Current and next-generation bluetongue vaccines: Requirements, strategies, and prospects for different field situations

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\section*{ABSTRACT}
Bluetongue virus (BTV) causes the hemorrhagic disease bluetongue (BT) in ruminants. The best way to control outbreaks is vaccination. Currently, conventionally modified-live and inactivated vaccines are commercially available, which have been successfully used to control BT, but nonetheless have their specific shortcomings. Therefore, there is a need for improved BT vaccines. The ideal BT vaccine is efficacious, safe, affordable, protective against multiple serotypes and enables the differentiation of infected from vaccinated animals. Different field situations require specific vaccine profiles. Single serotype outbreaks in former BT-free areas need rapid onset of protection against viremia of the respective serotype. In contrary, endemic multiple serotype situations require long-lasting protection against all circulating serotypes. The ideal BT vaccine for all field situations does not exist and balancing between vaccine properties is needed.

Many new vaccines candidates, ranging from non-replicating subunits to replicating next-generation reverse genetics based vaccines, have been developed. Some have been tested extensively in large numbers of ruminants, whereas others were developed recently and have only been tested \textit{in vitro} and in mice models. Most vaccine candidates are promising, but have their specific shortcomings and advantages. In this review, current and next-generation BT vaccines are discussed in the light of prerequisites for different field situations.

\section*{Bluetongue occurrence and control}
Bluetongue virus (BTV, family \textit{Reoviridae}, genus \textit{Orbivirus}) causes the hemorrhagic disease bluetongue (BT) in both wild and domestic ruminants and is one of the main veterinary diseases worldwide (Hutcheon, 1902; Spreull, 1905). In principle, BTV is non-contagious and is transmitted by bites of certain species of \textit{Culicoides} midges (Du Toit, 1944). At least 27 BTV serotypes are known (Hofmann et al., 2008; Jenckel et al., 2015; Maan et al., 2007, 2011) with widespread geographic distribution (Maclachlan, 2011), causing sometimes significant economic losses (Purse et al., 2005; Tago et al., 2014; Velthuis et al., 2010). The disease is emerging and outbreaks in former BT-free areas (Maclachlan, 2010), or outbreaks by serotypes exotic for that region are regularly reported (Hofmann et al., 2008; Maan et al., 2011). In addition, new serotypes have been identified recently (Hofmann et al., 2008; Jenckel et al., 2015; Maan et al., 2011). Historically, BTV is prevalent in tropical and subtropical regions between 35°S and 45°N, but outbreaks have now been reported further northwards. This emergence could be caused by climate change, since a high temperature contributes to both distribution and competence of the insect vector (Carpenter et al., 2011; Wittmann et al., 2002). BTV8 has caused huge losses in north western Europe in 2006–2008, which was historically BTV-free. Here, BTV8 was spread by an endemic insect vector (Rasmussen et al., 2010; Szmaragd et al., 2007; Wilson & Mellor, 2009). BTV4 caused problems in eastern Europe in the summer and autumn of 2014 (mail Promed, 2014), and BTV5 has recently been reported in northern Australia as an additional new serotype (mail Promed, 2015). Upcoming expansions and incursions to other areas by known serotypes, or by new serotypes are, therefore, expected and preparedness on BT outbreaks should be of high priority.

Control and eradication of BT by conventional methods such as movement restrictions or stamping out is hardly possible, due to asymptomatic infections,
prolonged viremia in cattle, and virus persistence in the midge population. Therefore, vaccination with effective vaccines against the right serotype is the preferred method to control BT (Maclachlan & Mayo, 2013; Papadopoulos et al., 2008; Roy et al., 2009). Indeed, BT vaccination campaigns have contributed to control outbreaks in the past (Baetza, 2014; Pioz et al., 2014 and references herein). There is a need for vaccine improvement, since available modified-live and inactivated vaccines have their specific drawbacks, such as residual virulence, congenital malformations, and onward transmission for modified-live vaccines, and high costs and the need for booster vaccinations for inactivated vaccines, whereas both lack DIVA potential. Recently, reverse genetics for BTV has been developed (Boyce et al., 2008), which has boosted BTV research, resulting in various types of next-generation vaccine candidates. Still, none of these vaccine candidates have been introduced to the market yet. Here, we discuss prerequisites of BT vaccines, the different field situations, and the pros and cons regarding those different field situations of currently marketed vaccines as well as of non-replicating and replicating vaccine candidates (Tables 1 and 2).

