Development and Evaluation of a Subunit DIVA Vaccine Against Bluetongue Virus Serotype 8 in Cattle

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Abstract

Bluetongue virus (BTV) causes the primarily vector-borne bluetongue disease of ruminants, which poses a permanent threat to Europe since new serotypes and strains are frequently introduced. Vaccination of cattle is essential to control BTV outbreaks. Commercial attenuated and inactivated vaccines are efficacious in reducing BTV spread and disease, but do not fulfil all safety, adaptability, or production requirements. Additionally, no current vaccines allow the differentiation of infected from vaccinated animals (DIVA). DIVA vaccines enable surveillance of BTV epidemiology and vaccine efficacy, and facilitate a quick return for countries to a BTV-free status. This thesis presents the development and evaluation of a novel subunit DIVA vaccine against BTV serotype 8 (BTV-8) in cattle.

Five His-tagged recombinant BTV proteins (VP2, VP5 of BTV-8; NS1, NS2, NS3 of BTV-2) were produced in baculovirus or *E. coli* expression systems. Purification protocols were optimized for all but VP5. Based on the feasibility of protein production and the capability of the remaining four proteins to induce humoral or cellular immune responses in mice, VP2, NS1, and NS2 were selected to formulate an experimental vaccine combined to an ISCOM-matrix adjuvant (SubV).

Next, cattle were immunized twice at a three-week interval with SubV, a commercial inactivated vaccine, or a placebo. SubV induced humoral immune responses, including virus-neutralizing antibodies, against all three proteins, as well as a cellular immune response directed against NS1. These responses were of similar type and comparable magnitude between both vaccines, suggesting that SubV might provide protection that is at least as effective as the commercial vaccine. Finally, the protective efficacy of SubV was evaluated and complete virological and clinical protection against virulent BTV-8 challenge was observed following vaccination in calves. This was likely due to the induction of virus-neutralizing antibodies directed against VP2 of BTV-8 and cross-serotype T cell responses directed against NS1 and NS2 of BTV-2. Furthermore, SubV was shown to be DIVA-compliant based on the detection of antibodies directed against VP7, by using commercially-available diagnostic assays. This novel BTV subunit vaccine is a promising candidate and should be further developed.

Keywords: Bluetongue, virus, cattle, vaccine, subunit, immunogenicity, humoral, cellular, protection, DIVA

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Dedication

To my family,
and to all friends who form my
Swedish family

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Anderson, Jenna; Bréard, Emmanuel; Lövgren Bengtsson, Karin; Grönvik, Kjell-Olov; Zientara, Stéphan; Valarcher, Jean-François; and Sara Hägglund (2014). Purification, stability, and immunogenicity analyses of five bluetongue virus proteins for use in the development of a subunit vaccine that allows the differentiation of infected from vaccinated animals. *Clinical and Vaccine Immunology* 21(3), 443-452.

II Anderson, Jenna; Hägglund, Sara; Bréard, Emmanuel; Lövgren Bengtsson, Karin; Pringle, John; Zientara, Stéphan; and Jean-François Valarcher (2013). Evaluation of the immunogenicity of an experimental subunit vaccine that allows differentiation between infected and vaccinated animals against bluetongue virus serotype 8 in cattle. *Clinical and Vaccine Immunology* 20(8), 1115-1122.

III Anderson, Jenna; Hägglund, Sara; Bréard, Emmanuel; Riou, Mickaël; Zohari, Siamak; Comtet, Loic; Olofson, Ann-Sophie; Gélineau, Robert; Martin, Guillaume; Elvander, Marianne; Blomqvist, Gunilla; Zientara, Stéphan; and Jean-François Valarcher. Strong protection induced by an experimental DIVA subunit vaccine against bluetongue virus serotype 8 in cattle (submitted manuscript).

The contribution of JMA to the papers included in this thesis was as follows:

I Shared author, laboratory work, and analysis of results. Participant in animal experiments.

II Main author and primary contributor to laboratory work. Participant in animal experiments. Shared analysis of results.

III Main author and primary contributor to laboratory work for vaccine preparations. Participant in animal experiments. Shared laboratory work with samples from vaccinated animals and shared analysis of results.

Abbreviations

BHK-21	baby hamster kidney (cells)
BSA	bovine serum albumin
BT	bluetongue
BTV	bluetongue virus
$CCID_{50}$	50% cell culture infectious dose
cELISAs	competitive enzyme-linked immunosorbent assays
CID	collision-induced dissociation
CLPs	core-like particle vaccines
COD	corrected optical density
CPE	cytopathic effects
Ct	threshold cycle
CTLs	cytotoxic T cells
CV	commercial inactivated vaccine
ddH_2O	distilled deionized water
DISC	disabled infectious single cycle
DIVA	differentiation of infected from vaccinated animals
DNA	deoxyribonucleic acid
ds	double-stranded
ECE	embryonated chicken eggs
ECs	endothelial cells
EDTA	ethylene diamintetraacetic acid
ELISAs	enzyme-linked immunosorbent assays
GST	glutathione S-transferase
His	histidine
IFN	interferon
IFN-γ	interferon gamma
$IFNAR^{(-/-)}$	type 1 interferon receptor-deficient
INRA	French National Institute of Agricultural Research
KC	*Culicoides*-derived (cells)
kDa	kilodalton
LPS	lipopolysaccharide

MEM	minimal essential medium
MHC	major histocompatibility complex
MLVs	modified live virus vaccines
MW	molecular weight
NS	non-structural
OD	optical density
PBMCs	peripheral blood mononuclear cells
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
RT-qPCR	real time quantitative-PCR
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLU	Swedish University of Agricultural Sciences
SPF	specific pathogen free
ss	single-stranded
SubV	experimental subunit vaccine
SVA	National Veterinary Institute (Sweden)
$TCID_{50}$	50% tissue culture infective dose
TE	Tris-EDTA (buffer)
three Rs	replacement, refinement, reduction
TMB	3,3',5,5-tetramethylbenxidine
UV	ultraviolet radiation
VIBs	viral inclusion bodies
VLPs	virus-like particle vaccines
VP	viral (structural) protein

1 Introduction

When Edward Jenner removed biological material from a cowpox sore on a milkmaid, inoculated the gardener's son with it, and showed that the boy was protected from serious smallpox infection, he brought the concept of vaccination to the forefront in Europe. He also highlighted the natural synergism between human and veterinary medicine. In contrast to bacteria, against which a wide array of antibiotics have been discovered, we have few tools to combat viruses, and in combination with biosecurity measures, vaccination remains one of our best methods for preventing and controlling many viral diseases. Virus outbreaks in livestock can cause huge economic losses and animal welfare concerns, as well as impact food quantity and quality. This is exemplified by the recent bluetongue virus (BTV) outbreaks within the Europe, which differed from previous experiences and resulted in the loss of tens of thousands of animal lives and over 200 million euros in the Netherlands alone (Elbers *et al.*, 2009; Velthuis *et al.*, 2010). Additionally, European countries that were previously considered BTV-free took on costly control measures necessary to regain their BTV-free status. Due to climate- and trade-driven factors contributing to the spread of this arbovirus (Purse *et al.*, 2005; Beer *et al.*, 2013), BTV is considered a persistently emerging threat to the region. In many parts of the world, BTV is endemic and controlled by vaccination, but in regions in which the virus is emerging, new considerations for vaccines are required since traditional approaches confound surveillance and control measures in these areas. In this introduction, I will present BTV as well as both traditional and new approaches to vaccination against this virus.

1.1 History

Bluetongue (BT) disease is thought to have long existed on the African continent, though it has only been described in scientific literature since the

late eighteenth century (Spruell, 1905). The virus became of interest following the introduction of foreign sheep breeds to southern Africa during the years of British and Dutch colonization. Since then, BTV has made numerous incursions onto every continent except Antarctica, including North America in the 1950s (Hardy & Price, 1952) and Australia in 1975 (Ward, 1994). Since outbreaks of the virus are often associated with large economic losses, BTV was included from the mid-1960s on the OIE's previous "List A" of notifiable diseases.

Advancements in virology, entomology, and vaccinology have arisen from research concerning BTV. For example, in the early 1970s researchers from the Onderstepoort Veterinary Institute, South Africa, showed that the BTV genome consisted of double-stranded (ds) ribonucleic acid (RNA) (Verwoerd *et al.*, 1970); a controversial observation at a time when viruses were thought to possess only dsDNA (deoxyribonucleic acid) or single-stranded (ss) DNA or RNA genomes (Palmarini, 2014). Furthermore, while trying to determine if *Culicoides* insects transmitted BTV or epizootic hemorrhagic disease virus, researchers at Onderstepoort improved the design of a light trap to better collect midges (Du Toit, 1944), thereby contributing to the field of entomology. Additionally, advances in egg-based vaccine attenuation, application of lyophilization to vaccine production, and understanding the concept of virus serotypes kept BTV on the cutting edge of vaccinology throughout the twentieth century (Verwoerd, 2009).

1.2 BTV classification and viral characteristics

Bluetongue virus is a non-enveloped RNA virus classified as a Group III virus of the family *Reoviridae* (International Committee on Taxonomy of Viruses, 2012). It is the type species of the genus *Orbivirus*, which includes other economically important viruses such as African horse sickness virus and epizootic hemorrhagic disease virus. Like other orbiviruses, BTV has a dsRNA genome composed of ten linear segments of different lengths, surrounded by a double-capsid icosahedral shell that is approximately 85 nm in diameter (Gouet *et al.*, 1999). The BTV virion consists of twelve known proteins, including seven structural viral proteins (VP1-7) that provide the virus's structure, and five non-structural proteins (NS1-4, NS3A), which are produced only during infection (Figure 1).

One hundred and eighty copies of VP2 (molecular weight, MW: 111 kDa) and 360 copies of VP5 (MW: 59 kDa) form 60 triskelion and 120 globular structures, respectively, that fit together to make up the virus's outermost capsid. The inner BTV capsid is composed of 780 copies (260 trimers) of VP7

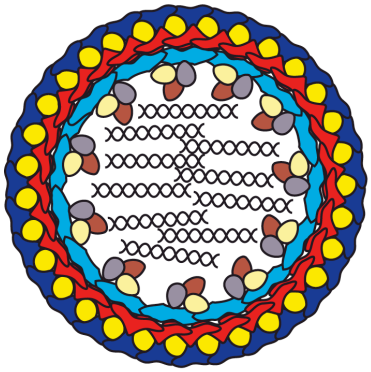
(MW: 39 kDa), organized as hexameric or pentameric rings and whose appearance provides the genus with its name (*orbi-* for "ring") (International Committee on Taxonomy of Viruses, 2012). Within this capsid lies an inner layer composed of 120 copies of VP3 (MW: 130 kDa), which in turn encloses the three minor structural proteins, VP1 (MW: 150 kDa), VP4 (MW: 76 kDa), and VP6 (MW: 36 kDa), as well as the virus's ten dsRNA genome segments.

Regarding genomic sequence, VP2 and VP5 are the most variable BTV proteins (Maan *et al.*, 2008). They act to facilitate attachment (Hassan & Roy, 1999) and entry into the host cell (Hassan & Roy, 1999; Hassan *et al.*, 2001). In particular, VP2 attaches to specific host cell receptors (including likely sialic acid and others (Zhang *et al.*, 2010)) to allow receptor-mediated endocytosis (Hassan *et al.*, 2001). VP5 has membrane-permeabilizing capabilities due to its many amphipathic helix regions, which can destabilize cellular membranes following attachment (Hassan *et al.*, 2001; Zhang *et al.*, 2010).

VP7 is an important structural protein of the virus as it contributes to BTV capside assembly (Limn *et al.*, 2000) and also attaches to cell receptors of *Culicoides* vectors (Xu *et al.*, 1997). The protein can bind dsRNA, and although the importance of this function is unknown, it may prevent the dsRNA from triggering apoptotic responses in infected cells (Diprose *et al.*, 2002). VP7 defines the BTV serogroup (Huismans & Erasmus, 1981) and is highly conserved across several orbiviruses (Oldfield *et al.*, 1990).

VP1, VP4, and VP6 function as RNA polymerase (Boyce *et al.*, 2004), the capping and methyltransferase enzyme (Ramadevi *et al.*, 1998), and helicase (Stäuber *et al.*, 1997), respectively. Together, these three proteins are called the transcription complex and are located at the vertices of the virion's inner core (Gouet *et al.*, 1999).

The five remaining BTV proteins are NS proteins. NS1 (MW: 64 kDa) forms tubules which are characteristic of orbivirus replication (Owens *et al.*, 2004), plays a role in viral morphogenesis and release from infected cells (Eaton *et al.*, 1988), as well as participates in the upregulation of viral protein synthesis (Boyce *et al.*, 2012). NS2 (MW: 41 kDa) helps form viral inclusion bodies (VIBs), binds ssRNA (Butan & Tucker, 2010), and aids virus replication and assembly (Horscroft & Roy, 2000). NS3 and its truncated version, NS3A, (MW: 25 and 24 kDa, respectively) are translated from the same genome segment and open reading frame (Van Dijk & Huismans, 1988) and work with NS1 to facilitate virion release from both insect and mammalian cells (Celma & Roy, 2009), perhaps through viroporin activities (Han & Harty, 2004). The functions of NS4 are less well elucidated since it has only recently been identified, but the protein seems to play a role in interactions between BTV and the host (Ratinier *et al.*, 2011).



Protein	Genome segment	Function
VP1	1	RNA polymerase
VP2	2	attachment, cell entry
VP3	3	structure, organization
VP4	4	capping/methyltransferase
VP5	6	membrane permeabilization
VP6	9*	helicase
VP7	7	capsid assembly
NS1	5	morphogenesis, release
NS2	8	binds ssRNA, viral assembly
NS3/A	10**	release (insect cells)
NS4	9*	host-virus interactions

*Different open reading frames; **same open reading frames

Figure 1. Schematic illustration of the BTV virion, including VP1-7 surrounding 10 dsRNA genome segments. The genome segment that encodes for each protein is indicated in the chart, as are the primary functions of the individual proteins.

Similar to other reoviruses including epizootic hemorrhagic disease virus, BTV is sensitive to temperature (inactivated by 3 h at 50°C or 15 min at 60°C), pH less than 6.0 or greater than 8.0, and certain chemicals and disinfectants including β-propiolactone, iodophores, and phenolic compounds (OIE, 2013). BTV is also sensitive to 254-nm UV radiation and can be inactivated after at least 20 min at 2.3 J/cm^2 (10-cm distance) (Ruscanu *et al.*, 2012). On the other hand, BTV is stable for years in the presence of protein, such as in blood or tissue samples, and is more stable at +4°C or -70°C than -20°C (Verwoerd & Erasmus, 2004). BTV can also be freeze-dried, for example for vaccine use.

1.3 Epidemiology and transmission

BTV epidemiology is described by the geographical distribution of different BTV serotypes as well as the presence of potential hosts and vectors. The virus neutralizing ability of antibodies that are produced against them determines the BTV serotype. This serotype-specificity of antibody production is attributed to the outer capsid protein VP2 (Huismans & Erasmus, 1981; Kahlon *et al.*, 1983; Roy *et al.*, 1990; Mertens *et al.*, 2007; Maan *et al.*, 2011b) and to some extent VP5 (Roy *et al.*, 1990). There are currently 26 BTV serotypes recognized worldwide (International Committee on Taxonomy of Viruses, 2012), two of which have been identified since the start of this project in 2010 (Hofmann *et al.*, 2008; Maan *et al.*, 2011b), plus a potential 27th serotype identified in Corsica just two months before printing this thesis (ProMED-mail, 2014). The BTV serotypes can be further divided into topotypes by genetic analysis of

certain RNA genome segments, such as segment 3 (encoding for VP3) or segment 10 (encoding for NS3). These topotypes indicate regional differences among serotypes and include "western" (the Americas, Africa, Europe) or "eastern" (Asia, Australia) genetic variations (Gould & Pritchard, 1990; Bonneau *et al.*, 1999; Balasuriya *et al.*, 2008), though it has been suggested that there are likely other topotypes corresponding to additional geographical lineages as well (Maan *et al.*, 2012).

1.3.1 Host and vector species

All ruminants are potential hosts of BTV, though species and breed, among other factors, can play a role in whether BTV infection manifests as clinical disease (please see section 1.5). BTV is typically transmitted among susceptible hosts through the bite of a competent *Culicoides* midge. Although there are over 1400 recognized species of *Culicoides*, only approximately 30 species are known to be competent for transmitting BTV (Aiello & Moses, 2012). Traditionally, *C. imicola* is considered the most important species for transmitting the virus in Europe, Africa, and the Middle East (Mellor, 2004), *C. sonorensis* (formerly referred to as *C. variipennis*) in North America (Tabachnick, 1996), and *C. insignis* in Central America (Mo *et al.*, 1994).

1.3.2 Routes of transmission

Since BTV is primarily considered a vector-borne virus, its epidemiology is strongly linked to the presence of competent vectors. However, in some cases, vertical (transplacental) transmission has been implicated for certain strains or serotypes (Luedke *et al.*, 1977a; Wouda *et al.*, 2008; Saegerman *et al.*, 2011), and there are even recent reports of direct contact transmission of some BTV serotypes in goats and cattle (Rasmussen *et al.*, 2013; Batten *et al.*, 2014).

Vector-borne transmission

Only female *Culicoides* midges feed on blood (Mellor *et al.*, 2000) and a single bite of an infected midge is sufficient to infect a susceptible sheep (Foster *et al.*, 1968). Conversely, the quantity of virus in the host blood considered necessary to infect a competent *Culicoides* midge is relatively low, at approximately 2.5-3 $\log_{10}$ $TCID_{50}$ (50% tissue culture infective dose) per milliliter (Fu *et al.*, 1999; Savini *et al.*, 2008). Based on studies primarily performed in the United Kingdom and United States using *C. variipennis*, the mechanisms of BTV infection and replication within its vector have been well elucidated (Figure 2). Briefly, the transmission of BTV to a susceptible ruminant begins with the bite of a competent female midge. The presence of trypsin-like proteins in the saliva of competent midges may aid the infectivity of BTV in insects, likely by cleaving VP2 from the virion to generate virus

subparticles that have been shown to be up to ten times more infectious to *Culicoides*-derived, but not mammalian, cells (Darpel *et al.*, 2011). The importance of these infectious virus subparticles in BTV transmission is unknown but they appear to facilitate virus entry into insect cells and may be linked to variations in the competencies of different *Culicoides* species (Mertens *et al.*, 1996).

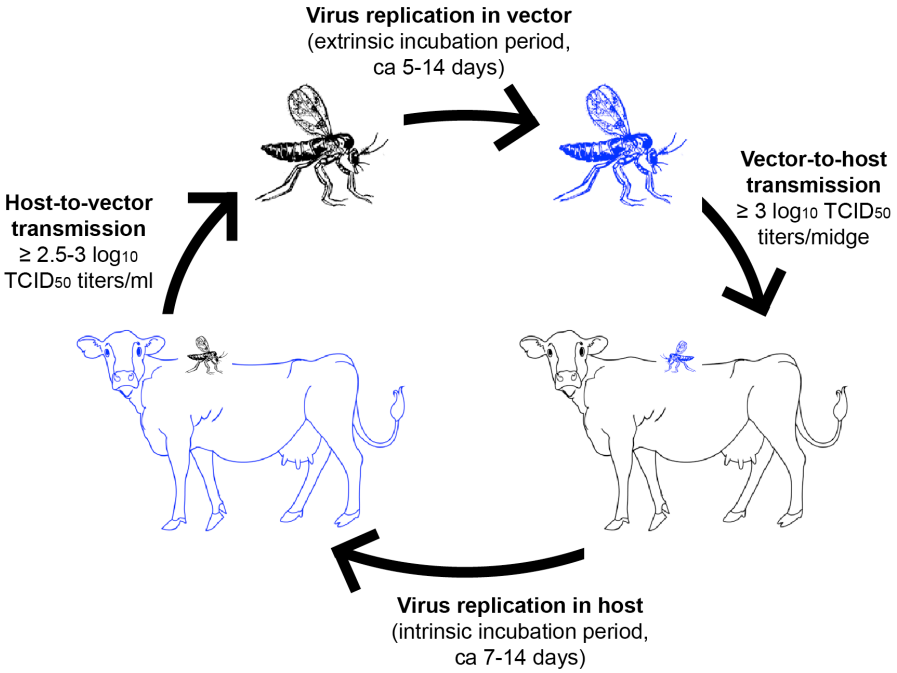


Figure 2. BTV vector-borne transmission cycle (adapted from (Purse *et al.*, 2005; Wilson *et al.*, 2009)).

