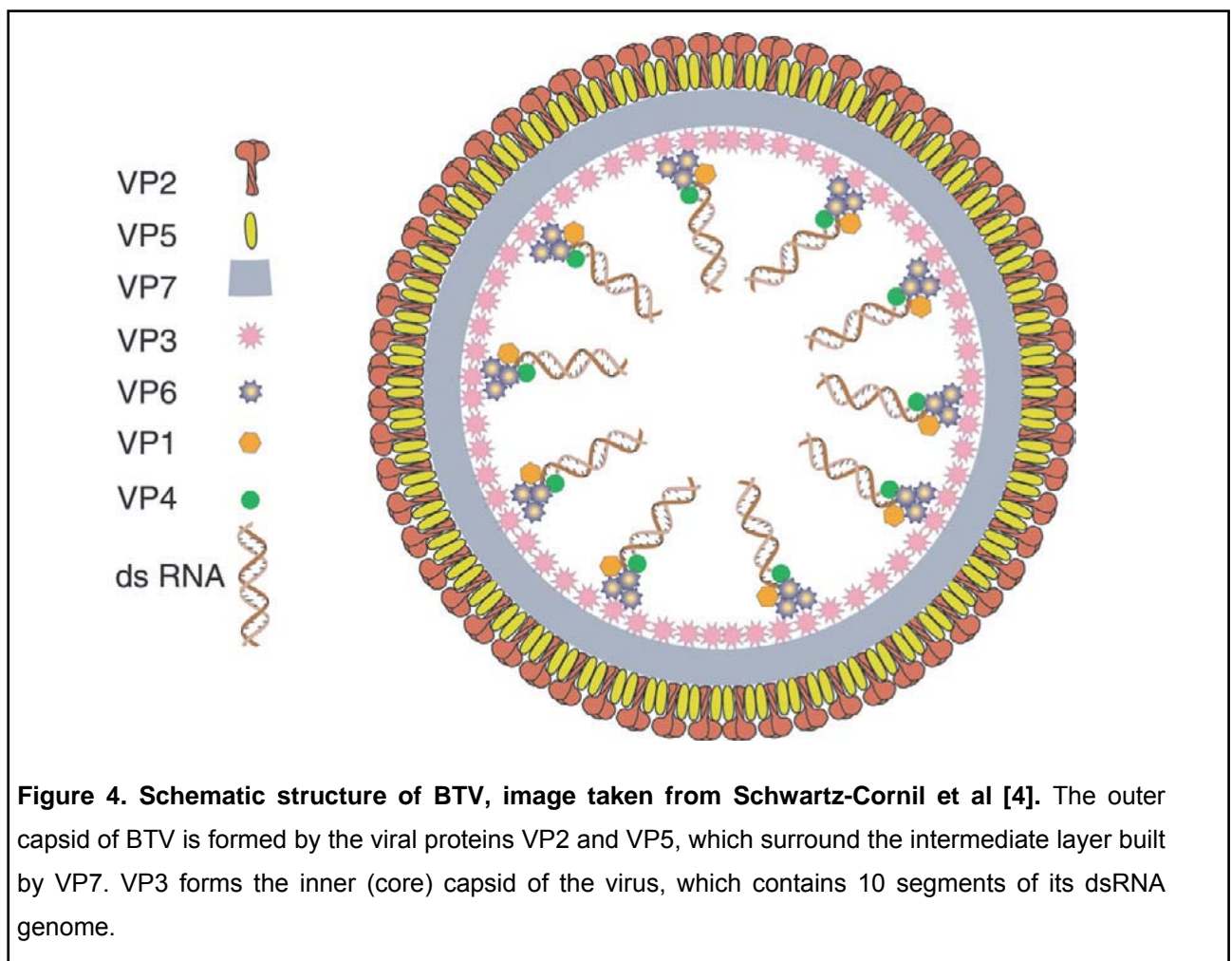


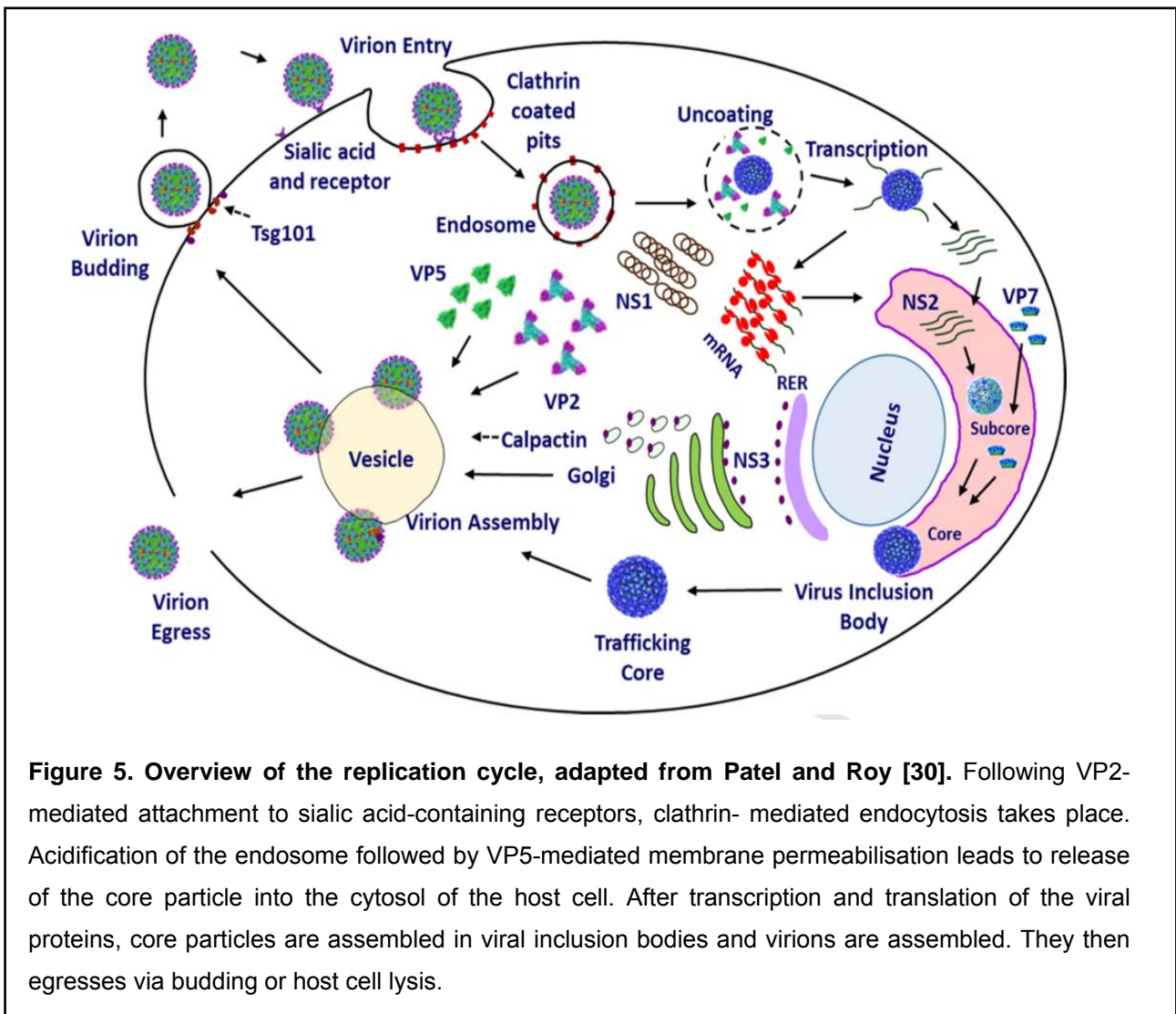
Figure 3. Acute disease and pathological findings in Bluetongue virus-infected sheep. Bluetongue disease gross lesions in BTV-8 infected sheep at 8 dpi; the animal displays severe facial oedema, nasal discharge and salivation (A). Pathological findings at 14 dpi (B) hard palate with linear hemorrhages following the tips of the rugae, (C) haemorrhagic focus at the base of the pulmonary artery, (D) several hemorrhagic foci covered with fibronecrotic material at the pilae ruminis, (E) hemorrhages at the papillary muscle of the heart, (F) petechiae of the coronary band.

Structure and replication cycle

The BTV genome consists of 10 segments of dsRNA, which encode 7 structural (VP1 - VP7) and 5 non-structural proteins (NS1 – NS4, NS3a) [19, 20]. The non-enveloped icosahedral virion is composed of three concentric protein layers that surround the inner core (**Figure 4**). The core includes the viral RNA, VP1 (RNA polymerase), VP4 (capping enzyme), and VP6 (helicase) [21, 22]. The inner capsid layer consists of a heterodimer of VP3 that forms a scaffold for VP7. The outer capsid is formed by the viral proteins VP2 and VP5, which are responsible for receptor binding in the mammalian host, hemagglutination, and membrane penetration, respectively [23, 24] (**Figure 5**). The large (110 kDa) attachment protein VP2 induces virus-neutralizing antibodies [25]. However, VP2 is highly variable and

currently 26 different BTV serotypes can be distinguished by antibodies that show little or no cross-neutralizing activity [4]. Binding of VP5 to VP2 leads to a VP2 conformational change, which may affect the epitopes of neutralizing antibodies [26, 27]. VP5, which is significantly more conserved than VP2, has been shown to be a membrane penetration protein and facilitates the release of the core from the endosome into the cytoplasm [28]. All other structural and non-structural proteins are relatively conserved among different BTV serotypes. As a consequence, most ELISAs for detection of group-specific -BTV antibodies employ the VP7 antigen [29].





Vaccine strategies

Currently available commercial vaccines against BTV are either attenuated or inactivated. Live-attenuated (MLV) vaccines have been used to control BT in sheep in Southern Africa, the United States and Israel as they induce solid immunity and can be produced relatively cheaply [31]. However, potential complications of MLV vaccines include transient viremia after vaccination – possibly leading to further distribution by vector midges - making them unsuitable candidates for use in non-endemic regions [32, 33]. The epizootic of BTV-8 which struck Europe between 2006 and 2008 was controlled by an extensive vaccination campaign using whole

inactivated BTV-8. Although these vaccines induced strong protection against BTV-8 infection and disease, they did not allow simple serological discrimination of infected from vaccinated animals (DIVA). Furthermore, vaccine production required the production of large amounts of infectious virus in cell culture, proper virus inactivation, and formulation of the antigen with adjuvant, all of which delayed production and added to the cost of producing the vaccine. Importantly, inactivated virus vaccines may not be suitable for the control of all serotypes of BTV as, for example, serotype 25 cannot yet be propagated in cell culture [34].

Much effort has been devoted to the development of alternative vaccine candidates, to overcome the drawbacks of conventional vaccines. Virus-like particles were produced using recombinant baculovirus-encoded BTV proteins that were expressed using baculovirus expression vectors [35, 36]. They proved to be promising candidates for the production of multi-serotype vaccines, but also caused concerns regarding potential replication in insect vectors in the field [37]. Other recombinant viruses encoding different BTV-antigens such as vaccinia viruses [38], canarypox viruses [39], capripox viruses [40] and herpes viruses [41] have also been proposed. These studies already revealed the importance of the two components of the outer BTV shell, VP2 and VP5, for protection [42]

INTRODUCTION TO VSV REPLICONS

Vesicular stomatitis virus (VSV) structure and replication

VSV is a non-segmented negative-strand RNA virus of the family *Rhabdoviridae*, and is known to trigger a robust humoral immune response in many different host species [43]. Virions are bullet-shaped particles with genomes which encode for the five viral proteins, namely nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the large RNA polymerase (L), resulting in a total size of approximately 11.2 kb. (**Figure 6**). The virus is characterized by a broad cell tropism with propagation to high titres and cytopathogenicity (**Figure 7**).