**Vaccine prerequisites**

In general, vaccines must be efficacious, safe, and affordable. In addition, differentiation of infected from vaccinated animals (DIVA) is supportive for veterinary vaccines to adequately monitor and control outbreaks.

The ideal BT vaccine protects against disease and blocks viremia of multiple serotypes in all ruminant species and also enables DIVA. The ideal vaccine is cheap to produce and only a single and low dose is required. Protection against viremia prevents vertical transmission in the pregnant animal and horizontal transmission by the vector. Immunity must be quickly induced and long lasting. The ideal BT vaccine does not revert to virulence and does not reassort or recombine with field virus. Market registration must also be feasible. Such an ideal vaccine is difficult to achieve and, therefore, BT vaccines will balance between efficacy and duration of immunity, safety for the vaccinated animal and the environment, costs, applicability for multiple serotypes, and possibility for DIVA.

**Immunity**

Both cellular and humoral immunity contribute to protection against BTV infection (Jeggo et al., 1984, 1985) and an effective vaccine should, therefore, aim to induce both.

The neutralizing antibody (nAb) response has been extensively investigated and is often described to be an important determinant of protection. NAbs are raised

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**Table 1. Properties of currently marketed BT vaccines.** Currently marketed BT vaccines have been scored for different important vaccine characteristics. Not fulfilling the criteria is indicated by −, partial fulfilling by + or ++, and if a vaccine clearly meets the described criteria, this is indicated by +++.

| Onset of immunity | Duration of immunity | Animal safety | Environmental safety | DIVA | Costs/dose |
|------------------|----------------------|---------------|----------------------|------|------------|
| MLV              | +++                  | +             | +++                  | −    | +++        |
| Inactivated      | +                    | ++            | +++                  | +++  | +          |

**Table 2. Properties of BT vaccine candidates.** Several different BT vaccine candidates have been described. Some have already been tested in small groups of ruminants, but others were only tested in vitro and mice models. Since none are near to market introduction, vaccine characteristics can only be estimated, based on the preliminary data and immunological and molecular knowledge of similar vaccine types in use for other viral diseases. Predicted scores for important vaccine characteristics are indicated. Not fulfilling the criteria is indicated by −, partial fulfilling by + or ++, and if it is predicted that a vaccine meets the described criteria, this is indicated by +++.

| Protected animal models | Onset of immunity | Duration of immunity | Animal safety | Environmental safety | DIVA | Costs/dose |
|-------------------------|------------------|----------------------|---------------|----------------------|------|------------|
| Serotyped inactivated   | Small groups of sheep | +                   | +++           | +++                  | +    | +          |
| Subunit                 | Small groups of sheep and cattle | +                 | +++           | +++                  | +++  | +          |
| VLP                     | Large groups of sheep | --                 | +++           | +++                  | +++  | +          |
| DNA vaccine             | IFNAR(−/−) mice combined with vector vaccines | +                 | +             | +++                  | +++  | ++         |
| Vector vaccine          | IFNAR(−/−) mice with only partial protection in small groups of sheep | ++            | ++            | +++                  | +++  | ++         |
| Serotyped live DISC     | Small groups of sheep | +++               | ++            | +                    | +    | −          |
| DISA                    | Small groups of sheep and cattle | +++             | ++            | ++                   | ++   | +          |
| DISA                    | Small groups of sheep | +++               | ++            | ++                   | ++   | ++         |
against outer-capsid protein VP2, while VPS enhances the VP2 directed nAb response (Huismans & Erasmus, 1981; Huismans et al., 1987; Legisa et al., 2015; Mertens et al., 1989). NABs are highly serotype specific, but significant cross protection can be induced after multiple vaccinations against different serotypes or after vaccination with multivalent vaccines (Verwoerd, 2012). However, modified-live vaccines for different serotypes do not replicate equally, being of concern when applied as a cocktail vaccine (Dungu et al., 2004a; Jeggo et al., 1983, 1986). Until 3 weeks after immunization with one serotype, serotype broad protection can be present in sheep, which becomes serotype specific after nine weeks (Feenstra et al., 2014a; van Gennip et al., 2012a). The mechanism of this short lasting broad protection is unknown. Obviously, studies on serotype specific protection should be performed by infection of animals later after vaccination, when the broad protection is not disturbing the outcome of the experiment anymore. Broadly protective vaccines are not available yet, which is one of the main challenges in the field of BT vaccine development.