The virus replicates first in the midgut cells (day 0), then in the fat body cells (day 1) and neural tissues (day 3) (Fu *et al.*, 1999). During these first three days following ingestion of the virus, an eclipse or partial eclipse phase occurs, such that either no BTV in the former case, or some BTV in the latter case, are detectable within the insect vector (Mellor *et al.*, 2009). It is thought that this phase occurs because the virus is being inactivated in the insect's gut lumen, or perhaps due to viral excretion from the gut cells before proliferation is evident (Mellor *et al.*, 2009). By day 5, BTV can be recognized in the salivary glands of the infected insect, where the virus replicates to approximately 1000 to 10000 times its day 0 titers and plateaus at these levels for the remainder of the insect's life (Foster & Jones, 1979). Researchers have

speculated that these final BTV titers may be limited by either the number of susceptible cells within a midge, or by a virus-vector balance that modulates the negative impact of virus replication on the vector (Mellor *et al.*, 2009). The lifespan of a *Culicoides* midge is generally only 20 days but under consistently mild ambient conditions, it can approach 90 days in the field (Mellor *et al.*, 2000) or laboratory (Goffredo *et al.*, 2004).

Following the first infectious bite from a female *Culicoides* midge, BTV transmission from ruminant host to vector is possible beginning between one and two weeks (Figure 2). Since females feed multiple times over their lifespan at three-to-four-day intervals, virus transmission may occur already at the third feeding (Mehlhorn *et al.*, 2007). It has been shown under both field and laboratory conditions that different midge populations demonstrate varying susceptibilities to different BTV serotypes, as well as to the same BTV serotype (Jones & Foster, 1978; Jennings & Mellor, 1987), at least partly due to species-specific saliva proteins. Furthermore, ambient temperature has also been shown to play a role in transmission, with transmission likelihood based on the balance between high temperatures that decrease vector lifespan but encourage an increase in vector bites, and conversely, low temperatures that increase vector lifespan yet result in a decrease of viral replication (Mellor *et al.*, 2009). For example, a study of experimental infection of competent South African *Culicoides* species demonstrated that in *C. bolitinos* held at 25°C and 15°C, titers of BTV-1 reached transmission potential (defined as ≥ 3 $\log_{10}$ $TCID_{50}$ per midge) two and eight days following infection, respectively, while in *C. imicola* held at 30°C and 23.5°C, BTV-1 titers reached this transmission potential four and ten days following experimental infection (Paweska *et al.*, 2002). At temperatures under 10-15°C, BTV replication is considered to cease entirely (Mullens *et al.*, 1995; Paweska *et al.*, 2002; Carpenter *et al.*, 2011), but like African horse sickness virus, appears to persist for at least three weeks in surviving infected insects and can resume replication with warmer temperatures (Mullens *et al.*, 1995; Wellby *et al.*, 1996).

Vertical transmission

Although vector-borne transmission is the most common mode of BTV spread, cases of transplacental transmission following infection of pregnant sheep and cattle with certain BTV serotypes or strains have been reported (Luedke *et al.*, 1977a; Wouda *et al.*, 2008; Saegerman *et al.*, 2011). Transplacental transmission was first documented in vaccine or laboratory strains, including BTV-1, -2, -4, -11, and -23, that had passage histories including chicken egg or cell lines (EFSA Panel on Animal Health and Welfare (AHAW), 2011; Rasmussen *et al.*, 2013). The BTV-8 strain which circulated

in Europe from 2006 is the only field strain to date that has been shown to be transplacentally transmissible (De Clercq *et al.*, 2008; Desmecht *et al.*, 2008; Saegerman *et al.*, 2011). BTV-8 RNA has additionally been detected up to ten days after birth in three calves (threshold cycle (Ct) 22, 27, and 27) that were born to two seropositive, but negative by reverse transcription-polymerase chain reaction (RT-PCR), heifers (Menzies *et al.*, 2008). Virus was also isolated from the calf with the lowest Ct (22). Furthermore, BTV has been isolated from two newborn calves following natural transplacental transmission (De Clercq *et al.*, 2008). While the duration of viremia in calves is unknown, there are indications that they are able to clear the virus and are not persistently infected (Maclachlan & Osburn, 2008).

In addition to viral strain, stage of gestation also likely plays a role in the transplacental transmission of BTV (Flanagan & Johnson, 1995; EFSA Panel on Animal Health and Welfare (AHAW), 2011; Rasmussen *et al.*, 2013). In particular, infection or vaccination of pregnant dams or ewes at early but not late gestation increases the risk of abortions or birth of abnormal or weak calves and lambs (Osburn, 1972; Thomas *et al.*, 1986; Waldvogel *et al.*, 1992b; Flanagan & Johnson, 1995). Moreover, as intramuscular inoculation of late-term fetuses with a virulent BTV strain, but not an avirulent strain, can result in premature delivery or abortion of weak calves (Waldvogel *et al.*, 1992a), it appears that BTV may not be able to cross the placental barrier during late gestation.

Some studies have been performed to determine the potential of BTV transmission by artificial insemination or semen from naturally- or experimentally-infected rams or bulls, as reviewed in (Wrathall *et al.*, 2006; EFSA Panel on Animal Health and Welfare (AHAW), 2011). As observed with transplacental transmission, some literature suggests that the probability of BTV excretion in semen may depend on whether the virus is a field or laboratory-adapted strain (Kirkland *et al.*, 2004). Although there has been debate about whether the virus can be transmitted in this manner, there are nonetheless regulations impacting the transport of semen originating from animals in BTV zones.

Potential direct transmission

New evidence suggests that BTV-26, a serotype identified in Kuwait in 2010 (Maan *et al.*, 2011a), appears to be transmissible in goats by close, direct contact (Batten *et al.*, 2014). There have also been reports of potential horizontal transmission of BTV-2 in sheep (Rasmussen *et al.*, 2013) and of BTV-8 in cattle following contact with BTV-infected placentas (Menzies *et al.*, 2008) or after ingestion of BTV-spiked colostrum (Backx *et al.*, 2009). Type 1

interferon (IFN) receptor-deficient (IFNAR$^{(-/-)}$) mice, used as a mouse model for BTV infection (Calvo-Pinilla *et al.*, 2009a), have also been shown to be orally susceptible to BTV-8 infection (Calvo-Pinilla *et al.*, 2010). However, these mice are also susceptible to BTV infection by other routes of infection such as subcutanous (Jabbar *et al.*, 2013) and intravenous routes (Calvo-Pinilla *et al.*, 2009b; Mohd Jaafar *et al.*, 2014). Since control measures are primarily based on vector-borne transmission of the virus, widespread direct transmission of specific BTV serotypes or strains could greatly impact virus spread as well as BTV research, diagnostic, and control strategies (Batten *et al.*, 2014), and should be further explored.

1.3.3 Geographical and seasonal distribution

As shown in Figure 3, BTV has been identified on every continent except Antarctica, though the geographical distribution of BTV serotypes differs by region. Typically, clinical BT disease can occur year-round in tropical regions, where the virus is endemic, and seasonally (late summer and fall) in temperate regions (Gerry *et al.*, 2001; Charron *et al.*, 2011; Coetzee *et al.*, 2012; Mayo *et al.*, 2012).

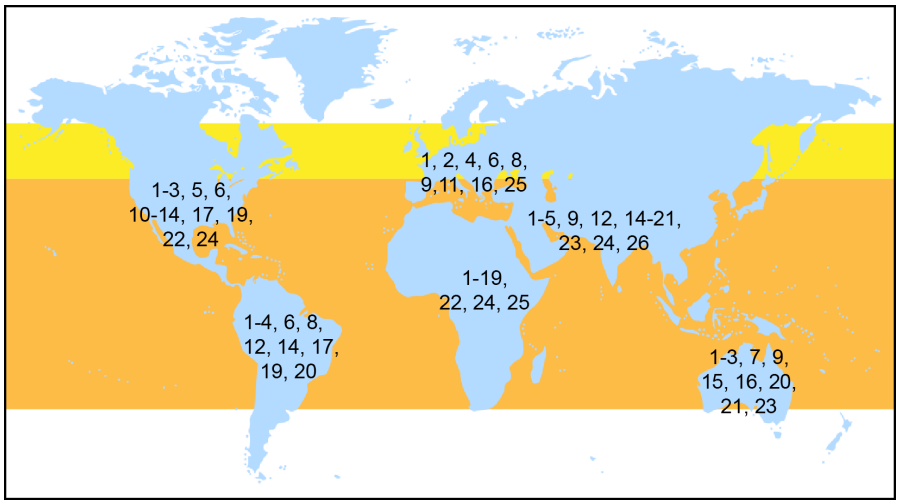


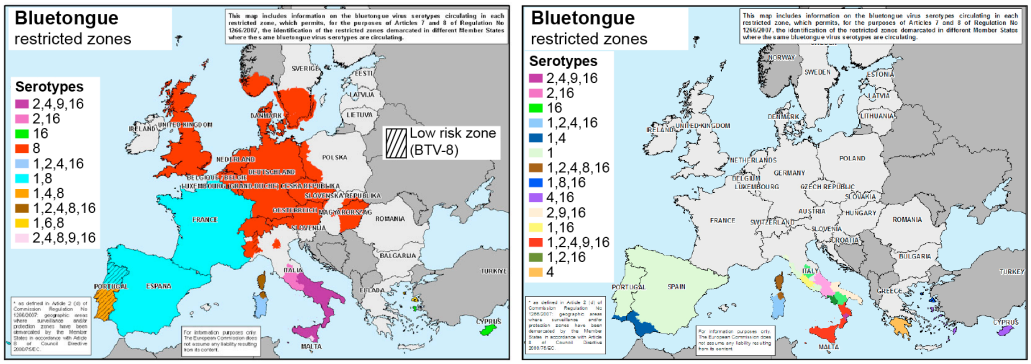
Figure 3. Global distribution of BTV serotypes (modified from (Wilson *et al.*, 2009; Tabachnick, 2010)). The recognized latitudinal range of BTV-competent *Culicoides* midges until 1998 is indicated by the orange band while the yellow band indicates the updated latitudinal range of competent vectors, following BTV-8 outbreaks in Europe.

BTV in Europe

Until recently, BTV was considered to be limited to the African continent and regarded as the cause of an exotic disease with some infrequent and short-lived incursions into European countries (Gibbs & Greiner, 1994). In 1998, BTV-9

was identified in Turkey, Bulgaria, and Greece, then spread to neighboring countries (including Italy) through 2001 (Zientara & Sánchez-Vizcaíno, 2013). BTV-1, -4, and -16 were also identified in southern Europe during that time, and in 2000, BTV-2 was detected in France and Spain (Saegerman *et al.*, 2008). These BTV serotypes continued to move westward and by 2005, the virus had been identified in over a dozen western and southern European countries and caused the death of over one million sheep (Saegerman *et al.*, 2008). In August 2006, a new outbreak of BTV occurred, this time due to serotype 8 and extending from central Europe (Luxembourg, northern France) to northern Europe (Belgium, Germany, and the Netherlands) (Saegerman *et al.*, 2008). This northward expansion of BTV was likely at least partly due to the climate change-related expansion of its insect vector (Purse *et al.*, 2005), but the exact mechanisms of the emergence of BTV-8 in northern Europe are unknown. The virus was eventually detected in southern Sweden in September 2008 (Lewerin *et al.*, 2010), and reached its northernmost-recorded latitude of 53°N in Vest-Agder county, Norway, in February 2009 (ProMED-mail, 2009). Around the same time, vaccine strains of BTV-6 and -11 were also identified in a limited area in northern Europe, but did not reach the same latitude as BTV-8 (De Clercq *et al.*, 2009; van Rijn *et al.*, 2012). Concurrent with the BTV-8 outbreaks, an outbreak of BTV-1 in southern Europe, including Spain, Italy, and France, also occurred from 2007, and resulted in similar clinical disease in sheep, but not cattle (Allepuz *et al.*, 2010).

Since then and following vaccination campaigns, much of northern and central Europe have regained BTV-free status, but BTV restricted zones remain in Spain, Portugal, southern Italy (including Sardinia), Corsica, Malta, Cyprus and several Greek islands (Figure 4). The BTV-8 which was first identified in the northern European outbreak remains present in certain areas of southern Europe but appears to have disappeared entirely from northern European countries.



A B

Figure 4. Bluetongue serotype distribution within the European Union in September 2007 (A) and June 2014 (B) (European Commission, 2014).

Overwintering

One mystery that remains to be solved regarding BTV is its apparent ability to "overwinter," or to survive during cold seasons in temperate regions where there is ostensibly no contact between the vector and its host. As reviewed by Wilson (Wilson *et al.*, 2008), three general principles suggest possible explanations for this phenomenon of BTV: i) the virus is able to persist in the vector; ii) the virus is able to persist in the host; or iii) the virus is able to persist in different vectors or hosts as yet undetermined.

If BTV is able to persist in the *Culicoides* vector, it should be able to be identified in either the adult (indicative of persistent infection) or larval stage (indicative of transovarial passage). Most *Culicoides* survive cold winter months as larvae (Kettle, 1962), but full BTV RNA has rarely been detected in the larval or pupal stage of midges and the virus has not been isolated from any of these samples (Mellor, 1990; White *et al.*, 2005). However, this potential mechanism of overwintering remains to be further evaluated. It is possible that under mild winter conditions or by moving indoors (European Food Safety Authority; Losson *et al.*, 2007; Lysyk & Danyk, 2007), some adult midges may also be able to survive beyond their usual 20-day lifespan. Although theoretically possible over mild winters, it seems unlikely that adult *Culicoides* are able to survive the three-to-nine months required for overwintering.

Alternatively, if BTV is able to overwinter in ruminant populations, it must accomplish this either through persistent infection where no detectable virus is present, through vertical (transplacental) transmission, or through horizontal (sexual) transmission. Non-infectious BTV RNA has been detected in blood several months after infection in cattle (Katz *et al.*, 1994), and some studies have been suggestive of persistent experimental BTV infection of cattle and

sheep (Luedke *et al.*, 1977b; Takamatsu *et al.*, 2003). However, these studies have not been repeated under natural conditions. Transplacental transmission has also been documented for certain BTV serotypes or strains (please refer to section 1.3.2) and there are indications that cows infected at an intermediate stage of gestation can give birth to viremic calves (Gibbs *et al.*, 1979; De Clercq *et al.*, 2008), which may potentially provide a mechanism for overwintering. On the other hand, it seems unlikely that horizontal (sexual) transmission would provide an overwintering opportunity, as trade regulations stipulate that semen must be tested for BTV before shipping and it is not certain that BTV can be transmitted horizontally (EFSA Panel on Animal Health and Welfare (AHAW), 2011).

Finally, transmission via ticks or sheep keds may also provide opportunities for overwintering (Luedke *et al.*, 1965; Stott *et al.*, 1985b; Bouwknegt *et al.*, 2010), but the potential ability of these insects to transmit BTV has only been shown under experimental conditions. Other ruminant hosts that may go unnoticed if subclinically infected with BTV, such as wildlife, may also provide an opportunity for BTV overwintering and should be further investigated.

1.4 Host-pathogen interactions

1.4.1 Pathogenesis and innate immune response to BTV

In typical cases, BTV enters the ruminant host through the bite of an infected *Culicoides* midge. This transmission is effective, as only 3 $\log_{10}$ $TCID_{50}$ of virus is required per midge to infect a susceptible ruminant (Fu *et al.*, 1999). Proteins present in the saliva of competent *Culicoides* may also play a role in BTV transmission, by recruiting inflammatory leukocytes, such as γδ T cells, in which the virus can replicate (Takamatsu *et al.*, 2003; Darpel *et al.*, 2011). BTV replicates in two phases called primary and secondary replication, respectively (Dal Pozzo *et al.*, 2009). Its primary replication occurs over approximately two to three days in conventional dendritic cells at the inoculation site and in regional lymph nodes (Barratt-Boyes & MacLachlan, 1994; Hemati *et al.*, 2009). Subsequently, BTV-infected lymphocytes and monocytes, dendritic cells, and macrophages from the lymph nodes (Barratt-Boyes & MacLachlan, 1994; MacLachlan *et al.*, 2014) enter circulation and are carried to the spleen, lungs, liver, other lymph nodes, or other organs (MacLachlan *et al.*, 1990) for secondary replication in mononuclear phagocytes and endothelial cells (Barratt-Boyes & MacLachlan, 1994; MacLachlan, 2004). Secondary replication occurs over the subsequent four to twenty days. During this time, BTV is physically associated with platelets,

mononuclear cells, and erythrocytes (MacLachlan *et al.*, 1990). Depending on the species, age, and virus serotype, viral RNA can be detected by RT-PCR following secondary replication for up to 167 days or 222 days in sheep and cattle, respectively (Richards *et al.*, 1988; Vögtlin *et al.*, 2013). However, it is important to note that although BTV RNA can be detected in the blood of infected cattle for long periods of time, the infectious virus itself has only been isolated for 39-56 days (MacLachlan *et al.*, 1994; Di Gialleonardo *et al.*, 2011), and any PCR-detected BTV after this point has not been shown to be infectious *in vivo* (Katz *et al.*, 1994). Furthermore, BTV's association with erythrocytes, which protects the virus from clearance by neutralizing antibodies, may explain its prolonged viremia (MacLachlan *et al.*, 1990), but viruses cannot replicate in red blood cells due to their lack of cell machinery. Because BTV can be isolated from most blood cell fractions, with the highest viral titers detected in the most common cell types, its association with blood cells appears to be non-specific (Barratt-Boyes & MacLachlan, 1995), and perhaps achieved through attachment to sialic acid (Zhang *et al.*, 2010).

Although the pathogenesis in sheep, cattle, and other ruminants is quite similar, there are clear differences in the pathogenicity and virulence of certain BTV strains in different ruminant species and breeds. These differences are largely attributed to species-specific variations in the susceptibility of endothelial cells (ECs) to BTV infection and to associated proinflammatory cytokine production (Coen *et al.*, 1991). For example, it has been shown that ovine ECs more rapidly produced higher levels of proinflammatory cytokines than bovine ECs, despite lower levels of BTV replication (Russell *et al.*, 1996; DeMaula *et al.*, 2001, 2002a; b). In particular, the increased ratio of plasma thromboxane, a vasoconstrictor, to prostacyclin, a vasodilator, observed in sheep but not cattle may help explain the dissimilarities in BT disease among ruminant species, including hemorrhage and edema. Furthermore, BTV infection has been associated with the induction of type I IFNs (as reviewed by (MacLachlan *et al.*, 2014; Vitour *et al.*, 2014)), which are important for stimulating and shaping adaptive immune responses, and the absence of type I IFNs may impede the development of a protective immune response against BTV (Rodríguez-Calvo *et al.*, 2014). NS3 in particular has been shown to interfere with the production of type I IFN (Chauveau *et al.*, 2013) and therefore may play an important role in BTV pathogenesis. It has also been noted that the balance of early CD4+ and CD8+ lymphocyte proliferative responses differed between sheep and cattle following BTV infection, with a significant, and potentially protective, increase in both CD4+ and CD8+ T cell responses detected in infected cattle, but an increase in primarily CD4+ T cells in sheep (Ellis *et al.*, 1990).

1.4.2 Protein-specific humoral immune responses

As noted above, BTV infection is marked by species and breed differences in clinical signs and immune responses (Neitz, 1948; Berry *et al.*, 1982). The main results concerning species-specific and protein-specific humoral immune responses to BTV have been summarized in Table 1 as well as in the following text.

VP2 induces virus neutralizing antibody responses in mice and ruminants (Huismans & Erasmus, 1981; Inumaru & Roy, 1987; Roy *et al.*, 1990). Since BTV serotype is defined using virus neutralization assays, it is also considered to be the serotype-determining protein (Huismans & Erasmus, 1981). These antibodies are detected approximately two weeks after natural infection and can persist for up to at least four to six years (Eschbaumer *et al.*, 2012). Neutralizing antibody presence is highly linked with protection (Jeggo *et al.*, 1984b; Roy *et al.*, 1990; Oura *et al.*, 2009), but no precise minimum protective titer is defined, presumably because cell-mediated immune responses also play an important role. However, it has been indicated that BTV-8 neutralizing antibody titers of at least 1-1.5 $\log_{10}$ $TCID_{50}$ may be required for long-term protection in lambs (Oura *et al.*, 2010). In addition to VP2, it has been suggested that VP5 may play a role in inducing virus neutralizing antibodies, likely through support of VP2 conformation (Roy *et al.*, 1990), and VP5 has additionally been shown to influence the specificity of neutralizing antibodies (Cowley & Gorman, 1989; Mertens *et al.*, 1989; DeMaula *et al.*, 2000). However, recent experiments based on virus neutralizing antibody assays using reassortants between BTV-1 and BTV-8 have suggested that VP2 alone is responsible for determining BTV serotype (Shaw *et al.*, 2013) and for inducing neutralizing antibodies (Kochinger *et al.*, 2014). Besides the outer capsid proteins, no other BTV proteins are thought to induce or influence virus neutralizing antibody production as defined by standard BTV neutralization assays (Huismans & Erasmus, 1981; Kahlon *et al.*, 1983; Huismans *et al.*, 1987; Inumaru & Roy, 1987; Roy *et al.*, 1990).