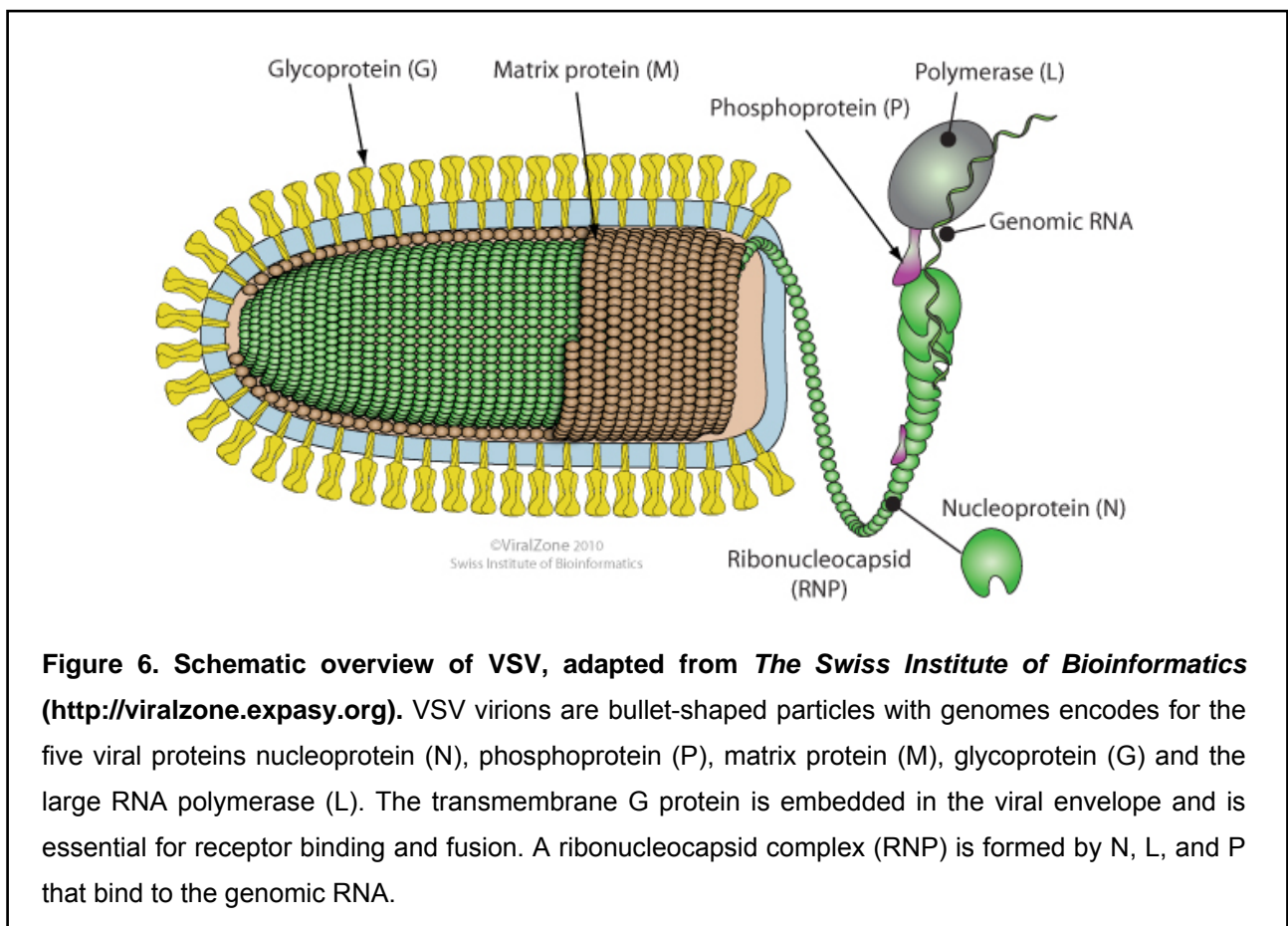


Figure 6. Schematic overview of VSV, adapted from *The Swiss Institute of Bioinformatics* (<http://viralzone.expasy.org>). VSV virions are bullet-shaped particles with genomes encodes for the five viral proteins nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the large RNA polymerase (L). The transmembrane G protein is embedded in the viral envelope and is essential for receptor binding and fusion. A ribonucleocapsid complex (RNP) is formed by N, L, and P that bind to the genomic RNA.

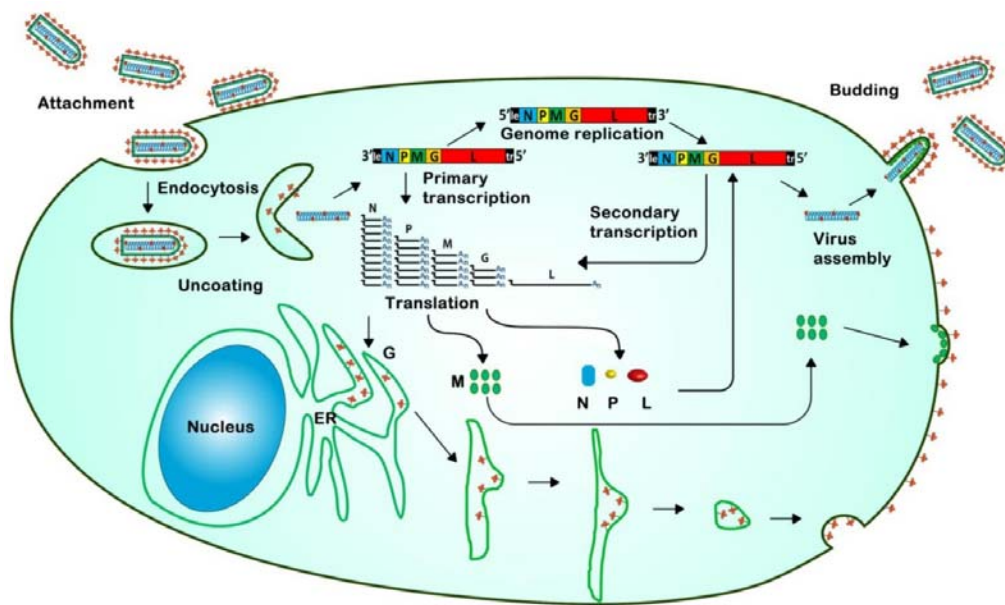


Figure 7. Replication cycle of VSV, adapted from Jianrong Li and Yu Zhang [44] VSV attachment is mediated by the outermost G to host cell receptors, which leads to a clathrin-mediated endocytosis. The ribonucleoprotein complex (RNP) is released into the cytoplasm, where transcription and replication takes place. Finally, the virus assembles and buds at the plasma membrane, releasing new virions.

Vesicular stomatitis virus replicons in experimental vaccines

At first, VSV with a deleted G gene, resulting in a propagation-incompetent VSV Δ G, was used for pseudotyping experiments with heterologous glycoproteins [45]. Later, recombinant VSV vectors have been frequently used for experimental vaccination of mammals against a number of different pathogens such as SARS [46], HIV-1 [47], human papilloma virus [48], hepatitis C virus [49], and respiratory syncytial virus [50]. A modified VSV vector in which the gene expressing VSV glycoprotein (G) was replaced by the influenza HA gene was shown to protect chicken from challenge infection with highly pathogenic avian influenza viruses [51, 52]. Due to the deletion of the G protein gene, the vector was restricted to a single round of infection contributing to the excellent biosafety profile of this viral vector. These single-cycle

virus vector vaccines were effective even though adjuvants were not employed [51, 52].

Generation of VSV replicon particles expressing foreign antigens

To generate replication-incompetent VSV replicon particles (VRPs) expressing high levels of foreign antigens, plasmids carrying the genes of interest instead of the viral glycoprotein G have to be designed, employing a reverse genetics system and helper cell lines, which are able to provide the lacking G protein *in trans*. (**Figure 8**) [53].

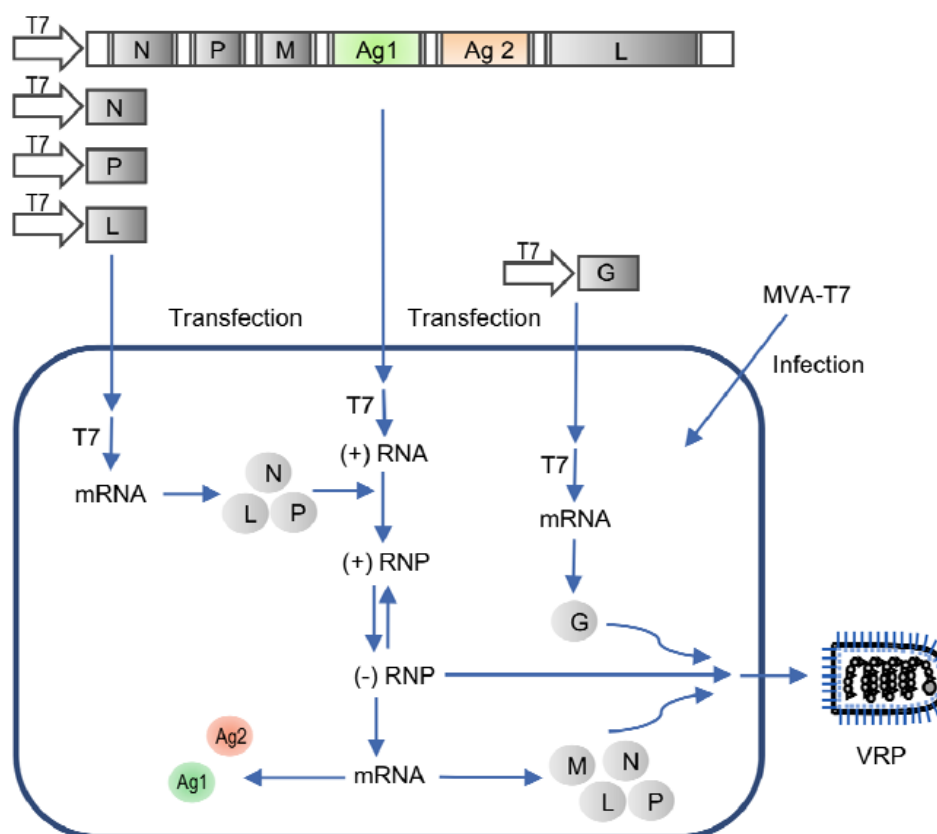


Figure 8. Generation of virus replicon particles encoding foreign antigens based on VSV, illustration taken from Zimmer [54]. The VSV the replicon RNA encodes for the N, P and L proteins, which constitute the viral RNA polymerase complex, the matrix protein M, and two heterologous antigens (Ag1 and Ag2). The replicon RNA lacks the gene encoding for the viral envelope glycoprotein G.

AIM OF THE PROJECT

Outbreaks of BT disease amongst domestic and wild ruminants can cause considerable economic loss that reflect high morbidity and mortality as well as indirect losses because of trade restrictions [10]. In the European outbreak of BTV-8 between 2006 and 2008, an inactivated vaccine was successfully employed. However, a serological discrimination between infected and vaccinated animals was not possible.

In this project, we aimed on overcoming these hurdles using propagation-incompetent VSV Δ G vectors as a new vaccine platform against BTV, which should be safe, efficacious and conferred the DIVA principle.

First, we generated VSV-virus replicon particles expressing different combinations of vaccine candidates and studied the expression of recombinant BTV antigens. Next, we assessed the immune response in a small number of sheep towards VSV VRPs compared to the inactivated vaccine. Immunized sheep were then infected with pathogenic BTV-8. Next, we immunized a small number of sheep to test the VRP's general suitability, using different combinations of vaccine candidates and comparing results to animals immunized with the inactivated vaccine before and after infection with infectious BTV-8 and monitored for viremia, clinical symptoms, and antibody production. Taking the results from these first experiments into account, we finally selected propagation-incompetent VSV Δ G vectors expressing the BTV-8 outer capsid proteins VP2 or VP5, or a combination of both, and performed a blind vaccine/challenge study with 24 sheep. The DIVA principle was validated using a commercially available VP7-based competitive ELISA.