The role of cellular immunity in BT protection is largely unknown, although some reports are claiming that the T-cell response is closer correlated to protection than the antibody response (Roy et al., 2009). BTV cytotoxic T-cells (CTLs) have been identified, with NS1 and VP2 being the major CTL targets and VPS, VP7, and NS3 being minor antigens (Andrew et al., 1995; Janardhana et al., 1999). Some efforts on induction of broadly protective CTLs have been performed, but no broad protection in the ruminant host has been shown after the serotype broad protective period of 3 weeks.

BTV is a potent inducer of interferon (IFN), therewith activating the innate immune system of the host after infection (Chauveau et al., 2012; MacLachlan & Thompson, 1985). However, viral non-structural (NS) proteins NS3 and NS4 have been described to be IFN antagonists (Chauveau et al., 2013; Ratnier et al., 2011, 2014). Lack of these proteins in a vaccine might lead to better induction of immunity. The function of VP2 and VP5 in humoral immunity and VP2 and NS1 in CTL response indicates that these proteins are important to induce immunity against BT.

**Field situation**

The objective of BT vaccination differs for different field situations. Vaccination could aim at prevention of morbidity and mortality in the vaccinated population, reduction of virus spread in the field, and eradication of BTV to allow safe movement between BT-affected and BT-free zones. Vaccination objectives are also influenced by the presence of predominant ruminant species in particular areas. Asymptomatically infected animals such as goats and cattle are vaccinated to control BT spread to more susceptible ruminants, but susceptible sheep breeds are vaccinated to reduce morbidity and mortality. The most extreme field situations that exist are the following: BT-free regions, sometimes adjacent to an endemic area, former BT-free countries affected by an outbreak of only one serotype, and endemic multiple serotype situations. For these different field situations with accompanying vaccination objectives, vaccines with different profiles are needed, and tailor made vaccines should, therefore, be available (Figure 1).

Of course, efficacy of BT vaccines is important. Aiming a rapid stop of virus transmission after an incursion, a quick onset of immunity is more important than duration of immunity. Safety of BT vaccines is important in case of preventive vaccination of a healthy population at risk compared with rapid protection in an emerging situation, or in an endemic situation with enormous losses. Preferably, a vaccine should never lead to any side effects, such as abortions, reduced growth of livestock or milk drop. However, minor side effects are acceptable when morbidity and mortality are high and when the infection burden can be reduced. Modified-live vaccines could be of risk due to uncontrolled spread of vaccine virus by the insect vector. However, in endemic situations aiming to reduce losses, this is a minor concern, whereas uncontrolled spread of vaccine virus could be very problematic if eradication of BT and regaining the BT-free status is the final goal. In general, veterinary vaccines should be cheap, because of the often low economic value of livestock, such as sheep and goats. In the developed world, with a high density of high-value cattle, vaccination costs can be higher, especially when aiming at a BT-free status. In poor, developing countries, a low vaccine price is very important, of course. DIVA-compliant vaccines potentially enable safe trade between free and affected countries, but also enable surveillance during outbreaks by preventing serological blindness after vaccination. Still, depending on the used DIVA vaccine, potential re-assembly with field strains should be evaluated. However, in endemic situations, DIVA is of less concern. Finally, in multiple serotype situations, vaccines should protect against all circulating serotypes in the area.

South Africa is an example of one of the many countries where multiple serotypes are endemic (Coetzee et al., 2012). Spread of modified-live vaccines is, therefore, not a major concern. However, vaccines must protect against multiple serotypes. Therefore, multivalent modified-live vaccines are used. Countries in northwestern Europe with a BT-free status may want to
introduce preventive vaccination. Spread of modified-live vaccines would have a large impact on animal trade and is, therefore, not acceptable. DIVA and safety are then main requirements of the vaccine. Emergency vaccination in a former BT-free country occurred for example in the UK in 2008, 1 year after BTV incursion. Vaccination reduced losses and was certainly cheap compared to the expected economic impact (Purse et al., 2005; Tago et al., 2014; Velthuis et al., 2010). In such a situation, eradication is the final aim, and uncontrolled spread of vaccine virus is unacceptable. Therefore, although expensive, inactivated vaccines were used.