Non-neutralizing antibodies against BTV are also induced by VP2, as well as by other VP and NS proteins. In particular, VP7 induces high titers of IgM antibodies in ruminants as soon as 7-10 days following BTV infection (Zhou *et al.*, 2001; Bréard *et al.*, 2011) or IgM and IgG antibodies 7-21 days after vaccination with live or inactivated vaccines (Monaco *et al.*, 2004; Gethmann *et al.*, 2009; Oura *et al.*, 2009; Bréard *et al.*, 2011; Modumo & Venter, 2012). The detection of VP7-specific antibodies quickly indicates BTV infection of any serotype and IgG antibodies directed against VP7 can be detected for up to 10 or even 13 months following vaccination in some ruminants (Hultén *et al.*, 2013; Zanella *et al.*, 2013a). As a result, VP7 is commonly used in diagnostic

assays (Zhou *et al.*, 2001; Hamblin, 2004; Mecham & Wilson, 2004; Anthony *et al.*, 2007). BTV antisera has also been shown to recognize VP3, the other protein of the BTV inner capsid (Inumaru *et al.*, 1987), and like VP7, VP3 and the proteins of the transcription complex (VP1, VP4, VP6) have also been shown to be serologically reactive across BTV serotypes (Mertens *et al.*, 2009).

Despite being largely associated with the induction of cellular immunity, NS1, NS2, and NS3 have also been shown to induce humoral immune responses following BTV infection. The roles of these antibodies in viral clearance or protection are not known. In the sera of lambs and calves collected after vaccination, NS2-specific antibodies were regularly detected in serological assays, while antibody production to NS1 was less consistently identified (Richards *et al.*, 1988). Additionally, NS1- and NS2-specific antibodies have been detected following natural infection in sheep or rams (Adkison *et al.*, 1987), and high titers of antibodies directed against NS1 and NS3 were also observed following BTV infection (Anderson *et al.*, 1993; López *et al.*, 2006; Barros *et al.*, 2009).

1.4.3 Protein-specific cellular immune responses

Since the mid-1960s, it has been suggested that cell-mediated immunity may play an important role in protection against BTV infection (Jochim *et al.*, 1965; Luedke & Jochim, 1968). However, it was not until the 1980s that the specific correlation between cellular immunity and BTV protection in sheep was more thoroughly examined (Jeggo & Wardley, 1982a; b; Jeggo *et al.*, 1984a; Stott *et al.*, 1985a). Identification of the specific viral proteins that induce these responses continues to be performed using samples primarily from mice or sheep (Table 1). Both VP and NS proteins have been shown to induce some level of T cell responses. In general, the NS proteins have predominantly been associated with cross-serotype cellular immune responses (Andrew *et al.*, 1995; Jones *et al.*, 1996; Janardhana *et al.*, 1999). The duration of cellular immune responses following BTV vaccination or infection is not yet known.

The protein-specificity of T cell responses induced by BTV infection or vaccination in cattle are poorly characterized. However, VP2 and NS1 have been shown to be strong inducers of cytotoxic T cells in sheep, followed by VP5 and NS3 (Andrew *et al.*, 1995). In the same study, VP7 did not induce cytotoxic T cells (CTLs), and those that were induced by NS1, but not VP2, were shown to be reactive across serotypes. Both NS1 and VP2 have been shown to induce cross-serotype and serotype-specific T cell responses, including helper T cells, in other studies (Takamatsu *et al.*, 1990; Janardhana *et al.*, 1999; Rojas *et al.*, 2014). VP7 has also been shown to provide protection in

the absence of neutralizing antibodies, presumably through the induction of non-neutralizing serum antibodies or cell-mediated immune responses (Wade-Evans *et al.*, 1996). This has also been shown for VP7 in combination with VP3 (Roy *et al.*, 1994; Stewart *et al.*, 2012). Janardhana *et al.* observed that neither NS2 nor NS3 induced CTLs in sheep (Janardhana *et al.*, 1999). This is in contrast to Andrew's study (Andrew *et al.*, 1995) and an earlier study in mice, in which NS1, NS2, and NS3 induced the highest amount of CTL responses (Jones *et al.*, 1996). In fact, vaccine studies in Balb/C and CBA/Ca mice have demonstrated that NS2 alone can provide partial protection against BTV infection by inducing CTL production (Jones *et al.*, 1997). In all of these studies, the protein-specific T cell responses have been variable among individuals and may be MHC (major histocompatibility complex)-restricted (Jeggo *et al.*, 1985; Takamatsu & Jeggo, 1989). MHC molecules present specific antigens for recognition by T cells and the genes that encode them are highly polymorphic in cattle (Amills *et al.*, 1998; Ellis & Codner, 2012) and other species. MHC-restriction of BTV protein-specific T cell responses could potentially impact vaccination, particularly for vaccines with protection based on cellular immunity, by resulting in variable levels of protection due to MHC diversity.

Table 1. *Selected reference list for protein-specific humoral and cellular immune responses induced by experimental or natural BTV vaccination or infection, excluding the results of this thesis work. Antibodies to VP2 (including virus-neutralizing antibodies) and VP7 have been reported in numerous studies, of which only a few references are provided here. Where no references are indicated, no relevant references exist (to the best of my knowledge).*

	Humoral immune responses			**Cellular immune responses**		
	Mice/Rabbits	**Sheep**	**Cattle**	**Mice/Rabbits**	**Sheep**	**Cattle**
VP2	(Huismans *et al.*, 1987; Inumaru & Roy, 1987; Franceschi *et al.*, 2011; Calvo-Pinilla *et al.*, 2012)	(Richards *et al.*, 1988; Odeón *et al.*, 1999; Oura *et al.*, 2009)	(Savini *et al.*, 2004a; Eschbaumer *et al.*, 2009; Celma *et al.*, 2013)	CD4+ (Franceschi *et al.*, 2011) CTL (Jones *et al.*, 1996)	CD4+ (T helper) (Takamatsu *et al.*, 1990) CTL (Andrew *et al.*, 1995; Janardhana *et al.*, 1999)	-
VP5	(Huismans *et al.*, 1987; Calvo-Pinilla *et al.*, 2009a)	(Richards *et al.*, 1988; Wang *et al.*, 1995, 2013; Odeón *et al.*, 1999)	(Odeón *et al.*, 1999)	-	CTL (Andrew *et al.*, 1995; Janardhana *et al.*, 1999)	-
VP7	(Calvo-Pinilla *et al.*, 2009a)	(Richards *et al.*, 1988; Wade-Evans *et al.*, 1997; Odeón *et al.*, 1999; Perrin *et al.*, 2007)	(Richards *et al.*, 1988; Barros *et al.*, 2009)	CD4+ (Rojas *et al.*, 2011) CTL (CD8+) (Rojas *et al.*, 2011)	No CTL (Andrew *et al.*, 1995) Possible (Wade-Evans *et al.*, 1997) CTL (Janardhana *et al.*, 1999)	-
NS1	(Calvo-Pinilla *et al.*, 2012)	(Adkison *et al.*, 1988; Richards *et al.*, 1988; Anderson *et al.*, 1993)	(Richards *et al.*, 1988)	CD4+ (Rojas *et al.*, 2014) CTL (Jones *et al.*, 1996, 1997; Rojas *et al.*, 2014)	CD4+ (Rojas *et al.*, 2014) CTL (Andrew *et al.*, 1995; Janardhana *et al.*, 1999)	-
NS2	(Mecham *et al.*, 1986)	(Adkison *et al.*, 1988; Richards *et al.*, 1988)	(Richards *et al.*, 1988)	CTL (Jones *et al.*, 1996, 1997)	No CTL (Andrew *et al.*, 1995; Janardhana *et al.*, 1999)	-
NS3	-	(López *et al.*, 2006; Perrin *et al.*, 2007; Barros *et al.*, 2009)	(Barros *et al.*, 2009)	CTL (Jones *et al.*, 1996)	CTL (Andrew *et al.*, 1995) No CTL (Janardhana *et al.*, 1999)	-

1.5 Clinical signs

Although the virus can infect all ruminants, clinical signs caused by natural BTV infection are traditionally observed only in sheep. Breed, age, sex, prior exposure to the virus, and environmental conditions can affect the nature of clinical disease following natural infection, as can the BTV serotype or strain (Ward *et al.*, 1994; MacLachlan *et al.*, 2009). In experimental infections, the route employed for inoculation (Umeshappa *et al.*, 2011) as well as the passage history of the virus stock (i.e. in cell culture or from infected ruminants) (Eschbaumer *et al.*, 2010) are important to consider when making conclusions concerning BTV infection, since differences, for example in virulence, can confound comparisons among studies (Coetzee *et al.*, 2014). In general, the clinical course of BTV infection occurs from two days to two weeks post-infection (Moulton, 1961).

1.5.1 Clinical signs in small ruminants

Clinical signs of BTV infection in sheep can range from mild to severe, with up to 90-100% morbidity possible for naïve populations of susceptible breeds (Moulton, 1961), such as the Merino and Poll Dorset breeds. BT disease has been shown to vary among individuals within the same breed following both natural and experimental infection (Backx *et al.*, 2007; Darpel *et al.*, 2007; Elbers *et al.*, 2008; MacLachlan *et al.*, 2008; Worwa *et al.*, 2010). However, clinical signs are generally characterized by hyperthermia, nasal secretions, edema of the lip, tongue, face, and lymph nodes, hyperemia or hemorrhage of the mouth and tongue, ulcers of the oral cavity (such as of the dental pads), and inflammation of the coronary bands combined with lameness or difficulty walking (Moulton, 1961; Erasmus, 1975a). In severe cases, cyanosis of the tongue can also occur, giving the disease its name.

BTV-8 infection of sheep has not been markedly different compared to infection with other BTV serotypes. However, clinical signs have generally been severe in selected breeds of animals in northern Europe (Backx *et al.*, 2007; Moulin *et al.*, 2012) and BTV-8 appears to have a high virulence irrespective of sheep breed (Worwa *et al.*, 2010).

BT disease has been observed in goats, but is less pronounced than the disease observed in sheep. Often, BTV infection in goats is marked by hyperthermia with or without slight hyperemia around the nose (Erasmus, 1975a). Experimental infection of goats with BTV-8 in some cases, however, has resulted in more severe clinical signs including dysphagia (difficulty swallowing), diarrhea, and lameness (Backx *et al.*, 2007). Furthermore, although no signs of BT disease were reported in goats infected with BTV-25,

stillborn or weak-born kids, possibly attributable to BTV infection, were observed in the field (Chaignat *et al.*, 2009) and the virus RNA was shown to persist in blood for at least 19 months (Vögtlin *et al.*, 2013). These results indicate that BTV might also be considered a pathogenic virus of goats, depending on the virus serotype, and like cattle, an important part of the picture for controlling BTV spread.

1.5.2 Clinical signs in cattle

In contrast with sheep, cattle are often not clinically affected by viral infection, yet demonstrate long viremia that peaks one to two weeks later, though at comparable titers, than the viremia observed in sheep (Richards *et al.*, 1988; Darpel *et al.*, 2007). Therefore, they act as amplifying hosts for BTV (MacLachlan *et al.*, 1994). The outbreak of BTV-8 in central and northern Europe, however, was distinguished by the appearance of clinical signs in cattle (as reviewed by (Dal Pozzo *et al.*, 2009)). Lesions of the nasal mucosa, nasal discharge, and conjunctivitis were observed first following natural infection, succeeded by lethargy, appetite loss, skin lesions, and a decrease in milk production (Zanella *et al.*, 2013b). Elbers and colleagues determined that dairy and nursing cows, as opposed to beef cattle, were most likely to be clinically affected by BTV-8 infection in the Netherlands (Elbers *et al.*, 2008). Importantly, BTV-8 infection in pregnant cows resulted in abortions, stillbirths, mummified or malformed fetuses, or newborn calves with developmental problems including abnormal posture, blindness, uncontrolled or circling gait, and other central nervous signs caused by hydroencephaly in so-called "dummy calves" (Wouda *et al.*, 2008; Dal Pozzo *et al.*, 2009; Worwa *et al.*, 2010).

1.5.3 Clinical signs in other ruminants

Since wildlife can play a significant role in the amplification or spread of certain viruses, clinical BT disease in wild ruminants has been investigated by experimental infection in numerous species.

BT disease similar to that reported in domestic sheep has been observed in mouflon (*Ovis aries musimon*) (Fernández-Pacheco *et al.*, 2008) and desert bighorn sheep (*Ovis canadensis*) (Robinson *et al.*, 1967) following natural infection, as well as in white-tailed deer (*Odocoileus virginianus*) following both experimental and natural infection (Falconi *et al.*, 2011). Clinical BT disease following experimental infection of European red deer (*Cervus elaphus*), North American elk (*Cervus elaphus canadensis*), and African blesbok (*Damaliscus pygargus*) was subclinical or mild (transient hyperthermia, conjunctivitis) and similar to that observed in cattle (Murray & Trainer, 1970; López-Olvera *et al.*, 2010; Falconi *et al.*, 2011).

In addition to wildlife, South American camelids such as llamas (*Lama glama*) and alpacas (*Lama pacos*, *Vicugna pacos*) have also been shown to be serologically or virologically positive for BTV infection and in a few cases, appear to have died of acute BTV infection (Henrich *et al.*, 2007; Meyer *et al.*, 2009; Ortega *et al.*, 2010). However, they are not considered important in the epidemiology of the recent BTV outbreaks in central and northern Europe (Schulz *et al.*, 2012).

1.5.4 BTV infection in carnivores

In line with evidence that BTV can be transmitted by direct contact of ruminants, some cases of BTV infection and clinical disease have been documented in Eurasian lynx (*Lynx lynx*) housed in a Belgian zoo (Jauniaux *et al.*, 2008) and several different species of African carnivores, including lions (*Panthera leo*) and spotted hyenas (*Crocuta crocuta*), have tested seropositive for BTV infection (Alexander *et al.*, 1994). All of these cases are thought to be caused by ingestion of infected ruminants or their organs, as has been similarly documented for African horse sickness virus (Van Rensberg *et al.*, 1981). However, there are also reports of canine abortions following use of a BTV-11-contaminated modified live virus vaccine administered during a late gestation period (Evermann *et al.*, 1994). These cases of BTV infection of carnivores have reported clinical problems such as abortion, fatality, and other clinical signs such as anemia and lung congestion with edema. However, the importance of clinical BT disease of carnivores in the field (if present) is not well understood.

1.6 Diagnosis

Clinical diagnosis is considered to be crucial during BTV outbreaks, as it can provide an opportunity for control measures to be quickly implemented (Mertens *et al.*, 2009). However, as detailed previously, it can be difficult to diagnose BTV infection in cattle or goats when the clinical signs are mild or even subclinical, or in any ruminant when the signs are unspecific. Furthermore, as a notifiable disease in many countries, an early and correct confirmation of a clinical BT suspicion by direct or indirect diagnostic tests is required. The identification of BTV RNA, isolation of BTV using eggs or cell culture, and the detection of BTV-specific antibodies, are common and key methods used to diagnose BTV infection. Depending on the method, BTV can also be identified at the serogroup or serotype level.

1.6.1 Virological diagnosis

BTV can be isolated in embryonated chicken eggs (ECE) or directly in cell cultures from blood, semen, or tissue samples (Clavijo *et al.*, 2000). An intravenous route of inoculation of ECE has been shown to be more rapid and effective than yolk sac inoculation for virus isolation (Goldsmit & Barzilai, 1985). Additionally, insect-derived cells, such as *Culicoides*-derived (KC) cells, have been shown to be more sensitive to BTV infection than many mammalian cell lines but they do not exhibit CPE (Mertens *et al.*, 1996). Virus neutralizing antibody tests are the most specific method for determining BTV serotype, but can take over a week to complete and rely on access to reference sera.

The OIE officially recommends the detection of segment 5, encoding NS1, by RT-PCR for the diagnosis of BTV (OIE, 2009) and it has been shown that real time quantitative-PCR (RT-qPCR) assays designed to detect this genomic segment are able to identify at least 24 of the 26 known BTV serotypes when using blood from infected ruminants (Polci *et al.*, 2007; Toussaint *et al.*, 2007). These assays, as well as a protocol identifying segment 1 (VP1) (Shaw *et al.*, 2007; Toussaint *et al.*, 2007), have been shown to enable BTV RNA detection in the blood of ruminants as early as two days post-infection (Batten *et al.*, 2008a). Several RT-PCR assays have also been developed to detect VP3 and results based on genetic sequences may indicate the virus's geographic origin (Gould & Pritchard, 1990; Harding *et al.*, 1995; Pritchard *et al.*, 1995).

Recently, RT-qPCR assays detecting segment 2 (VP2) of all 26 BTV serotypes have been developed by Maan and colleagues to facilitate rapid serotype determination using field and reference strains (Maan *et al.*, 2012). The use of such virological methods for identifying genotypes corresponding to BTV serotypes provides a faster alternative to traditional, time-consuming techniques for determining serotypes during BTV outbreaks, such as virus neutralizing tests.

1.6.2 Serological diagnosis

Classically, serum neutralizing antibody tests are the most specific method for identifying BTV-specific antibodies in serum samples. These tests rely on access to the reference strain of the specific BTV serotype concerned and can take over a week to complete. They are the gold standard for serological diagnosis. However, these tests are serotype-specific and may be difficult to perform on a large number of samples, such as when required for surveillance purposes. Therefore, enzyme-linked immunosorbent assays (ELISAs), which rely on the detection of BTV-specific antibodies in the sera of susceptible animals, are routinely used. ELISAs often take less than a day to complete and as they can detect specific IgM antibodies (Zhou *et al.*, 2001), recent BTV

infection can be identified. The use of competitive (c) ELISAs can additionally allow sera from several species to be analyzed using the same kit.

To minimize the number of ELISAs required to cover the 26 recognized BTV serotypes, detection of antibodies against proteins conserved among serotypes have been developed. VP7 has been selected since this protein induces a strong humoral immune response. ELISAs that detect IgM and/or IgG serum antibodies directed against VP7 have been shown to work well to identify BTV infection irrespective of serotype (Gumm & Newman, 1982; Zhou *et al.*, 2001; Hamblin, 2004; Mecham & Wilson, 2004; Vandenbussche *et al.*, 2008). Using a cELISA directed against VP7 of BTV is a prescribed test for international trade (OIE, 2009) and is widely used across Europe. Recently, the results of two inter-laboratory ring trials indicated that six different commercially-available cELISA kits targeting VP7 were able to detect, by 21 days post-infection, all of the BTV serotypes circulating in Europe at the time (Batten *et al.*, 2008a).

In addition to VP7, NS1 and NS3 have also been targets for BTV ELISAs, since they are only produced during viral infection in cells and therefore may indicate BTV replication (Anderson *et al.*, 1993; Barros *et al.*, 2009). Generally, some of the conserved BTV proteins, including VP7 and the NS proteins, may also be suitable targets for differentiating infected from vaccinated animals (DIVA). However, at present no DIVA vaccines are available on the market (please see section 1.7.2).

1.7 Prevention and control

Traditional prevention and control measures for viral livestock diseases include the restriction of animal trade movements or quarantine of sick animals, optimization of zoosanitary and other biosecurity approaches, treatment when available, vaccination, and eradication or pre-emptive slaughter. A combination of these measures is employed for some viral infections of livestock, such as classical swine fever in pigs (Moennig, 2000). For BTV, there is no specific treatment, and different approaches may be taken depending on whether the disease is endemic or epidemic in a particular region. Regardless of the control measure, reliable diagnostic tests and understanding of the epidemiological situation are essential to allow decision-making bodies to make informed choices (Wierup, 2012).

1.7.1 Biosecurity and animal movement control measures

In endemic areas such as South Africa, where non-indigenous or naïve sheep breeds can be severely afflicted, vaccination is considered to be the best method for preventing BT disease caused by viral spread (Dungu *et al.*, 2004).