RESULTS

Unpublished Results

Generation of recombinant VSV replicons expressing BTV antigens

The vaccine vector system is based on a vesicular stomatitis virus (VSV) deletion mutant that lacks the essential envelope glycoprotein G in the fourth transcription unit and expresses the enhanced green fluorescent protein at position 5 (VSV*ΔG). By inserting different BTV-8 antigens of interest VP2, VP5, VP3, VP7 and the non-structural protein NS1 and NS3 into the fourth transcription unit, we were able to design six different single antigen expression vectors (**Figure 9**). Three vectors, expressing two BTV-8 genes from the fourth and fifth transcription unit were also designed. Recombinant VSV*ΔG served as a control vector as it did not express any BTV antigen. All recombinant vectors were propagated on helper cell lines providing the VSV glycoprotein G *in trans*, which yielded infectious virus replicon particles (VPRs) of approximately 10^8 infectious units per ml of cell culture supernatant.

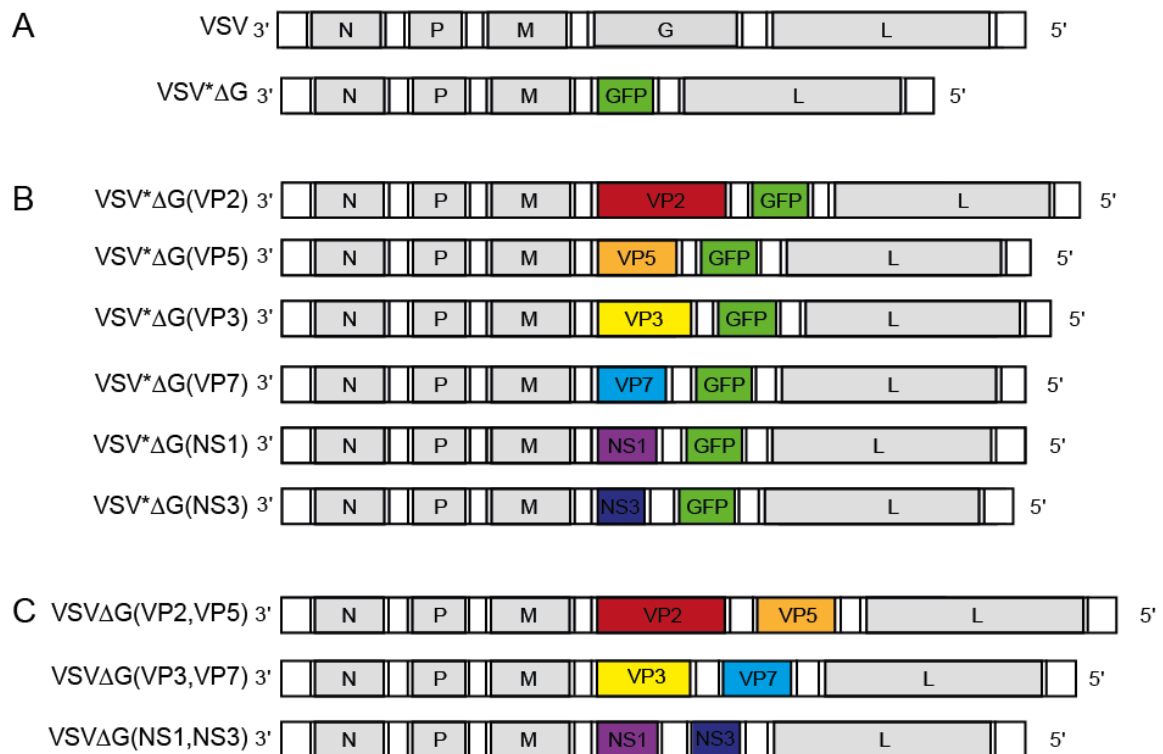


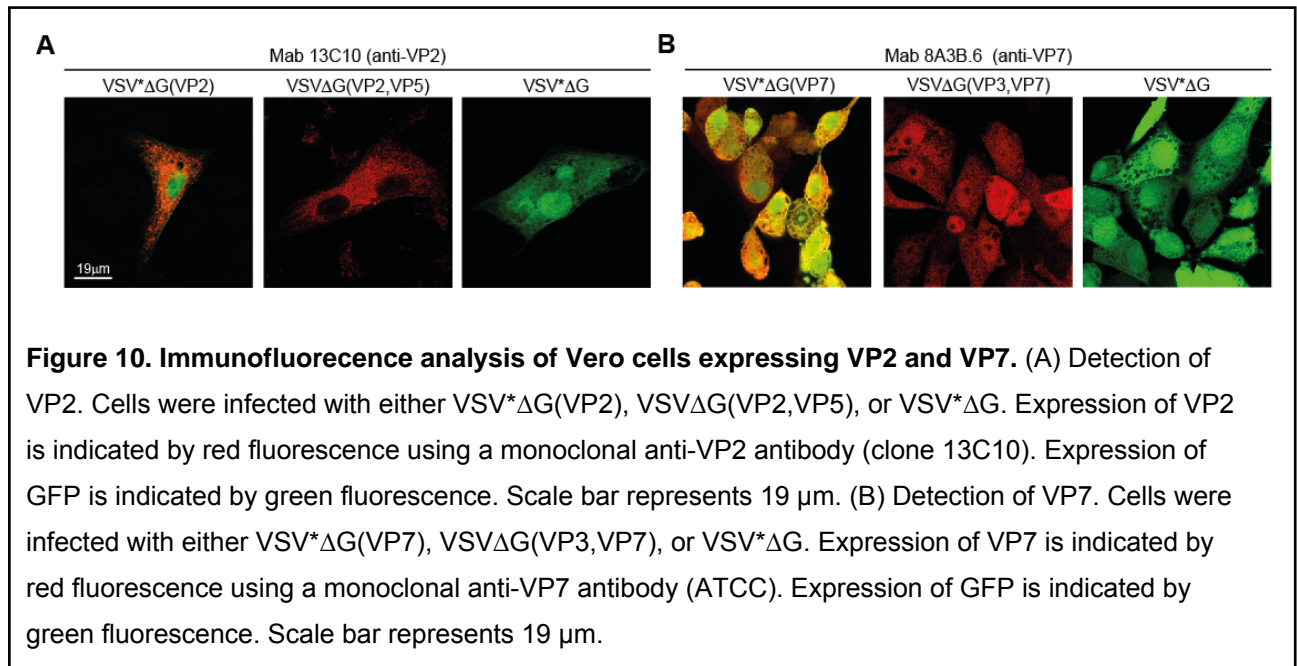
Figure 9. Genome maps of recombinant VSV. (A) VSV encodes for nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the large RNA polymerase (L). In the VSV*ΔG, the glycoprotein (G) has been replaced by the GFP gene (denoted by *). (B) The vectors VSV*ΔG(VP2), VSV*ΔG(VP5), VSV*ΔG(VP3), VSV*ΔG(VP7), VSV*ΔG(NS1), and VSV*ΔG(NS3) express individual antigens of BTV-8 from the fourth transcription unit while GFP (*) is expressed from an additional transcription unit downstream of the BTV antigen. (C) Double expression vectors VSVΔG(VP2,VP5), VSVΔG(VP3,VP7), and VSVΔG(NS1, NS3) express two BTV-8 antigens from the fourth and fifth position.

Characterization of antigen expression *in vitro*

In order to study the expression of BTV antigens VP2 and VP7, we performed indirect immunofluorescence analysis (IFA) with Vero cells infected with VRPs using an MOI of 1.

The BTV-8 VP2-specific monoclonal antibody reacted with cells infected with VSV*ΔG(VP2) and VSVΔG(VP2, VP5), whereas cells infected with VSV*ΔG were not recognized (**Figure 10A**). Expression of VP7 was detected by a VP7-specific

monoclonal antibody in cells infected with VSV*ΔG(VP7) and VSVΔG(VP3,VP7), whereas cells infected with VSV*ΔG were not recognized (**Figure 10B**).



Because specific monoclonal antibody were not available, expression of VP5 and NS1 was confirmed using modified antigens, containing a triple FLAG epitope at the C-terminus. The monoclonal anti-FLAG antibody reacted specifically with Vero cells infected with VSV*ΔG(VP5-FLAG), VSVΔG(VP2,VP5-FLAG) and VSV*ΔG(NS1-FLAG) but not with VSV*ΔG(VP5)-, VSV*ΔG(NS1)-, or VSV*ΔG-infected cells (**Figure 11**).

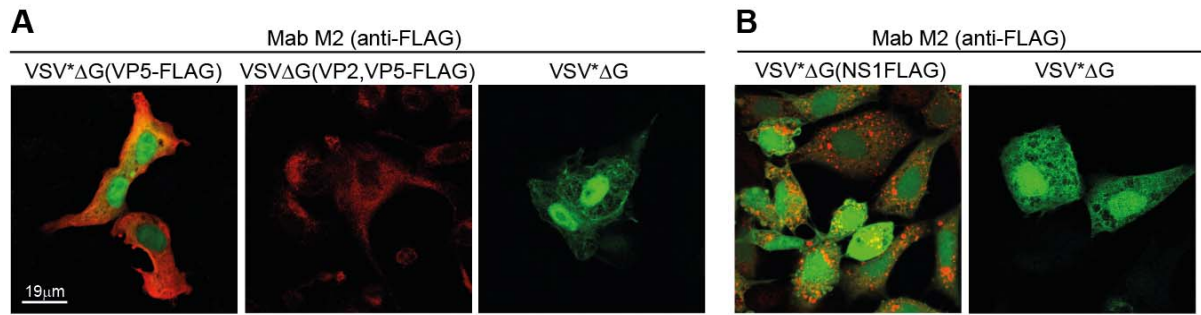


Figure 11. Immunofluorescence analysis of Vero cells expressing VP5 and NS1. (A) Cells were infected with either VSV*ΔG(VP5-FLAG), VSVΔG(VP2,VP5-FLAG), or VSV*ΔG. Expression of VP5 is indicated by red fluorescence using a monoclonal anti-FLAG antibody. (B) Cells infected with either VSV*ΔG(NS1-FLAG), or VSV*ΔG. Expression of NS1 is indicated by red fluorescence using a monoclonal anti-FLAG antibody.

For the remaining BTV antigens VP3 and NS3, monoclonal antibodies were not available. In order to obtain evidence for expression of these antigens, chickens were immunized with recombinant VSV replicons. Immune sera were then tested on Vero cells 24 h post infection with BTV-8 (**Figure 12**). All sera were found to react specifically with the infected cells.

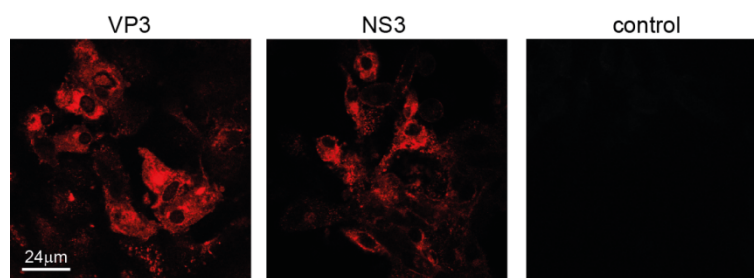


Figure 12. Immunofluorescence analysis of BTV-8 infected Vero cells stained with anti-VP3 and anti-NS3 immune sera. Sera obtained from chicken immunized with VSV-VRPs react with BTV-8 infected cells (red fluorescence). Scale bar represents 24 μm.

Taken together, these data show that infection of Vero cells with virus replicon particles (VRP) readily led to expression of BTV-8 antigens VP2, VP5, VP7 and NS1.

Evidence of expression of BTV-8 antigens VP3 and NS3 was obtained indirectly, since sera of immunized chickens reacted with BTV-8 infected cells. However, infectious VSV was not produced by the cells due to the lack of VSV G protein expression. Thus, we refer to the recombinant VSV vectors as virus replicon particles (VRP).

Analysis of antibody responses in VRP-immunized sheep

To evaluate the immunogenicity of recombinant VRPs in a natural BTV host, nine seronegative sheep were immunized three times at 3-week intervals. Three animals were immunized with cell culture supernatant containing at least 1×10^8 infectious units of each of the VRPs VSV Δ G(VP2,VP5), VSV Δ G(VP3,VP7) and VSV Δ G(NS1,NS3). Two animals were immunized with the VRPs VSV Δ G(VP3,VP7) and VSV Δ G(NS1,NS3) expressing four antigens in total. Four animals received VSV* Δ G as control. Furthermore, five animals that had been immunized with inactivated BTV-8 vaccine (BTVpur) two years ahead of the experiment were included in our analysis. No adverse side effects due to vaccination with VRPs were observed.