Commercially available vaccines

**Modified-live vaccines**

The first live-attenuated or modified-live BT vaccine (MLV) originates from 1906, and was a BTV isolate of serotype 4, passaged limited times in sheep, and has been used in South Africa for >40 years. Later on, MLVs were developed in South Africa by passaging for >100 times in embryonated chicken eggs to improve the attenuation. A vaccine containing 14 BTV serotypes was, therefore, developed; however, no adequate protection was induced in sheep (Van Dijk, 1993). Currently used MLVs have been generated by serial passaging of field isolates in embryonated chicken eggs and subsequently in tissue culture (Dungu et al., 2004a). Viruses have often been passaged >50 times in eggs and subsequently a couple of times in BHK21 cells. The only widely used MLVs currently available are produced by Onderstepoort Biological Products (OBP), Pretoria, South Africa. The broad protective approach consists of three pentavalent vaccines. Bottle A consists of serotypes 1, 4, 6, 12, and 14; bottle B of serotypes 3, 8, 9, 10, and 11; and bottle C of serotypes 2, 5, 7, 13, and 19. The vaccine must be administered at 3-week intervals in the correct order with annual booster vaccination. The most attenuated strains are included in bottle A, thereby preventing clinical signs and negative interference by better replicating strains, whereas under-attenuation of certain MLVs in the next bottles will not result in clinical signs due to previously induced partial protection. After two or three years, sheep are protected to all included serotypes, and are even protected to some more heterologous BTV serotypes. Pregnant ewes should not be vaccinated with these MLVs, since fetuses may develop congenital malformations. Further, temporary infertility in ewes and rams after the first vaccination has been reported. Still, this vaccination strategy demonstrates only limited side effects in sheep in South Africa, which commonly includes a transient febrile reaction (reviewed by Dungu et al., 2004b; Van Dijk, 1993). Although the OBP vaccine has not been registered for use in cattle, vaccine formulations containing MLVs for serotypes 2, 4, 9, and 16 have been used in cattle in southern Europe and the Middle East showing little adverse effects (reviewed in Savini et al., 2008). Monoserotype MLVs can be ordered at OBP, however, under-attenuation of MLVs of bottle B or C, and residual virulence for non-African sheep breeds could cause clinical signs. Hammoumi et al. determined that the protective dose of MLV for serotype 2 is $10^{1.3}$ TCID$_{50}$/ml in sheep (Hammoumi et al., 2003), and the minimal recommended dose by OBP of this vaccine is $10^4$ TCID$_{50}$ (Ronchi et al., 2003).

MLVs are relatively cheap and highly effective due to the induction of both humoral and cellular immune responses using a low dose. Vaccination prevents clinical signs, and strongly reduces BTV circulation (Caporale et al., 2004; Patta et al., 2004). After the incursion of BTV into Mediterranean Europe, Spanish, French,
Italian, and Portuguese authorities have mandated compulsory vaccination campaigns with MLVs of OBP in 2000. Their use is, however, controversial due to the above described disadvantages. MLVs often show a viremia with a virus titer \(>10^3\) TCID50 ml \(^{-1}\), which is sufficient for onward transmission by midges (Monaco et al., 2004, 2006; Savini et al., 2004; Veronesi et al., 2005).