However, biosecurity measures targeting vectors or vector-access to susceptible animals may also be employed (Erasmus, 1975b; Coetzee *et al.*, 2012), as well as trade restrictions and control of animal movement. Since competent *Culicoides* species are ubiquitous and because there is little interest in vaccinating indigenous sheep breeds (Dungu *et al.*, 2004), eradication seems unlikely in these areas. Additionally, many BTV serotypes co-circulate in South Africa (Niekerk *et al.*, 2003). This makes vaccination of non-indigenous sheep breeds the most practical and effective control method but also requires the use of multivalent vaccines.

In epidemic areas such as Europe, animal movement controls and trade restrictions are often the first line of defense. During the BTV outbreaks in 2000, this began with the establishment of protection and surveillance zones (3 and 10 km radii, respectively) surrounding infected farms and the pre-emptive slaughter of all susceptible animals on those farms (Caporale & Giovannini, 2010). No animals in the protection zones were allowed to leave and vaccination was acceptable as a complementary control strategy in these zones only (European Council, 1992). When it quickly became apparent that these tactics were insufficient against vector-borne BTV, larger protection and surveillance zones were demarcated and extra surveillance measures, including regular veterinary visits to confirm BT disease, were added (European Council, 2000). Widespread vaccination was implemented as a response to the outbreaks (Caporale & Giovannini, 2010) and was generally considered successful in preventing further BTV disease and spread (Zientara & Sánchez-Vizcaíno, 2013).

1.7.2 Vaccines

Currently, there are two types of vaccines against BTV that are commercially available (Table 2): i) modified live virus vaccines, which are attenuated forms of BTV; and ii) inactivated vaccines, which are composed of whole killed BTV plus an adjuvant such as aluminum and/or saponins. Each vaccine type has advantages and disadvantages, as reviewed below, and therefore the development of novel BTV vaccines using new technologies is an expanding area of research.

Modified live virus vaccines (MLVs)

The first vaccines against BTV were MLVs, developed by Arnold Theiler and colleagues at Onderstepoort, South Africa, in the early twentieth century (Verwoerd, 2009). Today, there are MLVs targeting a large number of BTV serotypes. The vaccine most commonly used in South Africa consists of three formulations of five different BTV serotypes each, attenuated by passage in both ECE and baby hamster kidney (BHK-21) cell culture (Coetzee *et al.*,

2012). This vaccine regimen has been designed to provide long-lived protection across all included serotypes with minimal immunologic interference among serotypes.

During the recent BTV outbreaks in Europe, MLVs were the only available vaccines up till 2004 (Di Emidio *et al.*, 2004) (Table 2). Monovalent MLVs targeting BTV-2 and BTV-16, as well as multivalent vaccines directed against BTV-2/-4, BTV-2/-9 and BTV-2/-4/-9, were employed in France (Corsica), Italy, Portugal, and Spain in the early 2000s, depending on the epidemiological situation of the target region (Savini *et al.*, 2008). Safety concerns related to the use of specific MLVs including BTV-2 and/or BTV-16 were raised due to adverse reactions observed in some vaccinated ruminants in certain regions (Monaco *et al.*, 2004; Ferrari *et al.*, 2005; Veronesi *et al.*, 2010). As a result, the use of monovalent BTV-16 was discontinued. As with natural infection, species differences in the clinical signs of sheep, goats, and cattle were observed following the use of these MLVs, with clinical signs more severe in sheep (as reviewed by (Savini *et al.*, 2008)).

MLVs can be produced and administered in a cost-effective manner and have several benefits associated with their use. For example, only one dose is required to induce virus neutralizing antibodies (Monaco *et al.*, 2004), small amounts of attenuated virus are enough to stimulate a protective immune response (Modumo & Venter, 2012), and the addition of an adjuvant is not necessary. However, although MLVs continue to be used with success in BTV-endemic areas such as South Africa (Dungu *et al.*, 2004) and North America (The Center for Food Security and Public Health, 2014), they have generally been associated with drawbacks, such as clinical disease in some breeds (including teratogenic effects and abortion when used during early gestation (Waldvogel *et al.*, 1992b)), reduced milk production, viremia, potential reversion to virulence, reassortment with field strains, as well as undesirable trade restrictions since their use cannot be differentiated from natural BTV infection (Monaco *et al.*, 2004, 2006; Savini *et al.*, 2004b; c; Ferrari *et al.*, 2005; Veronesi *et al.*, 2005, 2010; Batten *et al.*, 2008b). Therefore, due to safety concerns as well as to lack of a DIVA characteristic, MLVs are generally less favored within the European Union compared to inactivated vaccines, despite their recent success in controlling BTV-2 and BTV-9 outbreaks in parts of southern Europe (Patta *et al.*, 2004).

Inactivated vaccines

Classic inactivated vaccines are produced as killed whole virus, often using heat, ultraviolet radiation (UV), or chemical methods, including hydroxylamine and binary ethylenimine (Campbell, 1985; Di Emidio *et al.*, 2004; Ramakrishnan *et al.*, 2005, 2006; Savini *et al.*, 2007; Umeshappa *et al.*, 2010).

The first inactivated BTV vaccines became available in Europe in 2005, and targeted BTV-2 (Zientara *et al.*, 2010). Today, there are inactivated mono- and multivalent vaccines against BTV-1, -4, -8 and -9 as well, as summarized in Table 2. Compared to MLVs, inactivated vaccines are widely considered to be safer because they are not associated with viremia and do not allow the reassortment between field and vaccine strains. However, they are more expensive to produce and may be more costly to administer as they require two immunizations rather than one in order to provide a comparable duration of immunity (Rogan & Babiuk, 2005). Additionally, inactivated vaccines need to be formulated with an adjuvant so as to induce sufficient immune responses (Singh & O'Hagan, 2003). Common adjuvants used in inactivated veterinary vaccines include aluminum hydroxide, saponins, and emulsions. Inactivated vaccines against BTV-8 have been evaluated in Europe under experimental and natural conditions regarding their safety and protective efficacy. In brief, after two immunizations, they have been shown to be safe and induce protective immunity against experimental clinical and virological BTV-8 infection in primarily sheep (Gethmann *et al.*, 2009; Hamers *et al.*, 2009a; Oura *et al.*, 2009; Bartram *et al.*, 2011; Bréard *et al.*, 2011; Moulin *et al.*, 2012; Pérez de Diego *et al.*, 2012) for at least one year (Hamers *et al.*, 2009b). In particular, the immune responses induced by vaccination with commercially available inactivated vaccines includes neutralizing antibodies, serum antibodies, and CD8+ T cells (Umeshappa *et al.*, 2010; Pérez de Diego *et al.*, 2012). Some mild localized reactions have also been observed following their use (Hamers *et al.*, 2009a; Vetvac, 2014). Revaccination with inactivated vaccines is recommended after one year by manufacturers (Vetvac, 2014).

Table 2. *List of commercially available BTV vaccines, as adapted from (The Center for Food Security and Public Health, 2014; Vetvac, 2014).*

Vaccine name	Manufacturer	Hosts	Pathogens	Adjuvants	Countries of distribution
Blue Tongue Virus Vaccine	Veterinary Vaccines Production Centre (KARI, Kenya)	Goat, sheep	polyvalent BTV (attenuated)	None	Kenya
Bluetongue Vaccine	Onderstepoort Biological Products Ltd (South Africa)	Goat, sheep	polyvalent BTV (attenuated)	None	Namibia, South Africa
Bluetongue Vaccine	Colorado Serum Company (USA)	Goat, sheep	BTV-10 (attenuated)	None	USA, Canada
Bluevac BTV1	CZ Veterinaria S.A. (Spain)	Cattle, sheep	BTV-1 (killed)	Aluminum hydroxide, saponin	Europe*
Bluevac BTV4			BTV-4 (killed)		
Bluevac BTV8			BTV-8 (killed)		
Bluevac BTV1+4			BTV-1,-4 (killed)		
Bluevac BTV1+8			BTV-1,-8 (killed)		
BlueVac-10	PHL Associates Inc. (USA)	Sheep	BTV-10 (attenuated)	None	USA
BlueVac-11			BTV-11 (attenuated)		
BlueVac-17			BTV-17 (attenuated)		
Bluvax	Veterinary Vaccines Production Centre (KARI, Kenya)	Sheep	BTV	None	Kenya
BLUVAX	Kenya Veterinary Vaccines Institute (Kenya)	Sheep	BTV-1,-2,-3,-4,-8,-12,-134 (attenuated in embryonated chicken eggs)	None	Kenya
Bovilis BTV8	Merck Sharp & Dohme Ltd (MSD Animal Health, United Kingdom)	Cattle, sheep	BTV-8 (killed)	Aluminum hydroxide, saponin	Europe*
BTVPUR AlSap 1	Merial (France)	Cattle, sheep	BTV-1 (killed)	Aluminum hydroxide, saponin	Europe*
BTVPUR AlSap 8			BTV-8 (killed)		
BTVPUR AlSap 1+8			BTV-1,-8 (killed)		
BTVPUR AlSap 2+4		Sheep	BTV-2,-4 (killed)		
Freeze dried monovalent Bluetongue vaccine	Central Veterinary Control and Research Institute (Turkey)	Sheep	BTV-4 (live)	None	Turkey

Syvazul 1 Syvazul 1+8 Syvazul 8	Laboratorios SYVA S.A. (Spain)	Cattle, sheep	BTV-1 (killed) BTV-1,-8 (killed) BTV-8 (killed)	Oil	Spain, United Kingdom
Syvazul-4		Sheep	BTV-4 (killed)		
Zulvac 1 Ovis Zulvac 1+8 Ovis Zulvac 8 Ovis	Zoetis/Pfizer (United Kingdom)	Sheep	BTV-1 (killed) BTV-1,-8 (killed) BTV-8 (killed)	Aluminum hydroxide, saponin	United Kingdom
Zulvac 8 Bovis		Cattle	BTV-8 (killed)		

*Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, The Netherlands, Poland, Portugal, Romania, Slovak Republic, Slovenia, Spain, Sweden, United Kingdom

New vaccine designs

New vaccine designs rely on the same basic approach as classic vaccines: utilizing live antigens or killed antigens. As discussed, classic vaccines against BTV have the advantage of working well to prevent and control virus spread, and therefore novel vaccines must match their efficacy. Additionally, the design of new vaccines should aim to improve upon the disadvantages of classic vaccines by enabling DIVA and by potentially targeting multiple serotypes of BTV. Both of these aspects are especially important for central and northern Europe, where the virus is not endemic but where BTV outbreaks appear to pose a permanent threat. Many of the experimental vaccines targeting BTV have been listed in Table 3, and the general advantages and disadvantages of the different approaches are addressed below.

Recombinant viral vector vaccines consist of a live attenuated virus that has been genetically modified to include genes encoding foreign antigens, so that those genes can be expressed within the host and subsequently induce protective immunity against the target virus. Often, these antigens are produced in high number in host cells and therefore can induce strong immune responses (Calvo-Pinilla *et al.*, 2014). Antigen choice is especially important for inducing protective immunity, but other crucial considerations include recombinant vector stability, host range, expression and conformation of the foreign antigen, duration of immunity, cost of production, and safety (Yokoyama *et al.*, 1997). Potential pre-existing immunity and the location of the primary immune response to the viral vector itself can hinder the development of a protective immune response (Saxena *et al.*, 2013). Furthermore, since recombinant viral vectors are genetically modified, countries can be hesitant to implement their use. Capripox virus (Wade-Evans *et al.*, 1996; Perrin *et al.*, 2007), canarypox virus (Boone *et al.*, 2007), bovine herpes virus type 4 (Franceschi *et al.*, 2011), and vaccinia virus (Lobato *et al.*, 1997) vectors, among others, have all been

used with varying levels of success as the basis of recombinant viral vector vaccines against BTV (Table 3).

Additionally, disabled infectious single cycle (DISC) vaccines have been developed using reverse genetics technology, such that one essential gene product is missing from the produced virion and therefore the vaccine virus is only able to replicate once in target cells. This type of vaccine may provide a safer alternative to MLVs, though more virus or several doses are likely needed for a DISC vaccine to be equally effective since the amplifying effect of MLVs is prevented under this design. Promising VP6-deficient DISC vaccines have been produced and shown to provide protection against experimental BTV challenge in sheep (Matsuo *et al.*, 2011; Celma *et al.*, 2013).

Virus-like particle vaccines (VLPs) and subunit vaccines have also been shown to be promising vaccine candidates against BTV (Roy *et al.*, 1994; Stewart *et al.*, 2010, 2012). BTV VLPs are produced by infecting insect cells with recombinant baculoviruses that express genes encoding for VP2, VP5, VP3, and VP7, such that the produced proteins self-assemble into empty double-shelled particles (French *et al.*, 1990). Due to their composition, VLPs are able to mimic the structure of native BTV and have been shown to effectively induce protective humoral immune responses (Stewart *et al.*, 2010, 2012). Like inactivated vaccines, which also consist of killed antigen, these vaccines require the use of an adjuvant in order to stimulate a sufficient immune response. Adjuvants provide the advantage of allowing potent immune responses to be quickly induced and can help to direct an immune response to be primarily humoral, cellular, or a combination of both (Petrovsky & Aguilar, 2004). Antigen choice, which is possible in subunit vaccine development, can also facilitate the induction and direction of immune responses to optimize a vaccine's protective efficacy, provided sufficient information is available to allow informed choices.

Table 3. *Selected reference list for recent experimental BTV vaccines, including expression system, BTV proteins included in vaccine formulations, target species, and resulting clinical and virological protection against BTV challenge of different severity.*

Reference	Expression system	Proteins (serotype)	Species	Clinical protection	Virological protection
Killed vaccines					
(Roy *et al.*, 1990)	Sf9-baculovirus	VP2 (50 µg) (BTV-10)	Sheep	Partial	Partial
		VP2 (100, 200 µg) (BTV-10)		*Full*	*Full*
		VP2, VP5 (BTV-10)		Full	Full
		VP1, VP2, VP5-7, NS1-3, (BTV-10); VP3 (BTV-17)		*Full*	*Full*
(Roy *et al.*, 1994; Stewart *et al.*, 2012, p 2)	Sf9-baculovirus	Virus-like particles (VLPs): VP2, VP5, VP3, VP7 (BTV-1, -2, -8, -13, 17)	Sheep	Full	Full
(Stewart *et al.*, 2012)	Sf9-baculovirus	Core-like particles (CLPs): VP3, VP7	Sheep	Partial	Partial
(Jabbar *et al.*, 2013)	Bacterial	VP2, VP5, VP7 (BTV-8)	IFNAR(-/-) mice	Partial	Partial
(Mohd Jaafar *et al.*, 2014)	C41 (DE3) *E. coli*	VP2, VP5 (BTV-4)	IFNAR(-/-) mice	Full	Partial
Live vaccines					
(Wade-Evans *et al.*, 1996)	Capripox virus	VP7 (BTV-1)	Lambs	Partial	Not tested
(Lobato *et al.*, 1997)	Vaccinia virus	VP2 (BTV-1)	Sheep	Partial	Partial
		VP2, VP5 (BTV-1)		*Partial*	*Full*
(Boone *et al.*, 2007)	Canarypox virus	VP2, VP5 (BTV-17)	Sheep	Full	Full
(Perrin *et al.*, 2007)	Capripox virus	VP2, VP7, NS1, NS3 (BTV-2)	Sheep	Partial	Partial
(Calvo-Pinilla *et al.*, 2009b, 2012)	Modified Vaccinia Ankara virus + DNA	(BTV-4)	IFNAR(-/-) mice	Partial	Full

(Franceschi *et al.*, 2011)	BoHV-4*	VP2 (BTV-8)	IFNAR(-/-) mice	Partial	Partial
(Ma *et al.*, 2012)	EHV-1**	VP2 (BTV-8)	IFNAR(-/-) mice	Partial	None
		VP2, VP5 (BTV-8)		*Partial*	*Full*
(Kochinger *et al.*, 2014)	Vesicular stomatitis virus (single-cycle)	VP2 (BTV-8)	Sheep	Partial	Partial
		VP5 (BTV-8)		*None*	*None*
		VP2, VP5 (BTV-8)		Full	Full
(van Gennip *et al.*, 2012)	Reassortants (reverse genetics)	BTV-6 backbone + VP2, VP5 BTV-1	Sheep	Partial	Full
		BTV-6 backbone + VP2, VP5 (BTV-8)		*Partial*	*Full*
		BTV-6		Partial	Full
(Feenstra *et al.*, 2014)	Reassortants with NS3/NS3A knockout mutation (reverse genetics)	BTV-1 backbone + VP2 (BTV-8)	Sheep	Partial	Partial
		BTV-6 backbone + VP2 (BTV-8)		*Partial*	*Full*
		BTV-8		Partial	Partial

*Bovine herpes virus type 4; **equine herpes virus type 1; ***disabled infectious single cycle

DIVA

DIVA is an increasingly important consideration for veterinary vaccine design because of the movement restrictions placed on BTV-positive ruminants during outbreaks (Bhanuprakash *et al.*, 2009). Countries face losing their disease-free status following widespread vaccination using conventional vaccines, which can have a devastating economic effect and in some cases has driven countries to slaughter vaccinated animals that could not be differentiated from infected animals, as in the case of the 2001 outbreak of foot-and-mouth disease in the Netherlands (Pluimers, 2004; Meeusen *et al.*, 2007). DIVA is also crucial for maintaining serological surveillance as a tool for monitoring changes in vaccine efficacy or local epidemiology, especially in regions with potential co-circulation of several strains or serotypes (Uttenthal *et al.*, 2009; Avellaneda *et al.*, 2010).

Currently, there are no commercially available DIVA-compliant BTV vaccines. Some studies have suggested that NS1 (Anderson *et al.*, 1993) or NS3 (López *et al.*, 2006; Barros *et al.*, 2009) can be detected in infected, but not vaccinated, animals following the use of classic inactivated vaccines. However, since current vaccines contain whole virus and when inactivated may contain some of these NS proteins, there is a strong risk that false positives for infection, especially after repeated vaccination, can complicate their use as DIVA targets. This has been observed with foot-and-mouth disease (Paton & Taylor, 2011). Therefore, new strategies must be employed to create companion DIVA tests for existing vaccines, or to create effective next generation DIVA vaccines based on existing diagnostic tests.

2 Aims of the thesis

The main aim of this research was to develop and evaluate a novel subunit DIVA vaccine against BTV-8 in cattle. The specific objectives were:

- In study I, to formulate and optimize a novel subunit DIVA vaccine against BTV-8 by performing protein purification and stability analyses and by evaluating protein-specific immunogenicity in mice (Paper I)
- In study II, to evaluate the safety and protein-specific immune responses induced by the experimental subunit vaccine in cattle, in comparison with a commercial inactivated vaccine against BTV-8 (Paper II)
- In study III, to evaluate the protective efficacy induced by the experimental subunit vaccine in cattle, as well as the VP7-based DIVA aspect of the vaccine, following a virulent challenge with BTV-8 (Paper III)

3 Materials and methods

This section provides descriptions of the materials and methods used in the three studies of this thesis work. Where methods are not described in detail in the publications, additional attention is provided here.

3.1 Recombinant protein expression and production

Two different expression systems were used to produce recombinant BTV proteins, by following the manufacturers' protocols. For VP2 and VP5 of BTV-8 (French strain, isolated in 2006; molecular weight (MW): 111 and 59 kDa, respectively) and NS1 and NS3 of BTV-2 (Corsican strain, isolated in 2001; MW: 64 and 35 kDa, respectively), the respective protein-encoding genes were inserted into individual "bacmids" using recombination. Following individual infections of *Spodoptera frugiperda* (Sf9) cells, each recombinant protein was expressed in Bac-to-Bac® Baculovirus Expression Systems (Invitrogen, United Kingdom). For NS2 of BTV-2 (Corsican strain, isolated in 2001; MW: 40 kDa), the protein-encoding gene was cloned into a pET28 vector and expressed in BL21-AI™ *Escherichia coli* (Invitrogen, United Kingdom) (NS2; MW: 40 kDa), following the manufacturers' protocols. All proteins were tagged with 6 Histidine (His) residues for later purification using nickel or cobalt affinity.

Sf9 cells (Invitrogen, United Kingdom) were propagated in Sf-900™ III SFM medium (Invitrogen, United Kingdom) and on day of passage, infected with different recombinant baculoviruses expressing individual recombinant proteins VP2, VP5, NS1, or NS3. These infected Sf9 cells were harvested after 48 to 96 h and then centrifuged for 10 min at 300 x g for storage as cell pellets.