Three weeks after the third immunization, sera were prepared from sheep and analyzed for the presence of BTV-specific antibodies by immunofluorescence. All sera of sheep immunized with VRPs containing BTV antigens and BTVpur reacted specifically with BTV-8 infected cells, indicating that antibodies had been produced (**Figure 13**). In contrast, sera from VSV* Δ G-immunized sheep did not react.

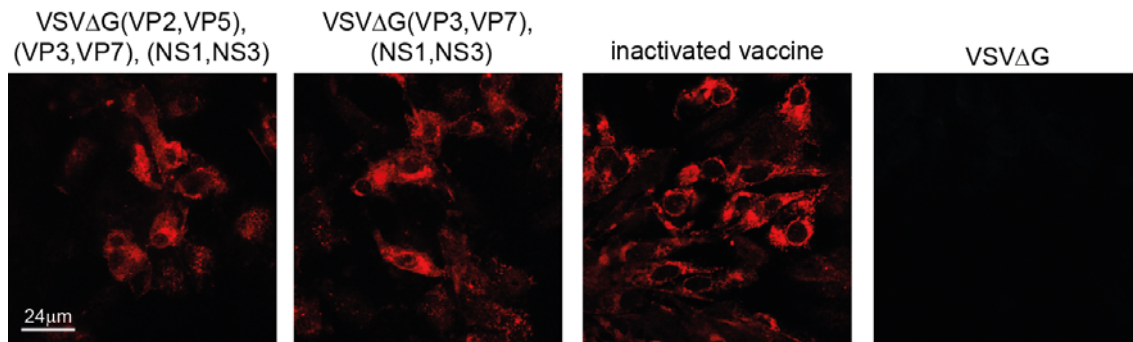


Figure 13. Immunofluorescence analysis of BTV-8 infected Vero cells at 24 hpi with BTV-8. Sera obtained from sheep after triple immunization with VSV-VRPs react with BTV-8 infected cells (red fluorescence). Scale bar represents 24 μ m.

Sera were tested for virus neutralizing activity against BTV-8. Sera of sheep before immunization with VSV-VRPs did not show any neutralizing activity confirming that the animals had no pre-existing immunity to BTV-8 (**Figure 14**). In contrast, sera of animals vaccinated with the commercially available inactivated vaccine (BTV_{pur}) had neutralizing activity. Sera collected 3 weeks after the first immunization showed some neutralizing activity at the highest serum concentration (1:4) tested. However, 3 weeks after the third immunization with the vaccine cocktail VSV Δ G(VP2,VP5), VSV Δ G(VP3,VP7), and VSV Δ G(NS1,NS3), all animals in the group developed high neutralizing antibody titres against BTV-8. The same sera did not neutralize BTV-1 or the VSV vector (data not shown). Sheep immunized with either VSV* Δ G or VSV Δ G(VP3,VP7) in combination with VSV Δ G(NS1,NS3) did not develop BTV-8 neutralizing antibodies. Taken together, these data demonstrate that the recombinant VRPs induced antibodies with neutralizing activity *in vitro*, when VP2 and VP5 were expressed from the VSV vector.

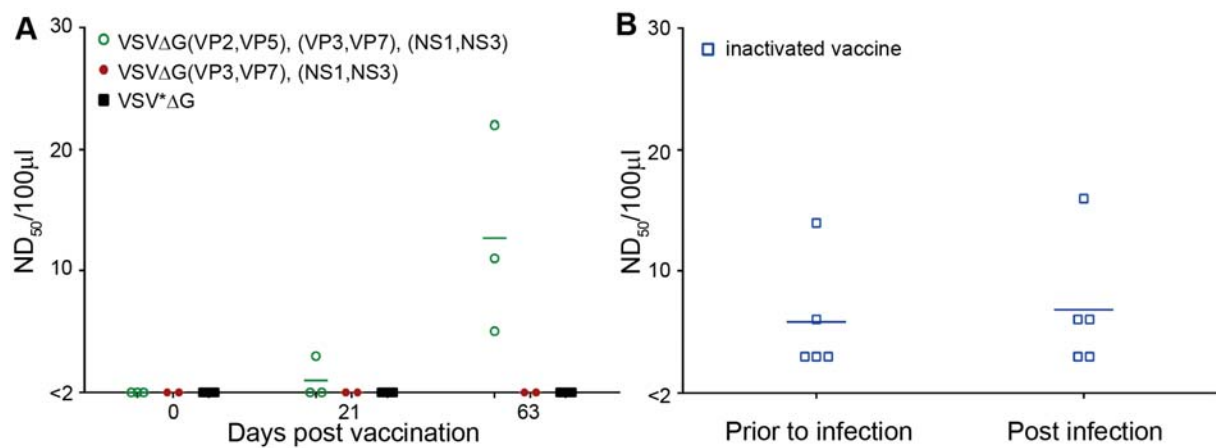
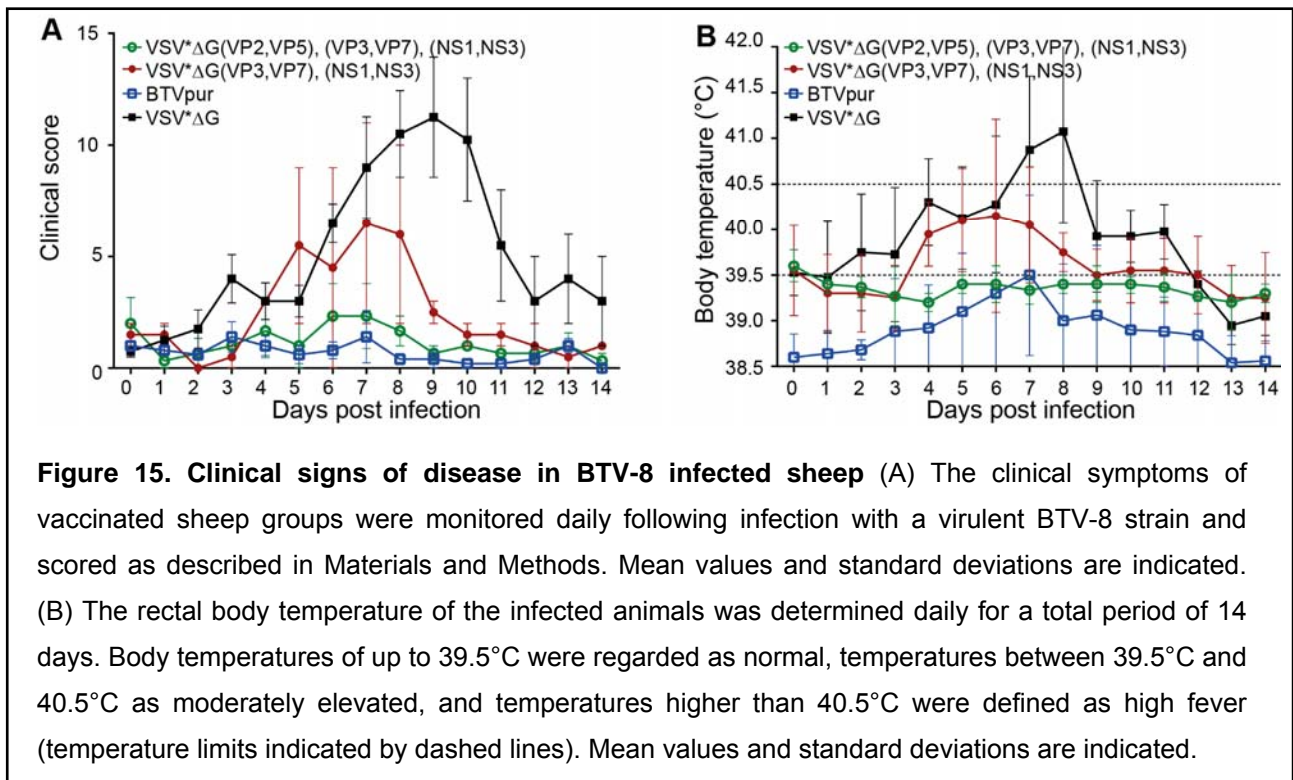


Figure 14. Neutralizing antibodies in sera of immunized sheep. Detection of BTV-8 neutralizing serum antibodies. (A) Sera were prepared from VRP-immunized sheep at day 0 (first vaccination), day 21 (second vaccination) and at day 63 (three weeks after the second immunization, prior to challenge infection). (B) Sera were prepared from sheep, immunized with inactivated BTV-8 vaccine (BTVpur) before and (14days) after infection with BTV-8. The infection or protection of cells was estimated 3 to 5 dpi according to the development of CPE. Neutralizing titres were calculated and expressed as ND₅₀/100 μ l. Values equal to or lower than 2 were regarded negative.

VRPs expressing a combination of VP2 and VP5 antigens protect sheep from challenge with pathogenic BTV-8

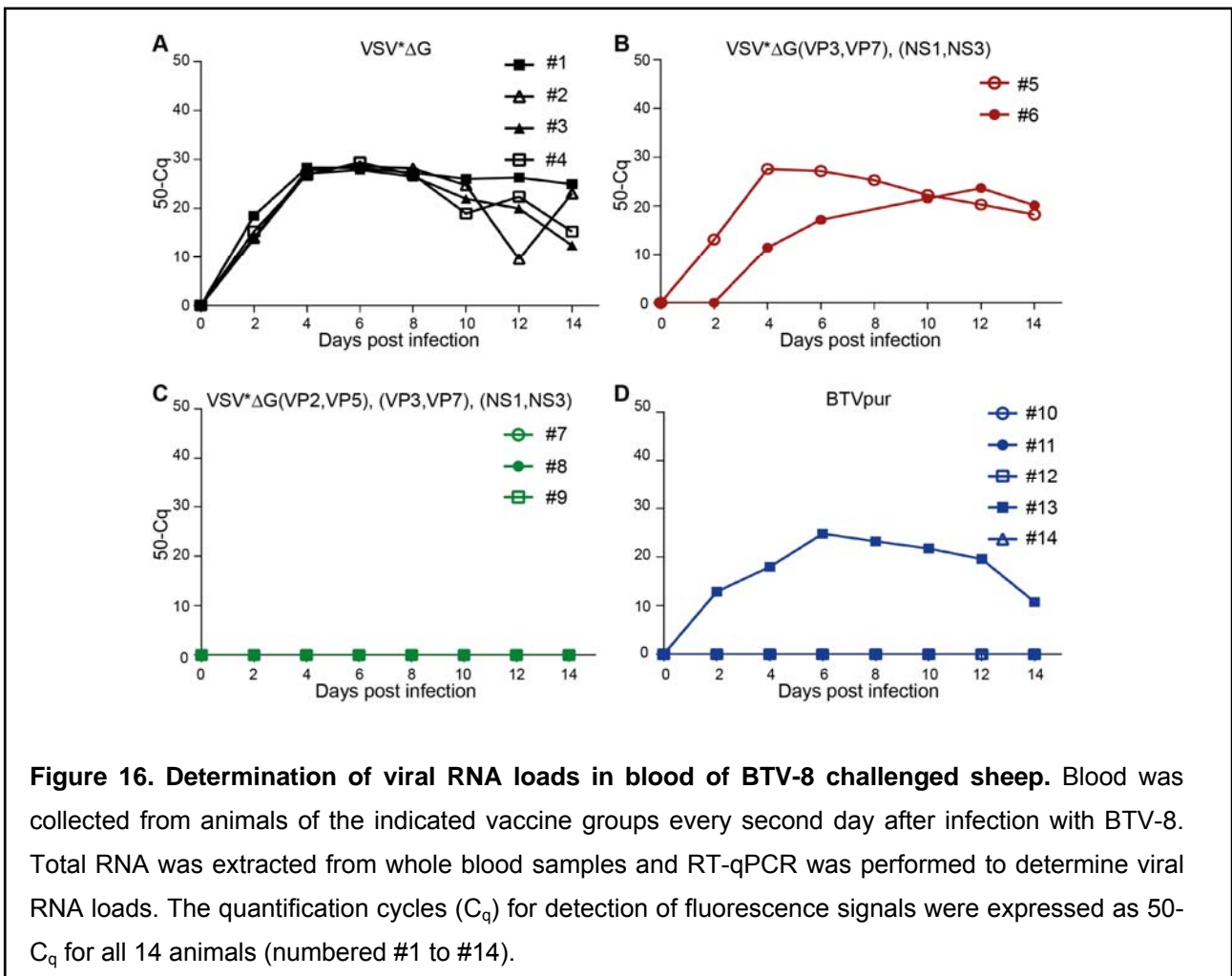
To evaluate the capacity of recombinant VRP vaccines to provide protection against pathogenic BTV-8, sheep were inoculated i.v. with erythrocyte suspension containing BTV-8 (C_q value of 18) 3 weeks after the second booster immunization. Sheep immunized with VSV* Δ G showed characteristic signs of bluetongue disease beginning at 5 dpi (**Figure 15A**). Typical symptoms were facial edema, nasal discharge, and lethargy. Two animals from this group were euthanized for humane reasons at day 11 post infection, as they developed severe lung edema and respiratory distress. All animals of this groups developed high fever, which peaked at day 8 after infection (**Figure 15B**). Animals immunized with the combination of VSV Δ G(VP3,VP7) and VSV Δ G(NS1,NS3) developed milder clinical symptoms (**Figure 15A**) and recovered from moderately elevated inner body temperature

(**Figure 15B**), sooner. Animals immunized with either the combination of VSV Δ G(VP2,VP5), VSV Δ G(VP3,VP7) and VSV Δ G(NS1,NS3) or the inactivated vaccine BTVpur did not develop BTV-specific clinical signs of disease (**Figure 15A**) and had normal rectal temperature ($< 39.5^{\circ}\text{C}$) throughout the experiment (**Figure 15B**).



The capacity of the vaccine-induced immune response to suppress viremia was evaluated by determining viral RNA loads in blood by quantitative RT-PCR (**Figure 16**). Animals immunized with either VSV* Δ G or combined VSV Δ G(VP3,VP7) and VSV Δ G(NS1,NS3) had high levels of viral RNA in blood, starting from day 2 post challenge and lasting until the end of the experiment at day 14. In contrast, BTV RNA was not detected in blood of sheep immunized with either the vaccine cocktail VSV Δ G(VP2,VP5), VSV Δ G(VP3,VP7) and VSV Δ G(NS1,NS3) or the inactivated

vaccine (BTVpur). Thus, recombinant VRPs expressing VP2 and VP5 induced protective immunity in sheep that prevented both viremia and bluetongue disease.



RESEARCH

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Vesicular stomatitis virus replicon expressing the VP2 outer capsid protein of bluetongue virus serotype 8 induces complete protection of sheep against challenge infection

Stefanie Kochinger, Nathalie Renevey, Martin A Hofmann and Gert Zimmer*

Abstract

Bluetongue virus (BTV) is an arthropod-borne pathogen that causes an often fatal, hemorrhagic disease in ruminants. Different BTV serotypes occur throughout many temperate and tropical regions of the world. In 2006, BTV serotype 8 (BTV-8) emerged in Central and Northern Europe for the first time. Although this outbreak was eventually controlled using inactivated virus vaccines, the epidemic caused significant economic losses not only from the disease in livestock but also from trade restrictions. To date, BTV vaccines that allow simple serological discrimination of infected and vaccinated animals (DIVA) have not been approved for use in livestock. In this study, we generated recombinant RNA replicon particles based on single-cycle vesicular stomatitis virus (VSV) vectors. Immunization of sheep with infectious VSV replicon particles expressing the outer capsid VP2 protein of BTV-8 resulted in induction of BTV-8 serotype-specific neutralizing antibodies. After challenge with a virulent BTV-8 strain, the vaccinated animals neither developed signs of disease nor showed viremia. In contrast, immunization of sheep with recombinant VP5 - the second outer capsid protein of BTV - did not confer protection. Discrimination of infected from vaccinated animals was readily achieved using an ELISA for detection of antibodies against the VP7 antigen. These data indicate that VSV replicon particles potentially represent a safe and efficacious vaccine platform with which to control future outbreaks by BTV-8 or other serotypes, especially in previously non-endemic regions where discrimination between vaccinated and infected animals is crucial.

Introduction

Bluetongue is a hemorrhagic disease of ruminants that is caused by bluetongue virus (BTV), a member of the genus *Orbivirus* within the family *Reoviridae* [1-3]. BTV is transmitted to livestock by blood-feeding *Culicoides* midges. In cattle, goats, and wild ruminants, BTV infection is typically asymptomatic despite prolonged viremia. These host species represent a potential reservoir for unnoticed dissemination of BTV in ruminant populations. In sheep, however, BTV infection often results in an acute disease with associated high morbidity and mortality, depending on the virulence of the virus and the sheep breed affected [4]. Typical symptoms of bluetongue in sheep include high fever, tissue edema, hemorrhages, and necrosis of the

upper gastrointestinal tract as well as of skeletal and cardiac musculature. Certain strains of BTV, notably the northern European strain of BTV-8, can cross the placental barrier, leading to infection of the developing fetus [5]. Hence, infection of pregnant animals with certain strains of the virus are frequently associated with abortions and malformations of offspring [6-8].

The BTV genome consists of 10 segments of dsRNA, which encode 7 structural (VP1 - VP7) and 5 non-structural proteins (NS1 - NS4, NS3a) [9]. The non-enveloped icosahedral virion particle contains an inner core which is formed by the viral RNA and the viral proteins VP1 (RNA polymerase), VP4 (capping enzyme), and VP6 (helicase) [10,11]. The inner core is surrounded by 3 concentric protein layers. The inner capsid layer consists of 120 copies of VP3 arranged as 60 dimers that form a scaffold for VP7. The outer capsid is composed of the viral proteins VP2

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and VP5, which are responsible for receptor binding, hemagglutination, and membrane penetration, respectively [12,13]. The large (110 kDa) attachment protein VP2 induces virus-neutralizing antibodies [14]. However, VP2 is highly variable and currently 26 different BTV serotypes can be distinguished by antibodies that show little or no cross-neutralizing activity [3]. Binding of VP5 to VP2 leads to a VP2 conformational change, which may affect recognition of epitopes by neutralizing antibodies [15,16]. All other structural and non-structural proteins are relatively conserved among different BTV serotypes. Therefore, most ELISAs for pan-BTV antibody detection employ the VP7 antigen [17].

A novel strain of BTV serotype 8 (BTV-8), which had not been detected in Europe before 2006, emerged as an epidemic wave in Western and Northern Europe [3,18,19]. This outbreak had a significant economic impact, not only because the disease caused morbidity and mortality in sheep and cattle but also because of restrictions imposed on livestock movement and trade [20]. The epizootic was controlled by a large-scale vaccination campaign using whole inactivated BTV-8. Although this vaccine induced strong protection against BTV-8 infection and disease, it did not allow the simple serological discrimination of infected from vaccinated animals (DIVA). Furthermore, vaccine production required the production of large amounts of infectious virus in cell culture, proper virus inactivation, and formulation of the antigen with adjuvant, all of which delayed production and added to the costs of producing the vaccine. Importantly, inactivated virus vaccines may not be suitable for the control of all serotypes of BTV as, for example, serotype 25 cannot be propagated in cell culture [21]. To overcome these hurdles, our goal was to develop and evaluate a generic vector vaccine in sheep, one that would comply with the DIVA principle. Although similar strategies using recombinant poxviruses (vaccinia, goatpox, and canarypox viruses), herpesviruses, and virus-like particles have been proven effective, we elected to evaluate the vesicular stomatitis virus (VSV) replicon system [22].

VSV is a non-segmented negative-strand RNA virus that is known to trigger a robust humoral immune response in many different host species [23]. Recombinant VSV vectors have been employed for experimental vaccination of mammals against a number of different pathogens such as human papilloma virus, hepatitis C virus, or influenza A virus [24]. A modified VSV vector in which the VSV glycoprotein (G) gene was replaced by the influenza A virus HA gene was shown to protect chickens from challenge infection with highly pathogenic avian influenza viruses [25,26]. This vector was propagated to high titers on helper cells providing the VSV G protein *in trans* and was able to infect a broad spectrum of different cell types. Due to the autonomous replication of the viral RNA, high-level

expression of recombinant antigens was achieved. However, due to the deletion of the VSV G protein gene, VSVΔG vectors are restricted to a single round of infection, contributing to their excellent biosafety profile. These novel vector vaccines were effective even though adjuvants were not employed [25,26].

In this study, propagation-incompetent VSVΔG vectors expressing the BTV-8 outer capsid proteins VP2 or VP5, or a combination of both, were generated. The expression of recombinant BTV antigens was studied and the immune response in sheep assessed. Immunized sheep were then infected with virulent BTV-8 and monitored for viremia, clinical symptoms, and antibody production. The DIVA principle was assessed using a commercially available VP7-based competitive ELISA.

Materials and methods

Cells

BHK-21 cells were obtained from the German Cell Culture Collection (DSZM, Braunschweig, Germany) and grown in Earle's minimal essential medium (MEM; Life Technologies, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS; Biowest Nuaillé, France). BHK-G43, a transgenic BHK-21 cell clone expressing the VSV G protein in a regulated manner, was maintained as described previously [27]. Vero cells (C1008) were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Glasgow's minimal essential medium (GMEM; Life Technologies) supplemented with 5% FBS.

Viruses

A German BTV-8 isolate (from 2008) was kindly provided by Bernd Hoffmann (FLI, Riems, Germany). A French isolate of BTV-1 was obtained from The Pirbright Institute (Pirbright, UK). Virus stocks were titrated on Vero cells. Virus infection was monitored taking advantage of the cytopathic effect apparent at day 5 post infection (pi). Infectious virus titres were calculated according to the Spearman-Kärber method and expressed as 50% tissue culture infectious doses per mL (TCID₅₀/mL).

In order to obtain virulent BTV-8 for challenge experiments, two adult BTV-seronegative Poll Dorset sheep were inoculated intravenously using erythrocytes prepared from a BTV-8 viremic calf. This virus was originally isolated in 2008 in Gummersbach, Germany. Following infection, blood was collected at daily intervals and analyzed for the presence of viral RNA by RT-qPCR [28]. The sheep were euthanized at day 5 pi, when a quantification cycle (C_q) value of 18 was determined in blood by RT-qPCR. Blood was collected from the animal, erythrocytes were washed and suspended in PBS, and frozen at -70 °C.

Construction of recombinant VRPs

Codon-optimized cDNA of VP2 (GenBank accession number AM498052) and VP5 (GenBank accession number AM498056) of the BTV-8 strain NET2006/04 was synthesized by GenScript (Piscataway, NJ, USA). For generation of recombinant VSV, the BTV-8 genes were inserted into the plasmid pVSV* using *MluI* and *BstEII* restriction sites upstream and downstream of the fourth transcription unit, thereby replacing the VSV G gene [25]. The resulting plasmids were designated pVSV*ΔG(VP2) and pVSV*ΔG(VP5), respectively. For generation of a dual antigen expression vector, the VP5 cDNA was inserted into pVSV*ΔG(VP2) using *XhoI* and *NheI* restriction sites located upstream and downstream of the fifth transcription unit, thereby replacing the green fluorescent protein (GFP) gene. The resulting plasmid was designated pVSVΔG(VP2,VP5). For expression of a modified VP5 containing a short peptide epitope at the C terminus, the VP5 gene without its Stop codon was inserted into the pCMV-3Tag-3 plasmid vector (Agilent Technologies, Inc., Santa Clara, CA, USA) upstream of and in frame with a triple FLAG epitope (DYKDDDDK)-coding region, which was followed by a Stop codon. The VP5-FLAG open reading frame was then amplified by PCR and inserted into either the fourth transcription unit of pVSV*ΔG or the fifth transcription unit of pVSV*ΔG(VP2). The resulting plasmids were designated pVSV*ΔG(VP5-FLAG) and pVSVΔG(VP2,VP5-FLAG), respectively. VSV replicon particles (VRPs) were generated and propagated on BHK-G43 helper cells as described previously [29].