Recombinant NS2 expression was induced for 5 h in medium containing 0.1% L-arabinose and 1 M IPTG, then centrifuged for 10 min at 500 x *g* for storage. All expressed proteins were aliquoted and stored at -80°C until

purified for use in purification and stability analyses or experimental immunizations and *ex vivo* immunological assays.

3.2 Recombinant protein purification

The purification method for individual recombinant BTV proteins was selected between His SpinTrap™ columns (GE Healthcare, United Kingdom) or HisPur™ Cobalt Spin Plates (Pierce, USA), and the corresponding manufacturers' protocols were then specifically optimized per protein regarding the lysis and elution buffers, as described in Figure 5 (final buffers shown in Table 4). Briefly, purification results following differences in imidazole concentration, pH, salt concentration, addition of detergent, working temperature, freeze-thaw cycle, and use of NP-40 lysis buffer were compared to the purification results following the manufacturers' protocols to determine an optimized lysis buffer per recombinant protein. Variations in imidazole concentration were tested to optimize protein-specific elution buffers in comparison with the manufacturers' protocols.

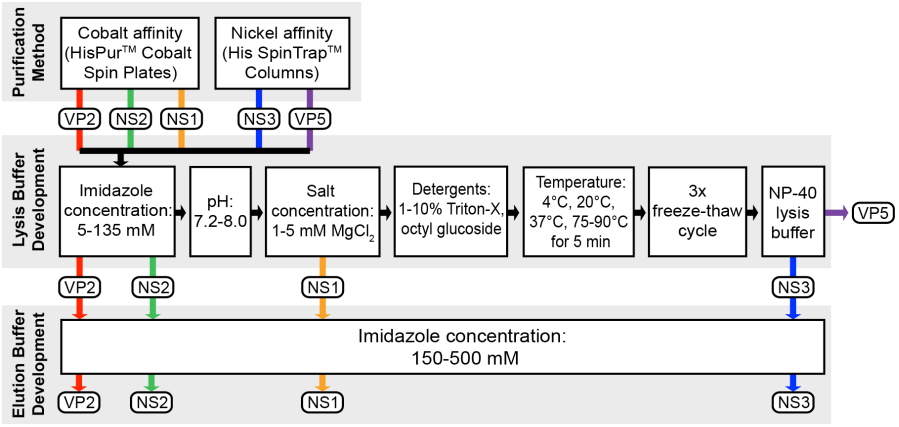


Figure 5. Optimization process of purification protocols for recombinant BTV proteins. Buffer variations were tried in sequence as indicated horizontally, until acceptable purity was achieved.

In addition to using optimized buffers for each recombinant protein, the manufacturers' protocols were followed with two exceptions: (1) lysed sample that flowed through the columns or plate wells was reloaded onto the same column or plate well, and incubation and centrifugation was repeated before washing; and (2) the total volume of optimized elution buffer was divided into four parts and added separately per column or plate wells, with an incubation step of 5 min at +4°C and centrifugation at 100 x *g* for 1 min (columns) or 500 x *g* for 3 min (plate wells) in between each addition.

Table 4. *Serotype, expression system, and optimized parameters for purification of His-tagged recombinant BTV proteins VP2, VP5, NS1, NS2, and NS3.*

	VP2	VP5	NS1	NS2	NS3
Serotype	BTV-8	BTV-8	BTV-2	BTV-2	BTV-2
Expression system	Baculovirus/Sf9 cells	Baculovirus/Sf9 cells	Baculovirus/Sf9 cells	BL21 *E. coli*	Baculovirus/Sf9 cells
Purification method (affinity)	HisPur spin plates[b] (cobalt)	His SpinTrap columns[c] (nickel)	HisPur spin plates (cobalt)	HisPur spin plates (cobalt)	His SpinTrap columns (nickel)
Lysis buffer	50 mM sodium phosphate, 300 mM sodium chloride, 5 mM imidazole	Not applicable	1 mM $MgCl_2$, 20 mM imidazole, Benzonase nuclease HC[d], in PBS	50 mM sodium phosphate, 300 mM sodium chloride, 5 mM imidazole, 100 µg/ml lysozyme[d]	NP-40 lysis buffer[e]
Elution buffer[a]	50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole	Not applicable	500 mM imidazole in PBS	50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole	50 mM sodium phosphate, 300 mM sodium chloride, 500 mM imidazole

[a]All buffers contained EDTA-free complete protease inhibitor cocktail tablets (Roche Applied Sciences, United Kingdom)
[b]Pierce, USA
[c]GE Healthcare, USA
[d]Sigma Aldrich, USA. HC, high concentration
[e]National Veterinary Institute (SVA), Sweden

3.3 Recombinant protein identification and quantification

The presence of each recombinant protein was determined by Coomassie staining of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and Western blot. All procedures were performed at room temperature unless otherwise indicated.

For SDS-PAGE, protein samples were diluted in Laemmli buffer (Bio-Rad Laboratories, USA), heated at 96°C for 5 min, then run on a 4-15% SDS-PAGE gel (Mini-PROTEAN® TGX™ precast gels, Bio-Rad, USA) at 200V for approximately 40 min. The gel was then washed in distilled deionized water (ddH_2O) and fixed for 30 min in fixing solution containing 25% isopropanol, 10% acetic acid, and 65% ddH_2O. Fixed gels were washed before incubating in Coomassie stain for 2 h, followed by de-staining in 10% acetic acid.

For Western blot, the gel was transferred at 50V for 1 h to a nitrocellulose membrane, blocked in 2% (w/v) BSA in PBS for 1 h, and then washed 3 times 5 min in PBS-Tween. Washed membranes were incubated in primary antibody for 2 h, washed again in PBS-Tween, and incubated in secondary antibody for 1 h. After a final washing step, visualization was performed using Stable DAB (Invitrogen, United Kingdom).

For mass spectrophotometry, selected protein bands for individual recombinant proteins were excised from Coomassie-stained SDS-PAGE gels and brought to SciLifeLab (Uppsala, Sweden) for identification. Proteins were in-gel digested using trypsin, then either individually resolved in 5 µl of 30% acetonitrile and 1% formic acid (VP2) or in 15 µl 0.1% formic acid (VP5, NS1, NS2, and NS3). Resolved VP2 was loaded onto an MTP 384 ground steel target using the dried droplet technique and an α-cyano-4-hydroxycinnamate matrix. Mass spectra were recorded in positive mode on an Ultraflex II MALDI TOF mass spectrophotometer (Bruker Daltonics, Germany) and then peptide mass mapping was performed in MASCOT (Mascot Science, United Kingdom). For resolved VP5, NS1, NS2, and NS3, peptides from individual recombinant proteins were separated using a reversed-phase C18-column and electrosprayed on-line to an LTQ Orbitrap Velos Pro ETD mass spectrophotometer (Thermo Finnigan, Germany). Collision-induced dissociation (CID) was applied to perform tandem mass spectrophotometry before using MASCOT to perform database searches against proteins in the NCBI Virus database (www.ncbi.nlm.nih.gov/genomes/VIRUSES/viruses.html).

Individual protein quantification was performed using a Bradford assay read at 595 nm. Briefly, purified recombinant protein or known quantities of bovine serum albumin (BSA) were diluted 1:50 in Bradford solution (AppliChem, Germany) and incubated, protected from light, for 5 min prior to measurement of absorbance at 595 nm. Protein concentrations (µg/ml) were calculated based on the optical density (OD) values of the BSA standard curve.

Digital analyses of protein band intensities were performed using the gel analysis method from the software program ImageJ (Schneider *et al.*, 2012) for comparative analyses of protein purity percentages.

3.4 Recombinant protein stability analyses

Recombinant BTV proteins VP2, VP5, NS1, NS2, and NS3 were purified according to the corresponding manufacturers' protocols by nickel or cobalt affinity using His SpinTrap™ columns (VP5, NS3) or HisPur™ Cobalt Spin Plates (VP2, NS1, NS2), respectively. Aliquots from before and after purification (called "crude" and "semi-purified," respectively) were stored at +4°C and -80°C and tested for the presence of proteins after storage for 0 days, 7 days (1 week), 14 days (2 weeks), and 35 days (5 weeks), as well as after storage for 616 days (88 weeks) at +4°C. Additional aliquots of VP2, NS1, NS2, and NS3 were prepared at different stages of optimized purification for experimental animal immunizations (purified, dialyzed, sterile-filtered VP2 and NS2; purified NS1) and stored at -80°C for testing after 210 days (30 weeks) and 560 days (80 weeks), respectively. Protein presence was determined by Western blot (as described in section 3.3).

3.5 Animals, clinical examinations, and study designs

Three different animal studies were performed throughout this thesis work and the vaccine design changed based on the results of these studies, as shown in Table 5. Clinical examinations, sampling, vaccinations, and viral challenge were performed in each study as indicated in Figure 6.

Table 5. Vaccines evaluated in each study.

	Study I	Study II	Study III
Species (age at start)	Mice (6-12 weeks)	Cattle (1.3-8.2 years)	Calves (0.6-1.1 years)
Vaccine components/dose	1.5 µg VP2 + 5 µg AbISCO-100[a]	150 µg VP2, 150 µg NS1, 150 µg NS2 + 600 µg AbISCO-300	150 µg VP2, 150 µg NS1, 150 µg NS2 + 450 µg AbISCO-300
	1.5 µg NS1, 1.5 µg NS2, 1.225 µg NS3 + 5 µg AbISCO-100	BTV Pur Alsap 8[b]	450 µg AbISCO-300 in PBS
	1.5 µg VP2, 1.5 µg NS1, 1.5 µg NS2, 1.225 µg NS3 + 5 µg AbISCO-100	PBS	
	5 µg AbISCO-100 in PBS		
Vaccine regimen	Subcutaneous immunizations on the back of the neck at a four-week interval	Subcutaneous immunizations on the left side of the neck at a three-week interval	Subcutaneous immunizations on the left side of the neck at a three-week interval

[a]Isconova AB, Sweden
[b]Merial, France. Each dose contains ≥ 7.1 times the 50% cell culture infective dose ($CCID_{50}$) of BTV-8 before inactivation (log_{10}), plus aluminum hydroxide and saponin (adjuvants)

3.5.1 Study I

To evaluate specific immunogenicities of each protein using a minimum number of animals, 24 six to twelve-week-old female Balb/C mice were divided into four groups of six mice each and housed at the Animal House of the National Veterinary Institute (SVA, Uppsala, Sweden). All mice were subcutaneously immunized in the back of the neck at a four-week interval with homologous vaccines prepared from purified recombinant proteins VP2, NS1, NS2, and NS3 (with respective purities of 95%, 51%, 85%, and 77%) combined in 3 different formulations: i) 1.5 µg VP2 and 5 µg AbISCO-100 (Isconova AB, Sweden) (name: vVP_2); ii) 1.5 µg NS1, 1.5 µg NS2, 1.225 µg NS3, and 5 µg AbISCO-100 (name: $vNS_{1/2/3}$); iii) 1.5 µg VP2, 1.5 µg NS1, 1.5 µg NS2, 1.225 µg NS3, and 5 µg AbISCO-100 (name: $vVP_2NS_{1/2/3}$), per dose; or iv) 5 µg AbISCO-100 diluted in PBS (name: Control). Due to difficulties

producing sufficient quantity of the purified protein, a lower amount of NS3 was used per dose. Each dose of experimental vaccine formulation was adjusted to 200 µl with sterile PBS.

Blood samples (approximately 50-100 µl) were collected from the tail of each mouse for protein-specific ELISAs before first immunization (week 0), before second immunization (week 4), and directly prior to euthanization by cervical spine dislocation (week 6). Following euthanization, spleens from all mice were surgically removed for immediate mononuclear cell isolation and subsequent lymphocyte proliferation assays. The Ethics Committee of Uppsala, Sweden approved this experiment (C237/10).

3.5.2 Study II

To test host-specific immunogenicity and safety of the experimental vaccine in cattle, fifteen healthy, bovine viral diarrhea virus-free, non-lactating Swedish red-and-white breed cows from a BTV-free region (range: 1.3-8.2 years of age; mean 4.3 years of age) were housed in the animal facilities of the Department of Clinical Sciences of the Swedish University of Agricultural Sciences (SLU, Uppsala, Sweden). The cows had not been previously vaccinated against BTV. All animals were divided into three groups of five cows each and immunized subcutaneously on the left side of the neck at a three-week interval with either: i) 150 µg each of purified VP2, NS1, and NS2, and 600 µg AbIsco®-300 (Isconova AB, Sweden), adjusted to 2 ml per dose with sterile PBS (name: SubV); ii) 1 ml dose of commercial inactivated vaccine BTV Pur Alsap 8 (lot L372815; Merial, France), which according to the manufacturer contains 7.1 times the 50% cell culture infective dose ($CCID_{50}$) of BTV-8 before inactivation (log_{10}) plus aluminum hydroxide and saponin as adjuvant (name: CV); or iii) 2 ml of sterile PBS (name: Control).

A BD Vacutainer system (BD Biosciences, USA) was used to collect blood samples (approximately 25 ml) from all animals in dry and heparinized tubes for antibody and T cell proliferation analyses, respectively. Samples were collected at 0, 3, 6, and 9 weeks following first immunization.

To monitor general and local adverse clinical reactions, clinical examinations including rectal temperature recordings were performed daily one day before to three days after each vaccination. Local swelling of injection sites were categorized by size and thickness as none, mild (<3 by 3 cm; flat), moderate (<10 by 10 cm; flat or diffuse), or severe (>10 by 10 cm; raised). The Ethics Committee of Uppsala, Sweden approved this experiment (C153/11).

3.5.3 Study III

To test protective efficacy of the experimental vaccine against BTV-8 infection, twelve healthy, conventionally-reared Holstein calves (range: 0.6-1.1 years of age; mean: 0.8 years of age) that had not been previously vaccinated or infected with BTV, were housed at the Biosecurity Level 3 animal facilities of the National Institute of Agricultural Research (INRA) Research Center (Nouzilly, France). All animals were divided into two groups of six calves each and immunized subcutaneously on the left side of the neck at a three-week interval with SubV, composed of the same amount of recombinant proteins VP2, NS1, and NS2 as study II (150 µg) but with a reduced amount of AbIsco®-300 (450 µg), or with 450 µg AbIsco®-300 in PBS (group name: Control). Three weeks after second vaccination, all animals were subcutaneously inoculated simultaneously with 2.5 ml each of two BTV-8 viral suspensions isolated from a BTV-8-viremic cow. The first viral suspension was isolated on ECE and passaged twice on BHK-21 cells (BHK suspension; $6x10^6$ $TCID_{50}$/ml) and inoculated on the right side of the neck, while the second viral suspension was isolated on KC cells and passed once more on the same cell line for virus amplication (KC suspension) and inoculated on the left side of the neck. Since no cytopathic effects (CPE) were observed following infection of KC cells, an RT-qPCR (Adiavet™ BTV Realtime ADI352, Adiagene, France) was performed using 10 µl of KC suspension and resulted in a Ct value of 14.1. Clinical examinations and collection of blood samples in ethylene diamintetraacetic acid (EDTA), dry, and heparinized tubes for analysis of viremia, humoral immune responses, and cellular immune responses, respectively, were performed as indicated in Figure 6.

Clinical examinations were performed after each vaccination, including recording rectal temperatures and any localized swelling at the injection site. Localized swellings were considered none (no swelling), mild (<3 cm), moderate (>3 cm and <10 cm), or severe (>10 cm). For clinical examinations performed after BTV-8 challenge, clinical scoring was performed as described previously (Perrin *et al.*, 2007), with minor modifications; respiratory signs were graded on a 3-point scale (serous, purulent, or necrotic nasal discharge) rather than a 2-point scale (mild or severe nasal discharge), and scores for rectal temperatures were included, as shown in Table 6.

This study was approved by the local ethical review board of Val de Loire (CEEA VdL, committee number n°19, file number 2012-08-01).

Figure 6. Timeline of study I (A), study II (B), and study III (C).

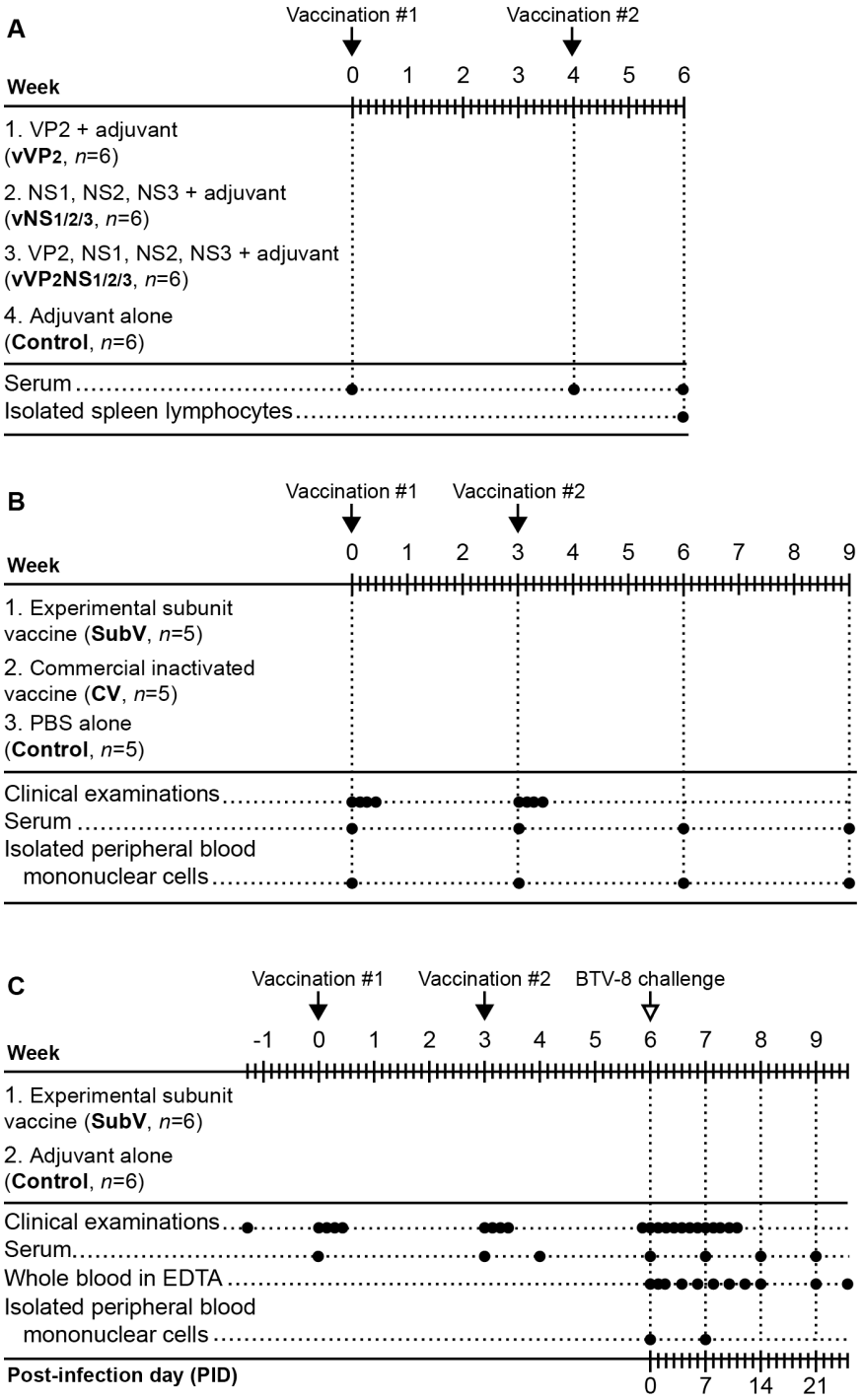


Table 6. *Clinical scoring for BTV-8 challenge, modified from (Perrin* et al.*, 2007).*

	Score
General State	
Good (normal behavior)	0
Apathy (separation from the group, slow)	1
Depression (lying down alone, still aware)	2
Prostration (recumbent, no movement)	3
Local Signs (edema or congestion)	
Face	1
Nose	1
Intermandibular space	1
Lips	1
Tongue	1
Skin or hooves	4
Right lymph nodes	
Normal (none)	0
Slightly swollen (< 3 cm)	1
Swollen (3-10 cm)	2
Very swollen (< 10 cm)	3
Left lymph nodes	
Normal (none)	0
Slightly swollen (< 3 cm)	1
Swollen (3-10 cm)	2
Very swollen (< 10 cm)	3
Locomotive Signs	
Stiffness	2
Lameness	2
Respiratory Signs	
Normal nasal discharge	0
Serous nasal discharge	1
Purulent nasal discharge	2
Necrotic nasal discharge	3
Cough	1
Other	
Diarrhea	1
Conjunctivitis	1
Ulcers	1
Plaintive bleating	1
Excessive salivation	1
Rectal Temperature (°C)	
38.0°C < T < 39.4°C	0
39.5°C < T < 40.0°C	1
40.1°C < T < 41.0°C	2
41.1°C < T < 42.0°C	3
T > 42.0°C	4

3.6 Virus detection

Virus detection by inoculation of ECE and RT-qPCR was performed on samples collected in study III as indicated in Figure 6.