Recombinant VSV*ΔG(VP2), VSV*ΔG(VP5), VSV*ΔG(VP5-FLAG), and VSV*ΔG [25] were titrated on BHK-21 cells taking advantage of the GFP reporter protein. Infectious titers were calculated and expressed as fluorescence-forming units per milliliter (ffu/mL). For detection of VSVΔG(VP2,VP5) and VSV*ΔG(VP2,VP5-Flag), infected cells were fixed with PBS containing 3% paraformaldehyde for 20 min at room temperature, washed with PBS containing 0.1 M (w/v) glycine, and permeabilized with 0.25% (v/v) of Triton-X100. The cells were incubated with rabbit anti-VSV serum and subsequently with a goat anti-rabbit horseradish peroxidase conjugate (dilution 1:500 in PBS; DAKO; Glostrup, Denmark) and finally stained with AEC/H₂O₂ substrate.

Immunofluorescence analysis

Vero cells grown on 12-mm-diameter cover slips were inoculated for 90 min with either VSV*ΔG, VSV*ΔG(VP2), VSV*ΔG(VP5-FLAG), VSVΔG(VP2,VP5) or pVSVΔG(VP2,VP5-FLAG) using a multiplicity of infection (MOI) of 3. At 8 hours pi, the cells were fixed with 3% paraformaldehyde for 20 min, washed with PBS containing 0.1 M (w/v) glycine, and incubated with PBS containing 0.25% (v/v) Triton X-100 for 5 min to permeabilize the plasma

membrane. The cells were incubated with a monoclonal antibody directed against either VP2 (clone 13C10, diluted 1:10; kindly provided by Dr Malte Dauber, FLI Riems, Germany) or a monoclonal antibody directed against the FLAG epitope (clone M2, diluted 1:50; Sigma-Aldrich, Deisenhofen, Germany) and subsequently with an anti-mouse IgG-Alexa 546 conjugate (dilution 1:500; Life Technologies, Carlsbad, CA, USA). Finally, the cells were washed with distilled water and embedded in Mowiol 4–88 (Sigma-Aldrich, Deisenhofen, Germany) mounting medium. The cells were analyzed using a Leica TCS-SL spectral confocal microscope (CFM) and Leica LCS software (Leica Microsystems AG, Glattpfugg, Switzerland).

To analyze sera from immunized sheep for the presence of virus-specific antibodies, Vero cells were grown on 12-mm-diameter cover slips, infected with BTV-8 (MOI of 1), and incubated for 48 h with medium containing 0.9% (w/v) methylcellulose. The cells were fixed, permeabilized, and incubated with serum (diluted 1:100 in PBS with 1% BSA) from immunized sheep and subsequently with anti-sheep IgG-Alexa 488 conjugate (dilution 1:500 in PBS; Life Technologies, Carlsbad, CA, USA). Fluorescence microscopy was performed using an Axio Observer Z1 inverted microscope (Zeiss, Jena, Germany).

Animal experiments

Animal trials were performed in compliance with the Swiss Animal Protection Law and approved by the Animal Welfare Committee of the Canton of Berne (authorization number 112/12). Twenty-four white Swiss White Alp sheep (12 to 24 months old), that were tested seronegative for BTV by ELISA (see below), were immunized intramuscularly with 2.5 mL of cell culture supernatant containing either VSV*ΔG, VSV*ΔG(VP2), VSV*ΔG(VP5), or VSVΔG(VP2,VP5), respectively. Three weeks (day 21) after the primary immunization (day 0), the animals received the same vaccine a second time. At day 42, 4 mL of erythrocyte suspension from BTV-8 infected viremic sheep (see above) were injected into the animals intravenously. Following inoculation, the animals were surveyed daily for clinical signs of disease which were scored according to a modified clinical scoring system [30]. All surviving animals were euthanized at day 14 post challenge.

Serological tests

Serum neutralization tests were performed as described previously [31]. Briefly, immune sera (heat-inactivated at 56 °C for 30 min) were serially diluted in FBS-free tissue culture medium and incubated for 1 h at 37 °C with 40 TCID₅₀/50 μL. Vero cells were added and incubated at 37 °C for 5 days. If not neutralized by immune serum, BTV caused a cytopathic effect 3 to 5 days pi. Neutralizing antibody titres were calculated according to the Spearman-Kärber method. For detection of VSV-

neutralizing antibodies, a recombinant VSV (serotype Indiana) expressing GFP was used [32]. A polyclonal anti-VSV serum was used as reference. For detection of VP7-specific serum antibodies, a commercially available competitive ELISA was used according to the manufacturer's protocol (VMRD, Pullman, USA).

RNA extraction and quantitative RT-PCR

Total RNA was extracted from whole blood samples using the Ambion MagMAX blood RNA isolation kit (Applied Biosystems, Foster City, California, USA) and stored at -70°C . For detection of viral RNA, a reverse transcriptase quantitative PCR (RT-qPCR) based on the amplification of segment 10 was performed in duplicates using *in vitro*-transcribed GFP RNA as internal control [28].

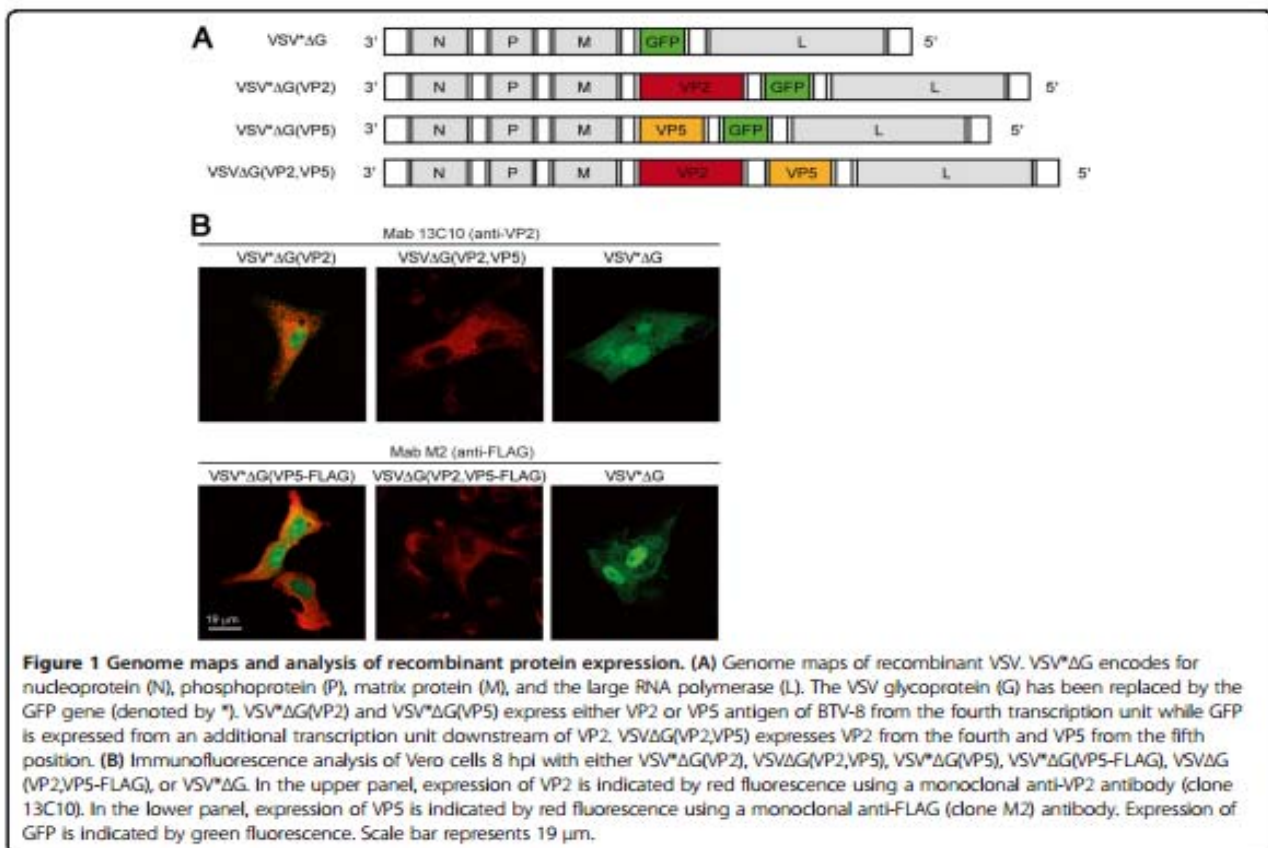
Statistical analysis

Statistical significance ($p < 0.05$) was considered using a two-way ANOVA and multiple comparisons were assessed using GraphPad Prism6 Sidak's post-hoc test.

Results

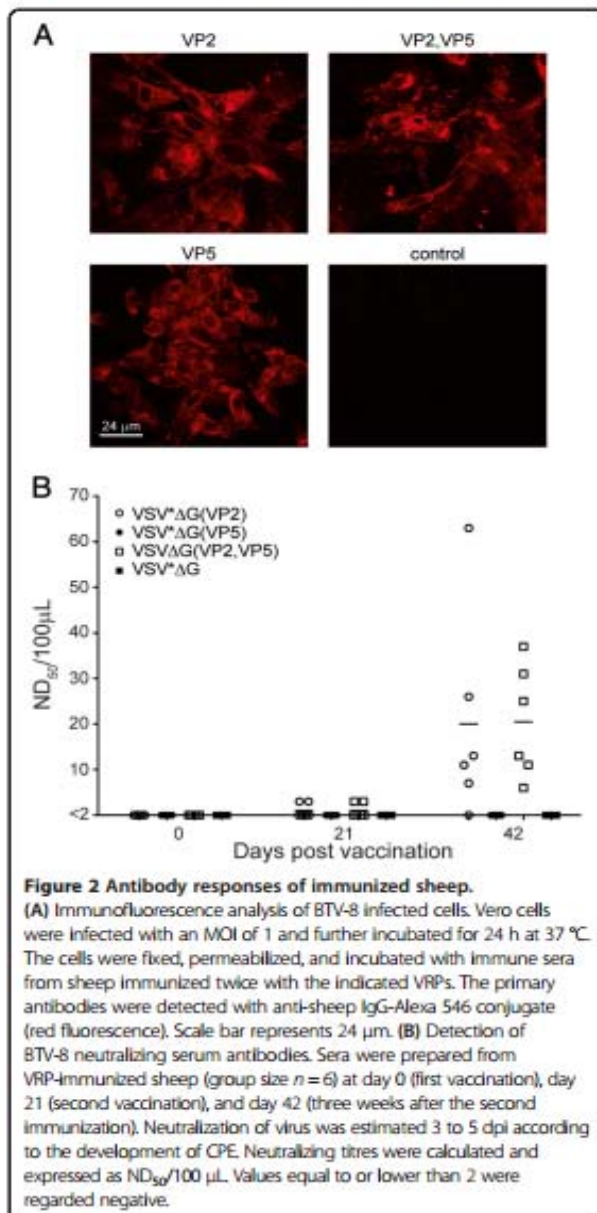
Generation of recombinant RNA replicon particles expressing BTV antigens

We previously generated a propagation-incompetent VSV vector by replacing the VSV glycoprotein (G) gene in the fourth transcription unit of the viral genome with genes encoding influenza virus antigens [25]. This vector contained an additional transcription unit at position 5, which was used to express the green fluorescent protein (GFP). Based on this vector platform we generated the recombinant viruses VSV Δ G(VP2) and VSV Δ G(VP5) containing the VP2 and VP5 genes of BTV-8 in the fourth transcription unit of the vector (Figure 1A). A vector expressing both antigens, VSV Δ G(VP2,VP5), was constructed by replacing the GFP gene in the fifth position of VSV Δ G(VP2) by the VP5 gene of BTV-8. VSV Δ G(VP5-FLAG) and VSV Δ G(VP2,VP5-FLAG) contained a triple FLAG epitope at the C terminus of VP5 and were generated to ease detection of this antigen. Recombinant VSV Δ G served as control vector as it did not express any BTV antigen [25]. All recombinant viruses were propagated on helper cells providing the VSV glycoprotein G *in trans*,



which yielded infectious titres of approximately 10^8 infectious units per mL of cell culture supernatant.