3.6.1 ECE inoculation

Virus inocula were prepared by diluting blood collected in EDTA tubes from all calves on PID8, 1:3 in PBS. A volume of 100 µl of diluted blood per calf (or of PBS for control eggs) was inoculated, in quintuplicate, in twelve-day-old embryonated specific pathogen free (SPF) chicken eggs (Håtunaholm, Sweden) by intravenous route, according to a general license for use of this technique (SVA, Sweden). All eggs were incubated at 37°C and monitored for seven days post-inoculation. Dead embryos were scored as positive if they showed hemorrhage characteristic of BTV infection. At completion of the study, embryos were incubated for 4 h at +4°C, homogenized, and stored at -70°C, and swabs of the thawed homogenates were stored in approximately 800 µl Tris-EDTA (TE) buffer before viral RNA extraction.

3.6.2 RT-qPCR

Viral RNA was extracted from whole blood samples and swabbed embyro homogenates using a Magnatrix robot at SVA (Uppsala, Sweden) and then incubated for 5 min at 95°C, to denature dsRNA to ssRNA. A pan-BTV RT-qPCR based on segment 1 (VP1) of BTV (Toussaint *et al.*, 2007) was performed on all extracted and denatured samples using 2 µl denatured RNA with 13 µl PCR mix from the AgPath-ID one-step RT-PCR kit (Ambion, USA).

3.7 Humoral immunity analyses

Analysis of humoral immunity induced by vaccination and/or viral challenge, including BTV-8 neutralizing antibody assays and protein-specific ELISAs against the purified recombinant BTV proteins, were performed in each study on serum samples collected as indicated in Figure 6.

3.7.1 Virus neutralizing antibody assay

Cattle sera were analyzed for the presence of specific BTV-8 neutralizing antibodies by classic virus neutralizing antibody tests in studies II and III.

Vero cells in 100 µl minimal essential medium (MEM, Gibco, United Kingdom) supplemented with 1% minimal essential amino acids (Gibco) and 1% HEPES (Gibco), were added per well to 96-well microtiter plates. Sera were heated for 30 min at 56°C and added in duplicate to wells at a range of

dilutions from 1:4 to 1:512 (study II) or 1:2 to 1:256 (study III). Virus was diluted in 50 µl and added to the plates. Plates were incubated for six (study II) or five days (study III) at 37°C and 5% CO_2, then examined for the presence of virus-specific CPE. In both studies, the neutralizing titer was defined as the highest dilution in which the cell monolayer was intact.

3.7.2 Detection of BTV-8 (VP2)-specific antibodies

Specific antibodies to VP2 of BTV-8 were analyzed using an indirect ELISA and Western blot (study I), or a cELISA (ID Screen bluetongue serotype 8 competition, ID VET, France) (studies II, III) according to the manufacturer's protocol. For studies II and III, detection of BTV-8 VP2-specific antibodies was also evaluated as an indicator of serotype-specific vaccination or infection as part of the DIVA analysis.

For study I, VP2 and background control proteins (lysate from SF9 cells) were coated onto 96-well ELISA plates (Maxisorp, Nunc, Denmark) and incubated at +4°C for 16 h before 3 h blocking in 2% (wt/vol) BSA in PBS at room temperature. Plates were washed three times with PBS and then sera samples, which had been diluted in background control protein for 1 h, were added to wells for 1.5 h incubation at 37°C. Plates were washed three times with PBS-Tween, incubated at 37°C with rat anti-mouse IgG1 heavy chain-HRP (MCA336P; AbDSerotec, United Kingdom) for 45 min, washed with PBS-Tween 3 times, and incubated with 3,3',5,5'-tetramethylbenxidine (TMB) substrate before addition of hydrogen peroxide stop solution. Corrected OD (COD = $OD_{protein}$-$OD_{background}$) values were calculated from absorbance values measured at 450 nm. Titers were calculated by doing a linear regression to a cut-off based on the COD value of negative control sera at a dilution factor of 50 and all data is presented as $\log_{10}$ values. For calculating means and performing statistical analyses, sera that were antibody-negative at the lowest tested dilution factor (50) were set to that threshold (dilution factor 50, i.e. 1.7 $\log_{10}$ titer).

Western blot, as described in section 3.3, was performed against VP2 and Sf9 cell lysate (background control), using cattle sera obtained three weeks after experimental infection with BTV-8, after two or eight immunizations with commercial inactivated vaccines against BTV-8, or from negative controls (non-infected and non-BTV-vaccinated animals). Diluted sera and sheep anti-bovine IgG:HRP (AAI23P; AbDSerotec, United Kingdom) were used as the primary and secondary antibodies, respectively.

For studies II and III, cELISAs were performed according to the manufacturer's protocol. The plates were read at 450 nm and validated

according to the manufacturer's specifications. All results are presented as 100 minus percent competition (($OD_{sample}/OD_{mean\ of\ negatives}$) x 100).

3.7.3 Detection of BTV-2 NS1-, NS2-, and NS3-specific IgG1 antibodies

Indirect ELISAs were used to detect NS1- and NS2-specific IgG1 serum antibodies (BTV-2) in all studies, and NS3-specific IgG1 serum antibodies (BTV-2) in study I only. Western blot was also performed in study I to detect bovine serum antibodies to NS1 and NS2.

For study I, individual test protein or background control proteins (lysate from SF9 cells for NS1, NS3; lysate from BL21 *E. coli* for NS2) were coated at +4°C for 16 h onto 96-well ELISA microtiter plates (Maxisorp, Nunc, Denmark), blocked for 3 h in 2% (wt/vol) BSA in PBS at room temperature, and then plates were washed three times with PBS. Sera samples were diluted in corresponding background control protein and incubated for either 1 h (NS2) or 1.5 h (NS1, NS3), then added to the plates for 1.5 h incubation at 37°C. Plates were washed three times with PBS-Tween, incubated with rat anti-mouse IgG1 heavy chain-HRP (MCA336P; AbDSerotec, United Kingdom) for 45 min (NS1, NS3) or 1 h (NS2) at 37°C, again washed three times with PBS-Tween, and then incubated with TMB substrate before adding hydrogen peroxide stop solution. COD values were calculated from absorbance values measured at 450 nm as described in section 3.7.2.

For studies II and III, NS1- and NS2-specific indirect ELISAs were performed as for study I, with minor modifications: plates were blocked for 60 min at room temperature, serum samples were incubated at 37°C for 75 min (NS2), and the secondary antibody used was HRP-conjugated mouse anti-bovine IgG1 monoclonal antibodies (Svanova Biotech, Sweden) for 45 min incubation at 37°C.

For studies I and III, antibody titers were calculated by doing a linear regression to a cut-off based on the COD value of negative control sera at a dilution factor of 50 (study I) and 10 (study III). For calculating means and performing statistical analyses, sera that were antibody-negative at the lowest tested dilution factor (50 or 10) were set to that threshold (dilution factor 50 or 10). Results are presented as $\log_{10}$ values.

For study II, COD values were calculated as described in section 3.7.2 and results are presented as percent positive (($OD_{sample}/OD_{mean\ of\ positives}$) x 100).

Western blot performed in study I, as described in section 3.3, was performed against NS1 and NS2 and relevant background controls (Sf9 cell and BL21 *E. coli* lysate, respectively), using serum samples described in section 3.7.2.

3.7.4 Detection of VP7-specific IgG or IgM antibodies for all BTV serotypes

For studies II and III, the differentiation between infected and vaccinated animals was performed by detecting specific IgG or IgM antibodies directed against VP7 of any BTV serotype, using a double antigen sandwich ELISA kit (ID Screen® bluetongue early detection one-step, study II) or a cELISA kit (ID Screen® bluetongue competition, study III) according to the manufacturer's protocols (ID Vet, France). The plates were read at 450 nm and validated according to the manufacturer's specifications. Results are presented as 100 minus percent competition (study II) or as percent competition (study III).

3.8 Cellular immunity analyses

Mononuclear cells from mouse spleens (study I) and peripheral blood mononuclear cells (PBMCs) from heparinized cattle blood samples (studies II, III) were isolated and restimulated *ex vivo* to evaluate protein-specific or virus-specific cellular immunological responses.

3.8.1 Lymphocyte proliferation analyses

For study I, mouse spleens were removed directly after euthanization and flushed individually with sterile PBS to create single-cell suspensions. Samples were centrifuged over Ficoll-Paque Plus™ medium (GE Healthcare, United Kingdom) at 1300 x *g* for 15 min at +4°C and the lymphocytes were removed and washed twice in PBS by centrifugation at +20°C, first at 500 x *g* for 10 min and then at 200 x *g* for 10 min.

After the second wash, lymphocytes were counted using Türks solution, and diluted to $2x10^6$ cells/ml ($2x10^5$ cells/µl) in RPMI medium supplemented with 10% fetal calf serum. Cells were plated at a final concentration of $2x10^6$ cells/ml onto sterile 96-well round-bottomed plates in a volume of 100 µl/well.

Isolated cells were restimulated, in quadruplicates, with individual proteins and relevant background controls at 0.03 µg/well then incubated for five days at 37°C and 5% CO_2. Eighteen hours prior to measurement of absorbance by spectrophotometry at 570 nm and 595 nm, 20 µl of alamarBlue® reagent (Invitrogen, United Kingdom) was added to each well. OD values at 595 nm were subtracted from OD values at 570 nm per well, and COD values were calculated among the different groups for BTV protein-specific stimulations.

For study II, PBMCs were obtained from heparinized blood samples from all cattle, as previously described (Taylor *et al.*, 1995). In short, heparinized blood samples were diluted 1:1 in room-temperature PBS and added to Ficoll-Paque Plus™ medium. Samples were centrifuged at 1100 x *g* for 30 min at +20°C and the lymphocytes were removed, washed, and restimulated as

described for study I (shown above). Restimulations were performed with 0.03-0.18 µg/well of individual test proteins and relevant background controls.

For study III, PBMCs were isolated from heparinized blood samples collected before challenge (6 weeks) and one week after challenge, as described for study II, then resuspended in bovine viral diarrhea virus-free fetal calf serum with 10% DMSO (Research Organics, USA) and frozen at -80°C for transport to Sweden. Cells were then stored in liquid nitrogen before analysis at $2x10^6$ cells/ml for stimulations. Stimulations were performed, in duplicates, with 0.3-1 µg/well of individual proteins and relevant background controls as well as $10^{3.5}$ $TCID_{50}$/ml of UV-inactivated BTV-8 (equivalent Vero cells as background control). AlamarBlue® reagent was added 7-16 h prior to measurement of absorbance by spectrophotometry at 570 nm and 595 nm and COD values were calculated as described above.

3.8.2 Detection of IFN gamma (IFN-γ) production of restimulated lymphocytes

In study II, the presence of IFN-γ in the supernatants of restimulated lymphocytes (described in section 3.8.1) was quantified using a sandwich ELISA kit (ID Screen® ruminant interferon gamma kit) according to the manufacturer's protocols (ID Vet, France). The plates were read at 450 nm and then validated by following the manufacturer's specifications. Results are expressed as COD values.

3.9 Statistical analyses

Due to the structure of the sample data, non-parametric Wilcoxon rank sum, Wilcoxon sign rank, and Kruskal-Wallis tests were used for analysis among two independent, two paired, or three independent immunized groups, respectively, in the statistical program R (R Development Core Team, 2012). Where relevant, Student's one-tailed t-tests were performed in Excel. Statistical significance of the tests was set to a p value of ≤ 0.05 (*) or ≤ 0.01 (**) unless otherwise specified and where applicable, values are provided as the indicated group mean plus-or-minus the standard deviation (mean±SD).

4 Results and discussion

4.1 Formulation and optimization of a novel DIVA subunit vaccine against BTV-8

To develop a novel vaccine against BTV that could be safe, potentially adaptable to different serotypes, and DIVA compliant, we chose to pursue a subunit vaccine design. The rationale behind the formulation of the experimental vaccine was to include a cocktail of BTV proteins that could induce both protective humoral and cellular immune responses in cattle. Based on available information, five proteins were selected, of which some could induce serotype-specific protection (VP2, VP5 of BTV-8) (Stewart *et al.*, 2012; Mohd Jaafar *et al.*, 2014) as well as potentially protect across multiple serotypes (NS1, NS2, and NS3 of BTV-2) (Calvo-Pinilla *et al.*, 2012), since several BTV serotypes were co-circulating in Europe when the project was designed.

4.1.1 Production, expression, and quantification of recombinant BTV proteins for subunit vaccine design

Recombinant BTV proteins VP2, VP5, NS1, and NS3 were produced in Sf9 cells using a baculovirus expression system, while recombinant BTV protein NS2 was produced in a BL21 *E. coli* expression system. Both of these systems have been successfully utilized previously for BTV recombinant protein production (Inumaru *et al.*, 1987; Jones *et al.*, 1997; Mecham & Wilson, 2004; López *et al.*, 2006; Jabbar *et al.*, 2013; Mohd Jaafar *et al.*, 2014) as well as for the production of commercial recombinant vaccines, such as Flubok® (influenza vaccine), Cervarix® (human papillomavirus vaccine), and SparVax® (anthrax vaccine). Additionally, the five target proteins were already expressed in these systems and available through collaboration in our laboratories. Baculovirus and *E. coli* production systems are commercially available and affordable, plus have the potential to rapidly produce large

quantities of functional recombinant protein when scaled up from laboratory to industrial settings, as reviewed by (Brun *et al.*, 2011). Baculovirus expression systems additionally allow post-translational modification of proteins, including glycosylation. Moreover, both systems do not require fetal calf serum and are therefore bio-safe in that aspect. However, no expression system is perfect and reported difficulties with the *E. coli* system include lack of post-translational modifications, potential misfolding of proteins, and risk of lipopolysaccharide (LPS) contamination. Similarly, baculovirus systems may not facilitate appropriate "mammalized" glycosylation of expressed proteins for use in vaccines (Harrison & Jarvis, 2006) and can be time-consuming when cloning the target genes into transfer vectors for generation of recombinant baculoviruses (Jayaraj & Smooker, 2009). In this thesis work, however, it was possible to use these systems to produce sufficient quantities of recombinant protein for practical use in vaccines and immunogenicity analyses. Adaptation of these systems to novel technologies, such as the use of baculovirus and silkworm larvae instead of insect cells (Kost *et al.*, 2005), or by utilizing other promising systems, such as yeast (Shin & Yoo, 2013) or plant-based (Guerrero-Andrade *et al.*, 2006) expression systems, may further enable their use for the production of greater quantities of recombinant protein.

In contrast to other BTV experimental vaccine studies that used cell lysate rather than purified protein (Roy *et al.*, 1990), we aimed to purify the recombinant proteins to be able to better evaluate protein-specific immunogenicities. Additionally, clearly-defined antigens such as purified proteins may be safer by inducing fewer secondary effects and are therefore highly desirable for commercial vaccine development (Clair *et al.*, 1999). Nickel and cobalt affinity systems are commonly used for purifying His-tagged proteins. Purification protocols, including lysis and elution buffers, were optimized for each of the five recombinant proteins by testing between the two affinity systems, in combination with modifying salt and pH concentrations, adding several detergents, and changing the temperature of lysis buffers. Differences in the optimized protocols were dependent on their degree of success for purifying each protein. Optimized protocols were identified for VP2, NS1, and NS2 in cobalt plates, for NS3 in nickel columns (although it remained difficult to purify in sufficient quantity), but not for VP5 in either system (*Paper I*). Recombinant baculovirus-expressed VP5 with a glutathione S-transferase (GST)-tag has previously been purified from detergent-soluble extracts of infected cells (Hassan *et al.*, 2001), but the recombinant protein used in the present project could not be solubilized despite many changes to the lysis buffer. VP5 has a hydrophobic region (Hassan *et al.*, 2001) that may hinder its solubility (Yasui *et al.*, 2010) under the conditions presented here

and may explain why the His-tagged VP5 could not be sufficiently and reproducibly purified. Modifying the recombinant protein, for example by using another affinity tag or by expressing a truncated version of the protein, could have potentially improved the solubility of VP5 and enabled its purification. However, recent studies have suggested that VP5 may not be essential for induction of BTV protection (Shaw *et al.*, 2012; Mohd Jaafar *et al.*, 2014) and as a result, it was decided to not pursue further investigations into purifying VP5.

The final buffer protocols used for purification are shown in Table 4 (section 3.2).

4.1.2 Stability of recombinant BTV proteins

The stability of the five recombinant BTV proteins was evaluated at different time points for different stages of purification, to determine how long batches of each of the recombinant proteins could be stored to perform the different experiments in this project. First, the stability of all five proteins, stored as semi-purified proteins at -80°C and +4°C, was evaluated after 0, 1, 2, and 5 weeks. With the exception of NS2 when stored at +4°C for 5 weeks, all proteins were detected by mouse anti-histidine tag Western blot at all measured time points and temperature conditions. Additionally, each protein did not appear to degrade, as determined by digital image analysis, throughout these five weeks.

Next, the protein stability in both crude and semi-purified extracts was evaluated after storage at +4°C for 88 weeks (termination of the part of the experiment that analyzed these samples). Whereas all five proteins were detected in crude extracts, only semi-purified VP2, NS1, and NS2 were detected under these storage conditions. These results indicate that the purity level of the proteins may influence their stability.

Since VP5 could not be purified and NS3 was difficult to purify in sufficient quantity, only purified VP2, NS1, and NS2 were evaluated after 30 and 80 weeks (endpoints of the individual experiments analyzing these samples) storage at -80°C. Additionally, dialyzed and sterile-filtered VP2 and NS2, but not NS1, were evaluated following 30 weeks storage at -80°C. NS1 could not be sterile-filtered due to protein loss during the process, likely because the protein bound to the membrane, and there was no dialyzed aliquot at this time point for evaluation. However, NS1 tested negative for bacterial contamination, which if present, would have otherwise been removed by the sterile filter. All proteins were detected using Western blot under these conditions and time points and each protein demonstrated similar integrity as that observed before storage.

Taken together, these results indicate that VP5 and NS3 can be stored long-term as crude aliquots and that VP2, NS1, and NS2 can be stored long-term in crude, semi-purified, or purified aliquots. Importantly for subunit vaccine development (Clair *et al.*, 1999), the results presented here suggest that VP2, NS1, and NS2 can be purified and stored with minimal degradation for at least 1.5 years at +4°C and therefore are suitable for vaccine use regarding shelf life. It would be interesting to further evaluate whether these proteins can be stored at room temperature, and for how long, as well as whether they can be stored in lyophilized forms. The storage of purified recombinant proteins as lyophilized aliquots at room temperature has previously been demonstrated (Diminsky *et al.*, 1999; Dutta *et al.*, 2001; Smallshaw & Vitetta, 2010) and would be desirable for commercial vaccine production.

4.1.3 Selection of BTV proteins based on their immunogenicity for inclusion in experimental subunit vaccine

Initially five recombinant BTV proteins were selected for evaluation but as noted above, recombinant VP5 could not be sufficiently and reproducibly purified. Therefore only the remaining four recombinant BTV proteins (VP2, NS1, NS2, and NS3) were included in different experimental formulations tested in a minimum number of mice, by following the three Rs (replacement, refinement, reduction) principle (CODEX, 2013). The formulations consisted of different protein combinations, rather than each of the proteins individually, in order to evaluate the potential stimulatory or suppressive interactions among the proteins in a minimum number of mice. Additionally, the different formulations were administered in combination with an adjuvant. Subunit vaccine formulations require adjuvants so that sufficiently high levels of immune responses are induced following vaccination. Adjuvants provide the added advantage of potentially directing the immune response, particularly towards a cellular immune response (Vogel, 2000). Neutralizing antibodies have been demonstrated to be crucial for serotype-specific protection against BTV (Huismans *et al.*, 1987; Roy *et al.*, 1990), but the importance of T cell responses in providing BTV protection has also been indicated (Jeggo *et al.*, 1984a, 1985; Jones *et al.*, 1997; Calvo-Pinilla *et al.*, 2012; Rojas *et al.*, 2014). Leading adjuvants such as ISCOM-matrices are understood to help stimulate CD8+ T cells in particular (Robson *et al.*, 2003), as well as CD4+ T cells (Pedersen *et al.*, 2012), and since we had expertise using these adjuvants within our group, the AbISCO product line (including AbISCO-100 for smaller animals such as mice; AbISCO-300 for larger animals such as cows) was selected for inclusion in the protein formulations. The individual immunogenicities of each protein in combination with the ISCOM-matrix

adjuvant was evaluated using protein-specific humoral and cellular immunity analyses.