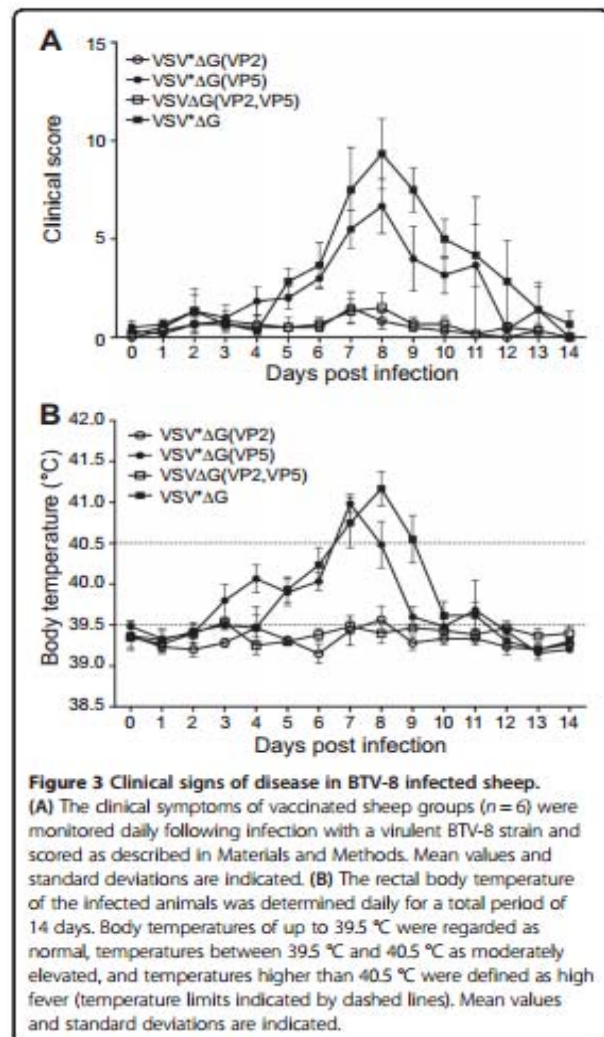
The expression of recombinant BTV-8 proteins was determined by immunofluorescence staining of Vero cells 6 hours pi with recombinant VSV. A BTV-8 VP2-specific monoclonal antibody reacted with cells that were infected with VSV Δ G(VP2) or VSV Δ G(VP2,VP5), whereas cells infected with VSV Δ G(VP5) or VSV Δ G were not recognized (Figure 1B). Since a VP5-specific antibody was not available, expression of VP5 was confirmed using a modified antigen with a triple FLAG epitope at the C terminus.



The monoclonal anti-FLAG antibody reacted specifically with Vero cells infected with VSV Δ G(VP5-FLAG) and VSV Δ G(VP2,VP5-FLAG) but not with VSV Δ G-infected cells (Figure 1B). Taken together, these data show that infection of Vero cells with virus replicon particles readily led to expression of BTV-8 antigens, both VP2 and VP5. However, infectious VSV was not produced by the cells since the VSV G protein was not expressed. Therefore, we refer to the recombinant VSV vectors as virus replicon particles (VRP).

Analysis of antibody responses in VRP-immunized sheep

To evaluate the immunogenicity of recombinant VRPs in a natural BTV host, 12 to 24 month-old Swiss White Alp sheep were divided into 4 groups of 6 animals each and immunized with cell culture supernatant containing at least 1×10^8 infectious units of either VSV Δ G(VP2),



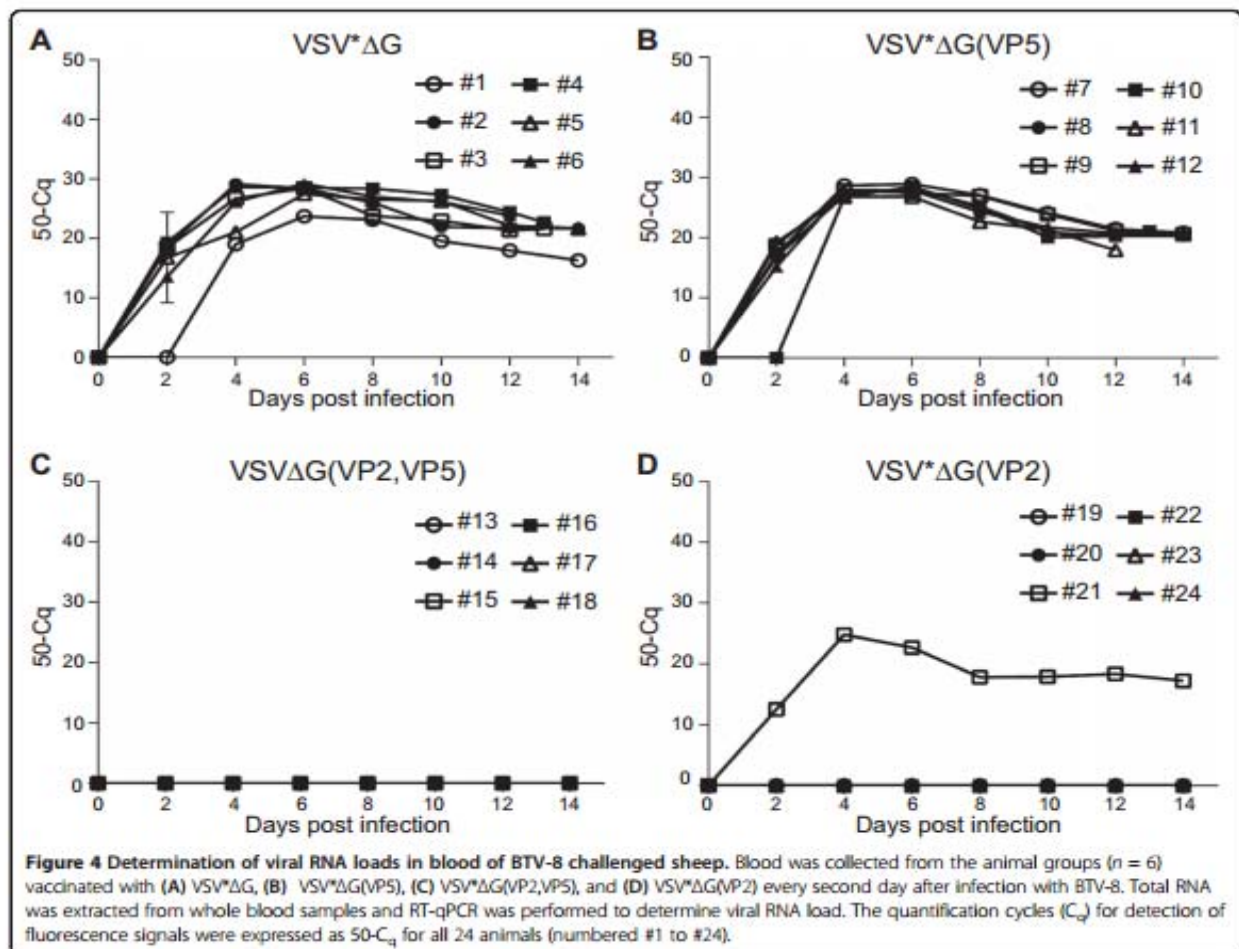
VSV*ΔG(VP5), VSVΔG(VP2,VP5), or VSV*ΔG. Adjuvants were not employed. After 3 weeks, the animals were immunized a second time with the same VRPs. No adverse side effects following vaccination were observed. Sera were analyzed 3 weeks after the second immunization for the presence of BTV-specific antibodies by immunofluorescence. Sera from sheep immunized with either VSV*ΔG (VP2), VSV*ΔG(VP5) or VSVΔG(VP2,VP5) reacted specifically with BTV-8 infected cells, indicating that all recombinant antigens were immunogenic and induced the production of BTV-8 specific antibodies (Figure 2A). Sera from VSV*ΔG-immunized sheep did not react.

The sheep immune sera were tested for neutralizing activity against BTV-8. Pre-immune sera did not show any neutralizing activity confirming that the animals had no pre-existing immunity to BTV-8 (Figure 2B). Sera collected 3 weeks after the first immunization showed limited neutralizing activity but only at the highest serum concentration used (1:4). However, 3 weeks after the second vaccination with VSV*ΔG(VP2) or VSVΔG

(VP2,VP5), all but one of the animals in the VSV*ΔG (VP2) group had developed high neutralizing antibody titres against BTV-8. The same sera did not neutralize BTV-1 or the VSV vector (data not shown). Sheep immunized with either VSV*ΔG or VSV*ΔG(VP5) did not develop BTV-8 neutralizing antibodies. Taken together, these data demonstrate that although recombinant VRPs induced antibodies against both VP2 and VP5, only anti-VP2 antibodies showed BTV-8 neutralizing activity *in vitro*.

VRPs expressing VP2 antigen protect sheep from challenge with pathogenic BTV-8

To evaluate the capacity of recombinant VRP vaccines to induce protective immunity against pathogenic BTV-8, sheep were inoculated intravenously 3 weeks after the second immunization with erythrocyte suspension from a BTV-8 viremic sheep (C_q value of 18). Sheep immunized with VSV*ΔG or VSV*ΔG(VP5) showed characteristic signs of bluetongue disease beginning at day 5 pi (Figure 3A).



Typical symptoms were facial edema, nasal discharge, and lethargy. Three animals from the VSV*ΔG group and two animals from the VSV*ΔG(VP5) group were euthanized for humane reasons at days 12 and 13 pi, respectively, as they developed severe lung edema and respiratory distress. All animals of the two groups developed high fever, which peaked at day 8 after infection (Figure 3B). In contrast, all animals immunized with VSVΔG(VP2,VP5) and 5 out of 6 animals immunized with VSV*ΔG(VP2) did not show any clinical signs of disease (Figure 3A) and had normal rectal temperature (<39.5 °C) throughout the experiment (Figure 3B). These differences were significant between 7 and 11 dpi in the clinical scoring and between 5 and 9 dpi for body temperature. The single animal in the VP2 group, which did not develop a detectable neutralizing antibody titre to BTV (see Figure 2B), showed moderate clinical signs of disease and fever for three days.

The capacity of the vaccine-induced immune response to suppress viremia was evaluated by determining viral RNA loads in blood by quantitative RT-qPCR (Figure 4). Animals immunized with either VSV*ΔG or VSV*ΔG(VP5) had high levels of viral RNA in blood, starting from day 2 post challenge and lasting until the end of the experiment at day 14, whereas BTV RNA was not detected in the blood of the 6 sheep immunized with VSVΔG(VP2, VP5). Likewise, 5 out of 6 sheep immunized with VSV*ΔG(VP2) did not develop any viremia. Thus, recombinant VRPs expressing VP2 alone or in combination with VP5 conferred protective immunity in sheep that prevented both viremia and development of disease.

VP7 antibodies used for differentiation of infected from vaccinated animals

At day 14 after infection, the animals were euthanized and sera tested for the presence of VP7-specific antibodies using a commercially available competitive ELISA (Figure 5). All animals seroconverted following challenge infection irrespective of the vaccine group tested, indicating that even in protected animals (groups VSV*ΔG(VP2) and VSV*ΔG(VP2,VP5)) limited replication of challenge virus must have occurred, which was sufficient to trigger an immune response against VP7. In contrast, all immunized sheep were tested negative for VP7 antibodies prior to infection, indicating that the VRP vaccine fully complied with the DIVA principle.

Discussion

The BTV outer capsid proteins VP2 and VP5 provide potential targets for neutralizing antibodies with all neutralizing epitopes recognized to date residing on VP2. The present study has demonstrated that recombinant VSV replicon particles expressing the VP2 protein of BTV-8 induce the production of serotype-specific neutralizing antibodies that protect sheep from disease and

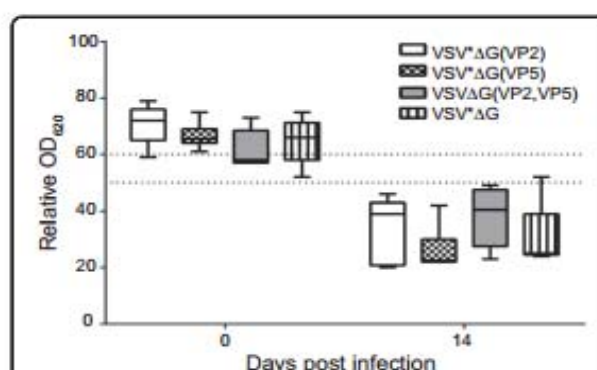


Figure 5 Detection of VP7 antibodies in serum of immunized sheep before and after challenge infection with BTV-8.