Of these four proteins, NS3 was the most difficult to purify in sufficient quantity, and was thus ultimately included in the different formulations in a slightly lower concentration than the other proteins (1.225 µg/dose compared to 1.5 µg/dose). Using assays that were limited by low quantities of purified protein, it was not possible to determine if NS3-specific humoral immune responses were induced in immunized mice, and cellular immune responses were not detected (data not shown), despite evidence from others that NS3 induces both humoral (López *et al.*, 2006; Perrin *et al.*, 2007; Barros *et al.*, 2009) and cellular (Andrew *et al.*, 1995; Jones *et al.*, 1996) immunity. For these reasons, NS3 was excluded from further analyses and from the final vaccine formulation used in the cattle studies.

For the remaining three proteins (VP2, NS1, and NS2), specific serum IgG1 antibodies directed against VP2 and NS2, but not NS1, were detected by indirect ELISA in immunized mice. Although not statistically significant, NS2 tended to induce stronger antibody response when formulated with the NS proteins alone, compared to the formulation that included VP2. These ELISA results were supported by complementary studies using Western blot analyses, in which serum IgG antibodies from cattle experimentally-infected or vaccinated against BTV-8 recognized purified recombinant VP2 and NS2. Consequently, an indication of the tertiary conformation of the individual proteins was obtained before proceeding to large animal experiments. The results indicated that VP2 and NS2 were correctly folded to be recognized by, and thus to detect, BTV-8 antibodies.

Regarding cellular immune responses, significantly higher specific spleen lymphocyte proliferative responses to VP2, followed by NS1 and NS2, were detected in samples from immunized mice, compared to control mice ($p \leq 0.05$ for all). These results support previously published conclusions about the protein-specificity of induced T cell responses in mice and sheep (Takamatsu *et al.*, 1990; Andrew *et al.*, 1995; Jones *et al.*, 1996, 1997). Taken together with the humoral immune results, these results suggested that the recombinant VP2, NS1, and NS2, when administered in combination with an ISCOM-matrix adjuvant, were immunogenic in mice and thereby potentially immunogenic in cattle. Therefore, these three proteins and the ISCOM-matrix adjuvant were included in the final formulation of the experimental subunit vaccine (called SubV).

4.1.4 Modulation of adjuvant composition of SubV for cattle

In study II, one dose of SubV consisted of 600 µg of AbISCO-300 (suitable for larger animals such as cows) and 150 µg each of VP2, NS1, and NS2 (Table 5), and was prepared just before administration by first mixing the proteins together and then adding the adjuvant. The final volume was adjusted to 2 ml by addition of PBS. Controls received 2 ml PBS alone. SubV induced increased rectal temperatures for 24 h following first vaccination (group means: 39.2±0.3°C and 38.0±0.4°C for SubV and Control groups, respectively), as well as mild-to-moderate injection site swellings. Higher rectal temperatures (group means: 40.0±0.8°C and 38.0±0.3°C for SubV and Control groups, respectively) and more pronounced injection site swellings were also observed following second vaccination with SubV in comparison to first vaccination (*Paper II*). However, localized swelling disappeared less than one week after vaccination and no change in behavior or reduction in appetite was observed in any animal throughout the study. Localized reactions with transient fever have also been reported following use of commercial BTV-8 vaccines, including Bluevac BTV8 (CZ Veterinaria S.A, Spain), Bovilis BTV8 (Merck/MSD Intervet, United Kingdom), and BTVPUR AlSap 8 (Merial, France) (Vetvac, 2014).

Both VP2 and NS2 were sterile-filtered to reduce the risk of contaminants before inclusion in the vaccines, and NS1 was tested and found negative for the presence of bacteria. ISCOM-matrices have been proposed to affect immune processes by accelerating and improving antigen uptake and presentation by antigen-presenting cells as well by inducing inflammation, including relevant cytokine production (Morein & Bengtsson, 1999; Morein *et al.*, 2004; Lövgren Bengtsson *et al.*, 2011). The inflammatory properties of ISCOM-matrices, likely due largely to the saponins included in their formulation (Smith *et al.*, 1998), can lead to some general and local reactions by inducing hyperthermia and moderate localized swelling at the injection site, as has been observed in other studies (Heldens *et al.*, 2009; Blodörn *et al.*, 2014). Therefore, it was hypothesized that the clinical signs observed following vaccination in study II could have been caused by the high amount of adjuvant included in the vaccine. Furthermore, in contrast to the cows vaccinated in study II, younger and smaller calves were to be immunized with SubV in study III and the adjuvant composition of SubV needed to be modulated accordingly. The adjuvant amount was reduced from 600 µg/dose in study II to 450 µg/dose for study III, using the same quantity of proteins. Consequently, milder secondary reactions and no statistically significant temperature increase was observed following vaccination with SubV compared to controls receiving adjuvant alone ($p=0.38$) (*Paper III*). Less localized injection site swelling was observed

following first vaccination with SubV, and swelling abated more rapidly following second vaccination compared to earlier (*Paper II*, data not shown). Improvements in protein purity (data not shown) may have also contributed to decreased localized swelling following vaccination. Notably, the reduction of adjuvant quantity in SubV did not seem to affect the induction of immune responses by the vaccine, as described in the following sections.

4.2 Humoral immune responses against BTV in cattle following vaccination and challenge

4.2.1 Induction of BTV-8 neutralizing antibody titers in cattle

Strong BTV-8 neutralizing antibody titers were detected three weeks after second vaccination in cattle immunized twice at a three-week interval with SubV. Titers were comparable to those induced by a commercial inactivated vaccine following the same vaccine regimen (2.7±0.2 and 2.9±0.5 $\log_{10}$ titers/ml for SubV and CV, respectively; p=0.17) (*Paper II)*. Neutralizing antibodies have been shown to be an essential component of the protective immune response against BTV (Huismans *et al.*, 1987; Roy *et al.*, 1990) and similar titers, ranging from 1.5-2.5 $\log_{10}$ $TCID_{50}$, have been observed following vaccination with killed commercial vaccines in ruminants that were ultimately protected from BTV-8 challenge (Bréard *et al.*, 2011). The induction of similar neutralizing antibody titers by both SubV and CV indicated that the experimental vaccine may induce a level of protection similar to that which has already been demonstrated for the commercial vaccine (Eschbaumer *et al.*, 2009; Gethmann *et al.*, 2009; Wäckerlin *et al.*, 2010; Bartram *et al.*, 2011; Bréard *et al.*, 2011). Of the three proteins included in the SubV formulation, VP2 is the only protein associated with the induction of virus-neutralizing antibodies (Huismans & Erasmus, 1981; Kahlon *et al.*, 1983; Roy *et al.*, 1990). Previous studies have demonstrated that VP5 may also play a role by supporting the tertiary conformation of VP2 (Cowley & Gorman, 1989; Mertens *et al.*, 1989; Roy *et al.*, 1990; DeMaula *et al.*, 2000). Therefore, it has been suggested that the inclusion of VP5 with VP2 would benefit any vaccine (Schwartz-Cornil *et al.*, 2008). However, since SubV induced neutralizing antibody titers that were equal to those induced by CV, the results indicated that VP2 alone and at the amount used in study II, may be sufficient to induce virus-neutralizing antibody titers equivalent to those attained with a commercial vaccine.

In study III, serum samples were collected before second vaccination and then almost weekly from this time point until three weeks after BTV-8 challenge, which enabled observation of the time course for antibody

development following vaccination and challenge. BTV-neutralizing antibodies were detected as early as one week after second vaccination in SubV-immunized calves, and continued to increase until one week after BTV-8 challenge, when they stabilized (*Paper III*). In contrast, virus-neutralizing antibodies were first detected in the sera of 1/6 controls only two weeks after BTV-8 challenge, and although all controls eventually seroconverted three weeks after challenge, they remained at significantly lower titers compared to those of the vaccinated calves ($p \leq 0.05$). Compared to other experimental vaccines, the peak neutralizing antibody titers observed in this study were similar to those observed in ruminants following vaccination with similar recombinant subunit vaccines or DISC vaccines (Roy *et al.*, 1990; Matsuo *et al.*, 2011). However, the DISC vaccine was able to induce neutralizing antibodies after just one vaccination, perhaps due to its ability to enable expression of the viral proteins at the natural site of infection, much like MLVs (Roy *et al.*, 2009).

4.2.2 Induction of serum antibodies directed against VP2 (BTV-8) and NS1, NS2 (BTV-2) detected by ELISA

Using cELISA, VP2-specific serum antibodies were detected after vaccination in animals immunized with either SubV (*Papers II, III*) or CV (*Paper II*). The quantity of detected VP2-specific antibodies induced by vaccination was greater in animals immunized by SubV than by CV. This may have been due to several factors, including the assay itself, which is based on the same protein used in SubV, to the high amount of VP2 antigen included in SubV in comparison with possibly lower amounts of VP2 antigen included in CV, or to conformational changes to the VP2 antigen during inactivation of the virus for inclusion in CV. The detection of VP2 antibodies is important for DIVA, because they can indicate serotype-specific infection or vaccination (as discussed in section 1.7.2).

Specific serum IgG1 antibodies directed against NS1 and NS2 were also detected in cattle immunized with SubV. Levels of NS2-specific antibodies increased in SubV-immunized animals compared to in controls as early as three weeks after first vaccination ($p \leq 0.05$ for both study II, III) and both NS1- and NS2-specific antibody levels peaked three weeks after second vaccination ($p \leq 0.01$ for NS1 for both study II, III; $p \leq 0.05$ for NS2 for both study II, III). Since the indirect ELISA used to detect antibodies herein was based on NS1 protein of BTV-2, and CV was of serotype 8, serotype divergence may explain the absence of NS1 antibodies following CV immunization in this study. However, both genetic and serological analyses of the protein and its encoding RNA segment (segment 5) have shown that NS1 is well conserved across

serotypes (Mecham *et al.*, 1986; Toussaint *et al.*, 2007; Maan *et al.*, 2008). Furthermore, it has been shown in previous studies that NS1 antibodies were not detected in sheep following immunization with inactivated vaccines (Anderson *et al.*, 1993). Therefore, it is unlikely that serotype differences could explain the lack of NS1 antibody response to CV. A more plausible explanation is that NS1-specific antibodies were simply not induced by CV, perhaps because NS1 was only present in low quantities in that vaccine. This latter explanation was supported by findings in study III, where, using a similar assay, specific serum antibodies to NS1 of BTV-2 were detected in 4/6 control calves three weeks after BTV-8 challenge, thus indicating that antibodies to NS1 of BTV-8 can recognize NS1 of BTV-2.

Similarly, though the magnitude of the specific antibody response directed against NS2 following vaccination with CV was much lower than those induced by SubV, it is unlikely that serotype-specific differences in the protein are responsible for the weaker antibody response to CV. This was supported by findings in study III, in which NS2 (BTV-2)-specific IgG1 antibodies were detected in the serum of 5/6 non-vaccinated controls three weeks after BTV-8 challenge. In comparison to high levels of NS2 included in SubV, the CV preparation likely included low original quantities of this protein since, like NS1, it is a primarily internal protein that is only produced during viral replication. Therefore, in contrast to challenge, CV induced only weak levels of NS2-specific antibodies. That NS2 induced any antibody production following immunization with CV, in contrast to NS1, may be due to several potential differences between the proteins, such as inherent dissimilarities in protein-specific immunogenicities or disparities in protein quantities following BTV replication for CV production. Additionally, it has previously been suggested that NS2 may associate with membrane proteins VP2 and VP5 (Mertens *et al.*, 1987), and therefore NS2 may have remained in higher final quantity than NS1 following clarification of BTV during CV manufacturing. To the best of my knowledge, the results presented in study II and III represent the first time that NS2-specific antibodies have been detected in vaccinated and infected cattle, though NS2 antibodies have been previously observed in the polyclonal sera of experimentally-infected rabbits (Mecham *et al.*, 1986).

Both NS1 and NS2 have induced specific serum antibodies in cattle, but the role that these antibodies may play in protection is not known. Studies in flaviviruses have shown that antibodies to NS proteins can provide protection against viral challenge, perhaps by inducing complement-mediated cytolysis or antibody-dependent cellular cytotoxicity through recognition of antigen expressed on infected cells (Kreil *et al.*, 1998; Calvert *et al.*, 2006; Chung *et al.*, 2006; Wan *et al.*, 2014), as reviewed by (Burton, 2002). However, whether

NS1 or NS2 antigens are expressed on the surface of BTV-infected cells, as well as other possible mechanisms of protection induced by non-neutralizing antibodies, should be further investigated.

4.3 Cellular immune responses against BTV in cattle following vaccination and challenge

Cellular immune responses induced by SubV and CV (study II, III) were measured in cattle by specific lymphocyte proliferation following *ex vivo* restimulation of PBMCs. In this work, AlamarBlue®-reagent was used to quantify the proliferation of cells following restimulation. This reagent works by changing absorbance in proportion to the number of living cells and when used in combination with background control stimulations, can provide low but specific values. The use of the alamarBlue®-reagent is simple and non-toxic, and the method has been demonstrated to be as reliable as alternative assays (Ahmed *et al.*, 1994). This method has also been successfully utilized in other studies (Hägglund *et al.*, 2011; Blodörn *et al.*, 2014).

4.3.1 Induction of VP2-specific lymphocyte proliferative responses

Specific lymphocyte proliferative responses directed against VP2 were not detected in cattle with either SubV or CV (study II, *Paper II*), nor after modifying the protocol by increasing protein concentrations fivefold for restimulation (study III, *Paper III*). This is in contrast to results of others, where strong but variable CTL responses directed against VP2 were reported in vaccinated sheep (Andrew *et al.*, 1995; Janardhana *et al.*, 1999). VP2-specific lymphoproliferative responses were also detected following restimulation of spleen lymphocytes in mice (study I, *Paper I*). As the tertiary conformation of VP2 has been shown to be important (White & Eaton, 1990), conformational issues, as well as differences in vaccine preparation (including adjuvant, antigen selection and/or quantity, and route of administration), cell origin for stimulation (for example, PBMCs or spleen lymphocytes) or species-differences among mice, sheep, and cattle, may provide an explanation for the observed differences between this study and previous reports. Although the lymphocyte proliferation assay was modified for study III by increasing protein concentrations for restimulation, some possible cellular cytotoxicity by the protein was observed in both studies (data not shown) which may have affected *in vitro* restimulation. Evaluation of whether experimentally-infected non-vaccinated control calves showed lymphoproliferative responses to VP2 after challenge with BTV might have enabled us to conclude if a T cell response was induced against this protein in cattle, but this was not possible due to poor

viability of cells isolated one week after challenge and then stored in liquid nitrogen (*Paper III*). This could as well be further investigated by restimulating isolated PBMCs from naturally infected cattle with purified VP2 expressed in different systems, or by restimulating PBMCs from cattle vaccinated with VP2 expressed in different systems with live or inactivated BTV.

While it cannot be excluded that the assay needs to be further improved, the disparity of the results between the murine and bovine assays also highlights the importance of evaluating a novel vaccine in the target species.

4.3.2 Induction of NS1-, NS2-, and UV-inactivated BTV-8 specific lymphocyte proliferative responses

In study II, significantly higher lymphocyte proliferative responses directed against NS1, but not NS2, were observed in cattle immunized with either SubV or CV three weeks after second vaccination compared to controls ($p \leq 0.01$ and $p \leq 0.05$ for SubV and CV, respectively) (*Paper II*). These results were verified by the detection of IFN-γ in supernatants from restimulated PBMCs. IFN-γ is a cytokine produced by cells of the innate and adaptive immune systems, including natural killer (NK) cells, helper T cells (CD4+), and CTLs (CD8+), and its production stimulates antigen-presenting cells and upregulates their antigen-processing and -presenting pathways (Schroder *et al.*, 2004). The detection of IFN-γ in supernatant following *ex vivo* restimulation of lymphocytes is used as an indicator of intracellular activation of type 1 T helper cells against viral infection (Allmendinger *et al.*, 2010; Hägglund *et al.*, 2011; Hund *et al.*, 2012).

In study III, in addition to confirming NS1-specificity of lymphocyte proliferative responses in SubV-vaccinated cattle, NS2-specific lymphoproliferative responses were also obtained after increasing the concentration of NS2 recombinant protein used to restimulate isolated PBMCs (*Paper III*). A significant T cell response directed against NS2 was detected in all vaccinated calves three weeks after second vaccination ($p \leq 0.05$ compared to controls). In line with these findings, NS2 has been reported to induce CTL production in mice or sheep following experimental BTV vaccination or infection in previous studies (Jones *et al.*, 1996, 1997).

Another advantage of study III over study II was the opportunity to evaluate BTV-8-specific lymphocyte proliferative responses in experimentally infected animals in the biosecurity level 3 laboratory facilities at SVA. Specific lymphocyte proliferation to UV-inactivated BTV-8 was detected three weeks after second vaccination (before challenge) in vaccinated calves ($p \leq 0.01$ compared to controls). Of the three proteins included in SubV, specific lymphoproliferative responses directed against only NS1 and NS2 (of BTV-2)

were detected. Therefore, these results suggest that a cross-serotype T cell response may have been induced by the NS proteins included in SubV. Based on the results of study II, where CV (of BTV-8) was shown to induce cross-serotype cellular immune responses directed against NS1 (as detected by using NS1 of BTV-2 for *in vitro* restimulations), it was hypothesized that such a response would be observed. Further evaluation is needed to characterize the nature of this cross-serotype immune response induced by the NS proteins included in SubV. For example, it would be of value to determine if the observed response was based on the proliferation of helper T cells or CTLs. Helper T cells are able to respond to killed antigens, such as those which were used in the T cell assays here, because they recognize exogenous antigens presented on MHC class II molecules. In contrast, CTLs better recognize intracellular antigens presented on MHC class I (Neefjes *et al.*, 2011). Therefore, helper T cells may be preferentially detected using such assays, even though cross-presentation of vaccine antigens or cross-priming stimulated by the ISCOM-matrix adjuvant (Duewell *et al.*, 2011) may have facilitated CTL induction *in vivo*. Other assays, such as flow cytometry following isolation of PBMCs restimulated with BTV or BTV-infected cells (Hemati *et al.*, 2009; Pérez de Diego *et al.*, 2012), may better predict the activation and functional ability of CTLs, as well as helper T cells, induced by SubV vaccination. Additionally, it would be of value to evaluate these responses against BTV-2 or other serotypes, to test the cross-reactivity of the SubV-induced T cell responses.

The initial inclusion of the NS proteins in the design of SubV was based on their potential to induce cross-serotype immune responses, which could contribute to protection against several BTV serotypes, as has recently been shown for BTV (Calvo-Pinilla *et al.*, 2012) and African horse sickness virus (de la Poza *et al.*, 2013) in IFNAR$^{(-/-)}$ mice. Additionally, the induction of cell-mediated immune responses by NS1 and NS2, in combination with neutralizing antibodies induced by VP2, may also contribute to broader vaccine efficacy as well as a potentially longer duration of protection, by stimulating diverse immune responses. Recent studies on swine influenza vaccine development have shown that DNA vaccines composed of variable antibody and conserved CTL epitopes provide greater protection against heterologous challenge than those composed of antibody epitopes alone (Wang *et al.*, 2012), which is crucial for viruses such as influenza or BTV in which reassortments can occur (Shaw *et al.*, 2012). Additionally, the induction of T cell responses can also contribute to the duration of protective immunity, as helper T cells can aid the maturation of B cells into long-lived plasma cells and memory B cells

(Slifka & Amanna, 2014) as well as the formation of activated CTLs into memory CTLs (Swain *et al.*, 2012).