Blood was collected from sheep three weeks after the second immunization (prior to challenge infection with BTV-8) and two weeks after challenge by the end of the experiment. Serum antibodies specific for the VP7 antigen of BTV were detected using a commercially available competitive ELISA. Optical density was recorded at 620 nm and expressed as percentage OD₆₂₀ relative to an internal negative control. OD₆₂₀ values < 50% were considered positive, values between 50% and 60% were regarded as doubtful, and values higher than 60% as negative (thresholds indicated by dashed lines).

viremia following challenge infection with BTV-8. Our data confirm that immunization with VP2 alone is sufficient to induce a protective immune response in vaccinated animals [14,33,34]. VP5 has been reported to enhance the protective immune response if present in the vaccine along with VP2 [16,35]. Employing a canarypox virus vector expressing VP2 and VP5 of BTV-17, protection in sheep was achieved, but the relative contributions of VP2 and VP5 to protection have not been addressed [36]. In a recent study, expression of both VP2 and VP5 by a recombinant equine herpesvirus vector was necessary to protect mice against BTV-8 infection, whereas VP2 alone was not fully protective [37]. In our study, VRP co-expressing VP2 and VP5 did not induce higher neutralizing antibody titers than VRP expressing VP2. However, one animal in the VSV*ΔG(VP2) group did not develop neutralizing antibodies and was not protected, whereas all animals in the VSV*ΔG(VP2,VP5) group remained completely healthy following challenge with BTV-8. The question whether this higher protection rate is attributable to the co-expression of VP2 and VP5 should be addressed by additional experiments with a larger number of animals. A critical parameter for the efficacy of vector vaccines could be the level of expression and conformation of the recombinant VP2 antigen. If expression levels are low, the proportion of properly folded VP2 might increase in the presence of VP5 given their extensive conformational interaction [13]. However, if high expression levels of VP2 are achieved with a

given vector system, the amounts of correctly folded VP2 might be sufficient to induce neutralizing antibodies. Antibodies directed to VP5 may not have neutralizing properties on their own, probably because this antigen is not readily accessible for antibodies on the virion surface. Accordingly, the VP5 primary sequence is much more conserved than that of VP2, indicating that VP5 is subject to less immune pressure.

Conventional BTV vaccines have to be properly inactivated and formulated with adjuvants before they can be applied to animals once (sheep) or twice (cattle). Neutralizing antibodies were shown to persist up to four years after immunization [38,39]. The present VRP vaccine did not depend on adjuvants to induce a protective immune response in sheep, probably because this RNA virus vector is cytotoxic and provides sufficient innate signaling to stimulate the immune system. Any adverse side effects which could be caused by adjuvants [40] are thereby eliminated. However, significant levels of neutralizing serum antibodies were detected only after the animals had been vaccinated with the VRP vaccine twice. Additional studies are required to determine the protective effect of a single immunization. In addition, it will be interesting to see for how long VRP-induced neutralizing antibodies will persist in sheep.

Despite their efficacy, live-attenuated BTV vaccines have inherent disadvantages including transmission to insect vectors, lack of attenuation, reassortment of gene segments with field strains of the virus, and potential to cross the placenta to cause reproductive losses and teratogenic effects [41]. In contrast, the propagation-defective VRP vaccine does not cause disease nor will it be able to revert to virulence. As the G protein, the major antigen of VSV, is not expressed by VRPs, neutralizing antibodies against the viral vector are not induced. Accordingly, VRP vaccines are effective when used repeatedly [25,26,42]. Since antibodies directed to VSV G protein are lacking, vaccinated animals will appear seronegative in VSV diagnostic tests [43].

Vector vaccines are superior to conventional vaccines, since they can serve as marker vaccines [22]. The VRP vaccine did not contain the VP7 antigen and therefore allowed the discrimination of infected from vaccinated animals using a commercially available VP7 ELISA. In contrast, conventional (inactivated) BTV vaccines, live-attenuated and propagation-defective BTV vaccines [44,45] do not comply with the DIVA principle. This has complicated the serological surveillance of BTV in those European countries that implemented a vaccination campaign to control the BTV-8 outbreak in 2006. Moreover, the inability to discriminate between infected and vaccinated animals had a significant impact on economy due to restrictions on animal trade [46]. The recombinant replicon particles eliminate this problem as they can be used as marker vaccines.

Currently, there is no universal vaccine available that would protect against all 26 known serotypes of BTV. The VRP technology is not restricted to BTV-8 antigens but may be used to express VP2 antigen of other BTV serotypes as well. Thus, in case a new serotype emerges in a non-endemic region, a recombinant VRP vaccine matching this serotype could be rapidly produced and used for emergency vaccination. This should be particularly valuable for BTV strains for which inactivated vaccines are not readily available because the corresponding viruses do not propagate well in cell culture. Furthermore, this generic vaccine platform may be also employed to protect horses against infection with African horse sickness virus, an orbivirus of which 9 different serotypes are known.

In summary, this study has demonstrated that propagation-incompetent VSV replicon particles can efficiently protect a natural host against bluetongue disease and viremia. This safe and adjuvant-free vaccine technology complies with the DIVA principle, can be easily adapted to other serotypes and viruses, and is rapidly available in case of an emerging BTV outbreak.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SK performed practical and writing work throughout the study in partial fulfillment of the requirements for the D.V.M.-Ph.D. degree from the University of Berne (Graduate School for Cellular and Biomedical Sciences). NR participated in serum neutralization assays, RT-qPCR and ELISA. MH contributed to study design, data analysis and manuscript editing. GZ conceived the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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CONCLUSION AND OUTLOOK

The BTV outer capsid proteins VP2 and VP5 represent potential targets for neutralizing antibodies. However, all neutralizing epitopes identified to date reside on VP2, and VP5 is thought to exert a conformational effect on VP2 (26). The present study demonstrates that recombinant VRPs that express the VP2 protein of BTV-8 induce the production of serotype-specific neutralizing antibodies that protect sheep from disease and viremia following challenge infection with BTV-8. Our data confirm previous reports that have demonstrated that immunization with VP2 alone is sufficient to induce a protective immune response [25, 62, 63]. VP5 has been reported to enhance the protective immune response if present in the vaccine along with VP2 [27, 38]. Employing a canarypox virus vector expressing VP2 and VP5 of BTV-17, protection in sheep was achieved, but the relative contributions of VP2 and VP5 to protection have not been addressed [39]. In a recent study, expression of both VP2 and VP5 by a recombinant equine herpesvirus vector was necessary to protect mice against BTV-8 infection, whereas VP2 alone was not fully protective [41]. In our investigations, VRPs co-expressing VP2 and VP5 did not induce higher neutralizing antibody titers than VRP expressing only VP2, although one animal immunized with VP2 was not protected nor did it develop neutralizing antibodies. This discrepancy between VSV-based VRPs and other vaccines might be due to the different vector systems, i.e. different expression levels and differences in the conformation of recombinant VP2, especially if yields of recombinant VP2 are low. Thus, the proportion of properly folded VP2 might increase in the presence of VP5 given their extensive conformational interaction [24]. If high expression levels of VP2 can be achieved with a given vector system, the amounts of correctly folded VP2 might be sufficient to induce neutralizing antibodies. Antibodies directed to VP5 may

not have neutralizing properties on their own, probably because this protein is not accessible for antibodies on the surface of the virion. Furthermore, VP5 is more conserved than VP2, suggesting that VP5 is subject to less immune pressure.

Conventional BTV vaccines have to be properly inactivated and formulated with adjuvants before they can be applied to animals once (sheep) or twice (cattle). Neutralizing antibodies were shown to persist up to four years after immunization [64, 65]. The present VRP vaccine did not depend on adjuvants to induce a protective immune response in sheep, probably because the RNA virus vector is cytotoxic and so stimulates the innate immune system via pathogen recognition receptor. Any adverse side effects that could be caused by adjuvants [66] are thereby eliminated. However, significant levels of neutralizing serum antibodies were detected only after the animals had been vaccinated with the VRP vaccine twice. Additional studies are required to determine the protective effect of a single immunization. In addition, it will be interesting to see for how long VRP-induced neutralizing antibodies will persist in sheep.

Despite their efficacy, MLV (live-attenuated) BTV vaccines have inherent disadvantages, including potential transmission to vector midges, lack of attenuation, reassortment of gene segments with field strains of the virus, and potential to cross the placenta to cause reproductive losses and teratogenesis [67]. In contrast, the propagation-defective VRP vaccine does not cause disease nor will it be able to revert to virulence. As the VSV G protein which is the major immunogenic VSV protein is not expressed neither by the VRPs nor in non-helper cells, antibodies directed against the viral vector are not induced. Accordingly, VRP vaccines are effective when used repeatedly [51, 52, 68]. Since antibodies directed to VSV G

protein are lacking, vaccinated animals will appear seronegative in VSV neutralization tests [69].

Vector vaccines are superior to conventional vaccines, since they can serve as marker vaccines [42]. The VRP vaccine did not contain the VP7 antigen and therefore allowed the discrimination of infected from vaccinated animals using a commercially available BTV VP7 ELISA. In contrast, conventional (inactivated) BTV vaccines, live-attenuated and propagation-defective BTV vaccines [70, 71] do not comply with the DIVA principle. This has complicated the serological surveillance of BTV in those European countries that implemented a vaccination campaign to control the BTV-8 outbreak in 2006. Moreover, the inability to discriminate between infected and vaccinated animals had a significant impact on economy due to restrictions on animal trade [31]. The recombinant replicon particles eliminate this problem as they can be used as marker vaccines.

There is no universal vaccine available that would protect against all 26 currently known serotypes of BTV. The VRP technology is not restricted to BTV-8 antigens but can be also used to express VP2 antigen of other BTV serotypes. Thus, in the scenario where a new BTV serotype emerges in a non-endemic region, a recombinant VRP vaccine matching this serotype could be rapidly produced and used for emergency vaccination. This should be particularly valuable for BTV serotypes and strains for which inactivated vaccines are not readily available because the corresponding viruses do not propagate well in cell culture. Furthermore, this generic vaccine platform may be also be relevant to protect horses against infection with African horse sickness virus. In summary, this study demonstrates that propagation-incompetent VSV replicon particles can efficiently protect a natural host against BT disease and viremia. This safe and adjuvant-free

vaccine technology complies with the DIVA principle, can be easily adapted to other serotypes or other viruses, and is rapidly available in case of an emerging BTV outbreak.

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INSTITUTE OF VIROLOGY AND IMMUNOLOGY

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Development of a novel marker vaccine platform against Bluetongue

August 2011 – Juli 2014

- Execution of BLV-granted project
- Worked in a BSL-4 facility
- Conducted reverse genetics, serology, animal trials, qRT-PCR, cell culture, pathology
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INSTITUTE OF ANIMAL WELFARE

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Research Intern

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- Evaluated breeding facilities for pigs and chickens

CLINICAL VIROLOGY

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Diagnostics and research assistant

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- Virological and serological testing of samples
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- Project: Canine Parvovirus type 2; evaluation of a novel qPCR for diagnostic use
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AGES INSTITUTE OF FOOD SAFETY

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Exploring new places • **Skiing** in winter • **Reading** books and papers

LIST OF PUBLICATIONS

Kochinger S., Renevey N., Hofmann M. A., Zimmer, G. 2014. Vesicular stomatitis virus replicon expressing the VP2 outer capsid protein of bluetongue virus serotype 8 induces complete protection of sheep against challenge infection. *Vet Res* 2014, 45:64.

DECLARATION OF ORIGINALITY

Last name, first name: KOCHINGER, Stefanie

Matriculation number: 11-127-297

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 69, of 7 June 2011.

Place, date

Mittelhäusern, 01.06.2014


Signature

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