4.4 Protective efficacy against BTV-8 challenge

4.4.1 Clinical signs following BTV-8 challenge

All non-vaccinated control calves showed mild clinical signs of BTV infection from two to fourteen days after BTV-8 challenge, including general depression with appetite loss, edema, nasal discharge, stiffness, and a biphasic rectal temperature pattern that peaked on PID4 and PID7. In contrast, three of six vaccinated calves demonstrated no clinical signs throughout the entire study and no increase in rectal temperature was detected in SubV-vaccinated animals after BTV-8 challenge (maximum rectal temperatures mean, SubV: 39.1±0.1°C; Control: 40.0±0.4°C; $p \leq 0.01$). In the remaining three calves immunized with SubV, limited and mild clinical signs were observed on one day each between PID4-6, including slight serous nasal discharge in one calf and a stiff gait for one day in two calves. Vaccinated calves had low mean clinical scores that never exceeded 0.5 (Table 6, section 3.5.3).

The clinical signs observed here were similar to those reported following natural or experimental infection of ruminants (Thiry *et al.*, 2006; Di Gialleonardo *et al.*, 2011; Rasmussen *et al.*, 2013), whereas other challenge studies have failed to induce clinical signs in control animals (Bréard *et al.*, 2011; Matsuo *et al.*, 2011). One of the BTV-8 suspensions in this study was passaged on KC cells, which may more closely mimic natural, *Culicoides*-derived infection, and may explain the occurrence of clinical signs following this challenge, in contrast to studies where the challenge virus was passaged in mammalian cell cultures (Flanagan & Johnson, 1995; OIE, 2009). This influence of viral passage on experimental pathogenicity has been previously observed for BTV (Moulin *et al.*, 2012) as well as other arboviruses, such as Schmallenberg virus (Wernike *et al.*, 2012).

4.4.2 Quantification of BTV RNA and detection of BTV in blood

Viremic titers similar to previous reports of experimental BTV challenge (Di Gialleonardo *et al.*, 2011; Rasmussen *et al.*, 2013) were detected by RT-qPCR analysis in the blood of all controls by PID6, peaked at PID10, and remained stable through PID25. These results were also verified by ECE inoculation, where 2-4 of 5 ECE inoculated with diluted blood samples from control calves showed characteristic hemorrhagic signs of BTV infection and were also RT-qPCR-positive (Ct range: 27-38) for BTV RNA.

In contrast, no BTV RNA was detected by RT-qPCR analysis in any SubV-vaccinated calf before or after BTV-8 challenge through to experiment termination (PID25). These results were confirmed by the absence of BTV isolation in ECE.

Taken together with results from clinical examinations, the experimental subunit vaccine presented here is a promising vaccine candidate. In contrast to other experimental and commercial vaccines studies, which provide or discuss only clinical (Calvo-Pinilla *et al.*, 2014; Mohd Jaafar *et al.*, 2014) or only virological (Matsuo *et al.*, 2011) protection, our results show that no clinical BT disease was observed in cattle following SubV vaccination. Furthermore, SubV completely prevented the systemic replication of BTV, and thereby would likely prevent vector-borne transmission of the virus and bar potential BTV recombination in cattle (Roy *et al.*, 2009).

4.5 DIVA compliancy based on serotype-specific and pan-BTV ELISAs

The companion DIVA tests of SubV are based on two parameters: i) the detection of VP2 antibodies, which identifies serotype-specific BTV infection or vaccination; and ii) the detection of VP7 antibody levels, which differentiates between infection (followed by BTV replication and potential transmission) of any serotype and vaccination with SubV (Figure 7).

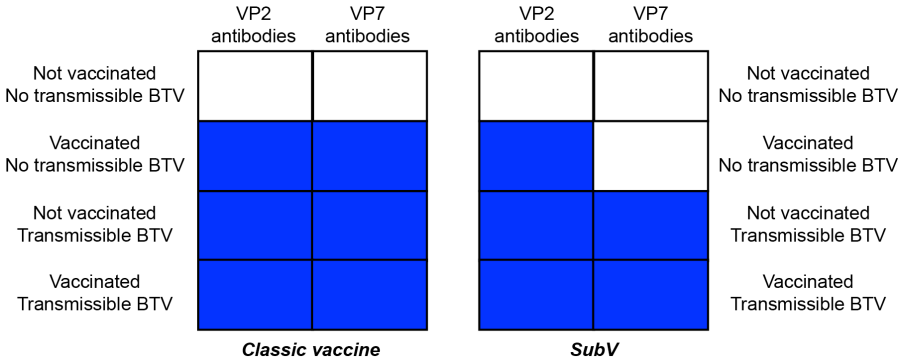


Figure 7. Schematic illustration of the DIVA concept for SubV compared to a classic vaccine. Blue rectangles indicate positive samples based on the VP2- or VP7-specific ELISA test used for evaluating corresponding antibodies and conditions. Animals with "transmissible BTV" are not protected against BTV infection (thus BTV replication and potential transmission can occur).

Based on VP2 antibody detection, calves were seropositive for BTV-8 infection within three weeks following vaccination with CV (*Paper II*) or

SubV (*Paper II, Paper III*), or following experimental BTV-8 infection (*Paper III*) (Figure 8).

In study II, VP7-specific serum antibodies were detected by sandwich ELISA, only in animals immunized with CV (*Paper II*). In study III, high levels of VP7-specific antibodies were detected by cELISA in the sera of all controls after BTV-8 challenge (*Paper III*, Figure 8). VP7-specific antibodies were also detected by cELISA in the sera of SubV-vaccinated calves after challenge, but these levels were low and could therefore be distinguished from non-vaccinated controls. Since antibodies to VP7 were detected following vaccination with a killed vaccine in study II (Figure 8), as also reported previously (Gethmann *et al.*, 2009), and because no live BTV or BTV RNA was detected in the blood of any SubV-vaccinated calf following BTV-8 challenge in study III, the presence of VP7-specific antibodies induced in the SubV-vaccinated calves was probably due to the presence of antigen in the challenge virus suspensions or to local replication at the site of injection. This contrasts the systemic virus replication detected in controls, which is likely required to enable virus transmission. Based on the results of study III, the cut-off was defined to be at ≥75% to suggest systemic BTV replication. This indicates that SubV is DIVA compliant and confirms results that animals in which BTV-8 can replicate can be identified using the cELISA as early as two-to-three weeks following infection (Gethmann *et al.*, 2009). This cut-off would likely be lower under field conditions or perhaps following experimental challenge with infected *Culicoides* midges (Pages *et al.*, 2014), and thus must be validated with samples from naturally-infected animals.

VP7 was chosen in this project as the DIVA marker because it has been used in Europe to indicate BTV infection irrespective of serotype and because the immune responses it induces can be detected at an early stage (Zhou *et al.*, 2001; Bréard *et al.*, 2011) and yet do not appear to be essential for protection (Roy *et al.*, 1990; Wade-Evans *et al.*, 1996; Mohd Jaafar *et al.*, 2014). There are several commercially-available VP7-based ELISAs available in Europe, including assays designed for use with bulk milk samples (Kramps *et al.*, 2008), which could be a quick and effective way to perform surveillance in non-endemic countries. In study II, a sandwich ELISA was chosen because it had been shown to be very sensitive, particularly for detecting antibodies induced by inactivated vaccines (Oura *et al.*, 2009) such as CV included in that study. However, VP7 antibody levels detected by these assays have been reported to decrease after two or three weeks in some experimentally-infected ruminants, likely due to a lower assay sensitivity to IgG compared to IgM (Eschbaumer *et al.*, 2011). Therefore, in study III, a cELISA was used instead in order to follow recommendations for safe serological diagnoses and to limit

specificity issues of the sandwich ELISA based on time of sampling (Eschbaumer *et al.*, 2011), which could be problematic under surveillance conditions.

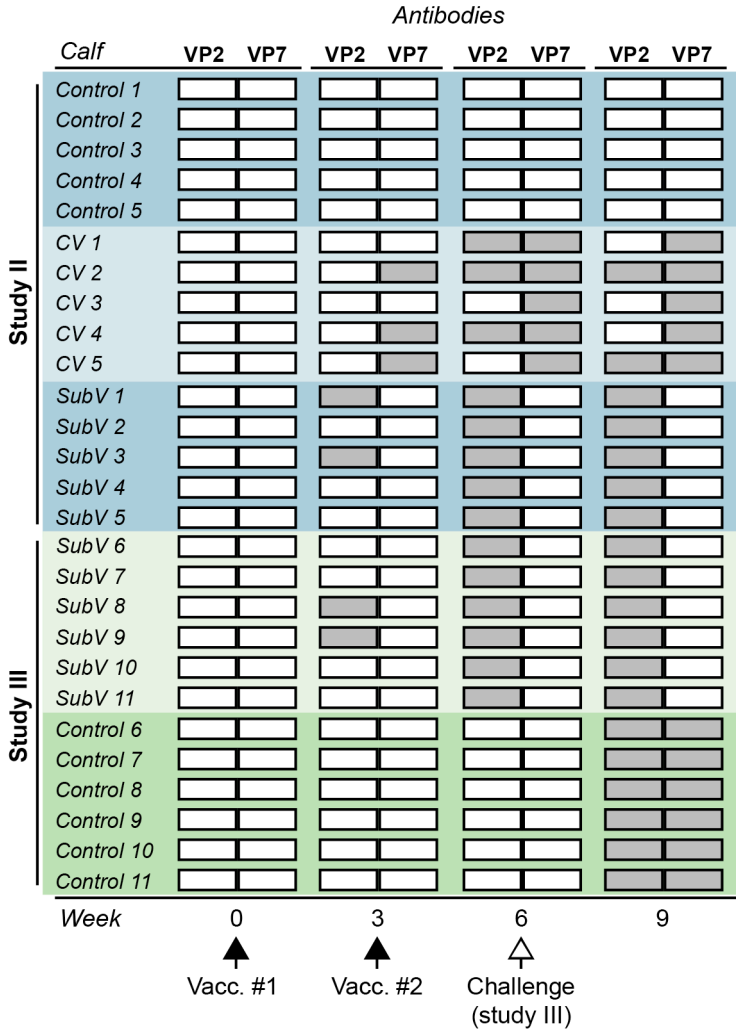


Figure 8. Specific serum antibodies against VP2 of BTV-8 and VP7 of BTV for DIVA compliancy of SubV in studies II and III. In study II (blue), animals were immunized at 0 and 3 weeks (black arrows) with PBS (Control 1-5), a commercial inactivated vaccine (CV 1-5), or the experimental subunit vaccines (SubV 1-5), but not challenged with virus. In study II (blue), no animals were challenged with virus. In study III (green), animals were immunized at 0 and 3 weeks (black arrows) with the experimental subunit vaccine (SubV 6-11) or adjuvant alone (Control 6-11), then challenged with BTV-8 at 6 weeks (white arrow). A cELISA was used to detect VP2-specific antibodies in both studies, while a sandwich ELISA and a cELISA were used to detect VP7-specific antibodies in study II and study III, respectively. Gray boxes indicate seropositive ELISA results while white boxes indicate seronegative ELISA results.

In addition to VP7, other proteins, such as NS1 and NS3, have also been suggested as potential DIVA markers, particularly in combination with inactivated vaccines, because they are only produced during BTV replication (Anderson *et al.*, 1993; Barros *et al.*, 2009). However, since these proteins are not actively removed from CV, as is performed for certain foot and mouth disease vaccines (Paton *et al.*, 2006), it is possible that enough antigen would remain such that some NS1- or NS3-specific antibodies would be induced by the vaccine; this could complicate or even impair any such DIVA capability. In fact, antibodies directed against NS1 were detected in 14 of 56 cows vaccinated with inactivated BTV vaccines in the northern area of vaccination in Sweden, where BTV was not known to circulate (J.F. Valarcher and L. Renström, personal observation).

In conclusion, this thesis work began with a pool of five immunologically-relevant recombinant BTV proteins, that was narrowed down to three proteins suitable for subunit vaccine production. In combination with an ISCOM-matrix adjuvant, these proteins were shown to induce humoral and cellular immune responses in cattle that were similar to those induced by a commercial inactivated vaccine. Finally, the experimental vaccine was shown to provide complete clinical and virological protection against virulent BTV-8 challenge in calves, while also satisfying the DIVA requirement through the use of existing diagnostic assays.

5 Concluding remarks

Veterinary and human vaccine development offers an avenue for increasing knowledge about the pathogen of interest, while simultaneously providing a potential real-life application for those results. Here, we present a promising subunit vaccine candidate against BTV-8, while also contributing to BTV knowledge regarding the protein-specificity of immune responses in cattle.

- The experimental subunit vaccine provided virological and clinical protection against BTV-8 infection in calves. This protection was likely mediated by the induction of strong neutralizing antibody titers directed against VP2 and cross-serotype cellular immune responses to NS1 and NS2. Serum antibodies to VP2, NS1, and NS2 were also induced by both the experimental subunit vaccine and by BTV-8 challenge.
- The measurement of specific serum antibodies to VP7 enabled the differentiation of infected animals in which the virus replicated to levels that were likely to be transmissible, from the vaccinated animals that were protected against infection. Therefore, the detection of VP2- and VP7-specific antibodies by ELISA could be used as DIVA companion tests with the experimental subunit vaccine developed in this thesis work. These companion tests will additionally allow the detection of BTV infection with a serotype against which SubV does not protect.
- The experimental subunit vaccine induced immune responses that were comparable to those induced by a classic commercial inactivated vaccine. By evaluating the experimental vaccine in comparison with the commercial vaccine, it was possible to get an indication of the potential protective efficacy of the experimental vaccine before performing a challenge study. Furthermore, these studies shed light on the specific immune responses induced by the inactivated vaccine in cattle; for example, the inactivated vaccine induced cross-serotype cellular immune responses directed against

NS1 of BTV-2 in cattle, as well as antibodies to VP2, NS2, and VP7, but not to recombinant NS1.

- Species differences in T cell responses induced by recombinant VP2 were observed. In particular, this VP2 induced specific lymphocyte proliferative responses in mice, but similar responses could not be detected in vaccinated cattle. These responses need to be evaluated in cattle following natural BTV infection or alternatively following BTV vaccination and restimulation of isolated PBMCs with VP2 produced in several different expression systems. The species differences observed in this thesis work highlight the importance of evaluating candidate vaccines in the target species.
- It was possible to produce and purify VP2 of BTV-8 and NS1 and NS2 of BTV-2 in sufficient quantity for vaccine use in experimental settings. Furthermore, these recombinant proteins were stable for at least 1.5 years at +4°C and -80°C, and each induced humoral and cellular immune responses in mice which indicated that they might be immunogenic in cattle. Therefore, they were suitable choices for rational subunit vaccine design.

6 Future perspectives

BTV is one of the most well characterized orbiviruses and many important, pioneering contributions to the study of veterinary infectious diseases have been made through BTV-related research. However, gaps in knowledge still remain. For example, much of the *in vivo* immunological studies have been performed in sheep. Sheep are important targets for vaccination as they are often most severely clinically affected by BTV infection. However, several studies have indicated the ease with which the segmented BTV genome can reassort (Oberst *et al.*, 1985, 1987; Stott *et al.*, 1987; Shaw *et al.*, 2012), and since cattle act as the virus's main amplifying host, they are also important targets for vaccination in areas where the virus is not endemic or where several BTV serotypes co-circulate. It is crucial that more immunological research is performed in cattle.

Through the work presented in this thesis, we have begun to study the immune responses induced by purified VP2, NS1, and NS2 in cattle. We have also demonstrated that an experimental subunit vaccine composed of these three recombinant proteins provided protection against BTV-8 infection. However, though the concept of this vaccine is working, it remains to be optimized, which may be achieved by answering the following questions:

- *Are each of the proteins necessary for a successful vaccine and can their quantities be reduced without losing protective efficacy?* To answer this question, the individual protective contributions of each protein, and particularly of NS1 and NS2, should be clarified and different formulations should be tested experimentally. For example, animals vaccinated with SubV (or with formulations including both or only one NS protein) could be challenged with BTV-2 or with another BTV serotype, and then the protective efficacy evaluated in order to verify if these proteins do indeed provide cross-serotype protection. Reducing

protein number or quantity could decrease the cost of vaccine production.

- *Can the production and/or purification of these proteins be optimized to give higher yields, without losing safety, stability, and immunogenicity?* This could also reduce the cost, as well as time, of vaccine production. Other systems that have recently been used with success in recombinant protein production for experimental veterinary vaccines include yeast (Shin & Yoo, 2013), plant-based (Guerrero-Andrade *et al.*, 2006), or silkworm-baculovirus (Li *et al.*, 2008) expression systems. Although it was possible to purify these three proteins using a His-tagged system, experimenting with other affinity tags, such as a GST tag, may yield better results.
- *Would a different adjuvant increase the efficacy of this vaccine, especially if protein quantities are reduced?* There is an increasing number of adjuvants available for veterinary vaccine use. The adjuvant effect might vary according to the antigens included in the vaccine (Blodörn *et al.*, 2014). For example, testing water-oil emulsions or CpG adjuvants (Singh & O'Hagan, 2003; Bode *et al.*, 2011) might induce strong T cell responses against the NS proteins and possibly also against VP2.
- *What is the length of protective duration provided by the experimental vaccine?* It has been demonstrated that the experimental vaccine produces diverse immune responses, which may provide a long duration of immunity, indicated by the magnitude of virus neutralizing antibody responses and the additional induction of T cell responses. This is especially important for cattle compared to other shorter-lived agricultural animals such as pigs or chickens and could not only reduce the cost of vaccination (by decreasing the required frequency of re-vaccination) but also potentially enable eradication programs based on vaccination. The duration of immunity following vaccination needs to be determined, both for the recombinant subunit vaccine presented in this thesis and for any optimized version of that vaccine.
- *Would this experimental vaccine be equally effective against BTV-8 challenge in sheep, as observed for cattle?* It is uncommon to design a vaccine against BTV specifically for use in cattle, rather than first evaluating the experimental vaccine in smaller (and less expensive) animals such as sheep. We chose to do so because cattle play an important role in maintaining BTV in circulation as its primary amplifying host and they are also present in higher numbers than sheep in Sweden. Furthermore, the BTV-8 outbreak in northern Europe was

unusual because in contrast to other BTV strains or serotypes, it caused clinical signs in cattle and goats. Nonetheless, as sheep remain an important piece of the BTV puzzle it would be essential to determine whether this experimental vaccine would be as equally efficacious against BTV-8 challenge in sheep as in cattle. It would also be of interest to decrease the quantity of protein in order to decrease the cost of vaccination in small ruminants.

In addition to optimizing the experimental vaccine, its design can also be further developed. One advantage of this experimental subunit vaccine's design is its potential adaptability to other BTV serotypes, based on the inclusion of serotypically-conserved NS1 and NS2 proteins. The adaptability of this subunit vaccine could be tested by simply exchanging the VP2 of BTV-8 with VP2 of another serotype, since it is possible that the vaccine may provide protection against other serotypes.

Developing a multi-serotype BTV vaccine, by including VP2 from different serotypes, and evaluating potential cross-protection against those serotypes, would be an important line of investigation for the future. Studies by Jeggo and colleagues, which show some cross-neutralization among BTV serotypes, have provided support for speculation that there may be a minimum number of BTV serotypes needed in a multivalent vaccine to provide broad protection (Jeggo & Wardley, 1982a; Jeggo *et al.*, 1983, 1984b). It would be interesting to evaluate this, first by identifying conserved epitopes, and then experimentally in vaccine studies. Additionally, it could be promising to experimentally study whether a multiple-formulation vaccine regimen, including several VP2 of different serotypes for each vaccination, could offer broad protection across several serotypes. It has recently been shown that VP2 from at least six different African horse sickness virus serotypes can be expressed in a mult-loci baculovirus expression system (Kanai *et al.*, 2013), which could be an advantageous approach for producing VP2 of BTV for a potential multiserotype subunit vaccine.

Finally, it was shown through the last two studies presented in this thesis that the experimental subunit vaccine is DIVA compliant under the study conditions using VP2- and VP7-specific ELISAs. The challenge conditions in the final study were strong, and as animals were subcutaneously inoculated with BTV-8, the challenge route differed from the natural route of infection. The DIVA compliancy of the experimental vaccine should be further evaluated in a larger number of animals under field conditions. Thereby, the cut-off defined in this thesis could be adjusted in the field following natural infection.

In summary, this thesis presents the first stages in the development and evaluation of a novel, rationally-designed recombinant subunit DIVA vaccine against BTV-8. These results provide a promising foundation for further optimizing and even developing the experimental vaccine, while simultaneously contributing to the basic science knowledge regarding the host-pathogen interactions required to develop efficient BTV vaccines.

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