Indeed, local transmission of MLVs for serotypes 2 and 16 has been demonstrated (Ferrari et al., 2005). Virulent BTV variants as a result of reversion to virulence or reasortment with field virus have been reported (Batten et al., 2008; Ferrari et al., 2005; Savini et al., 2014; Veronesi et al., 2005). For example, the MLV for serotype 2 used in 130 000 animals in Corsica and 400 000 in Italy caused no adverse reactions (Breard et al., 2004), whereas the MLV for serotype 16 used in Corsica in 2004 induced clinical signs and the vaccination campaign was terminated immediately. MLV for serotype 16 also caused problems in Sardinia (reviewed by Savini et al., 2008). Abortions and stillbirths have also been reported as well as lower milk production after vaccination with MLVs. Officially, MLVs for other serotypes were never used in Europe but have been detected, and are likely introduced via unknown introduction routes (De Clercq et al., 2009; van Rijn et al., 2012). In several parts of the world, such as the USA, Turkey, Africa, and India, MLVs are still used to protect the ruminant population against BT (Bhanuprakash et al., 2009; McVey & MacLachlan, 2015). However, uncontrolled spread of MLVs is not desired if BT eradication is the ultimate goal.

### Inactivated vaccines

Inactivated BT vaccines have been developed in the 1970s and 1980s, but were not commercially available at that time (Campbell et al., 1985; Parker et al., 1975; Stevens et al., 1985; Stott et al., 1985). The emergence of BT in Europe has led to the development of more and commercialized inactivated BT vaccines. These became the preferred type of vaccine in Europe, primarily because these are completely safe, although the completeness of inactivation has sometimes been questioned. The first commercially available inactivated BT vaccine was against serotype 2. Later, inactivated vaccines against serotype 4 and the bivalent vaccine against serotypes 2 and 4 have been used in southern Europe (Savini et al., 2009). Heterologous protection by inactivated BT vaccines can be induced, but cross protection is difficult to predict. Further, cross protection has often been investigated at three weeks after vaccination when there is still serotype broad immunity that disappears later on (Breard et al., 2015).

These inactivated vaccines were produced at industrial scale by Merial and used saponin/aluminum hydroxide as adjuvant. Inactivated vaccines against serotypes 1, 8, and 9 were later marketed by the same company (Savini et al., 2008) (reviewed in Zientara & Sanchez-Vizcaino, 2013). Mass vaccination in several countries against serotype 1, 2, 4, or 8 have resulted in complete eradication demonstrating the success of inactivated BT vaccines (reviewed in Savini et al., 2008).

Vaccination with inactivated vaccines against serotype 8 started in Spring 2008 in north western Europe, about 2 years after the first report of BTV8 in the Netherlands in August 2006 (Baetza, 2014; European commission, 2009). This devastating outbreak has highly contributed to the rapid production of inactivated vaccines by different companies. Several aluminum hydroxide/saponin adjuvanted BTV8 vaccines were used: BLUEVAC\(^\circ\) 8, CZ Veterinaria, Spain; BTVPUR\(^\circ\) AlSap 8, Merial, France; Bovilis-BTV-8, Intervet, The Netherlands; and Zulvac\(^\circ\) 8 Ovis or Bovis, Fort Dodge, The Netherlands. For most of these vaccines, details of production and formulation have not been published. The inactivated BT vaccines for serotype 8 are all highly effective, and have significantly contributed to eradication of BTV8 in many European countries (Eschbaumer et al., 2009; Moulin et al., 2012; Oura et al., 2009; Szmaragd et al., 2010; Wackerlin et al., 2010). Protection against clinical signs and viremia after virulent BTV8 challenge has also been shown at 12 months post-vaccination in sheep and cattle (Oura et al., 2009; Wackerlin et al., 2010). Booster vaccination induced higher antibody responses compared with single vaccinations, and especially in cattle repeated vaccination and a high dose is considered necessary for long term protection (Hund et al., 2012). The dose of antigen of the BTVPUR\(^\circ\) AlSap 8 Merial vaccine is for example equal to \(10^{7.1}\) TCID\(_{50}\) for sheep and the double for cattle (Merial, 2015). NAbs persist for >3 years in cattle and for >2.5 years in sheep (Batten et al., 2013; Oura et al., 2012). Thus, the use of inactivated vaccines to control BT needs a high and repeated dose, but is very effective.

Potentially, inactivated BT vaccines enable DIVA based on the absence of non-structural proteins. However, stringent virus purification is required to remove traces of these non-structural proteins. ELISA tests detecting NS1 or NS3 antibodies have been described (ID Screen Blue Tongue DIVA, IDVET, France) (Barros et al., 2009; Laviada et al., 1995), but have not yet been used in practice.
Experimental vaccines

A large variety of approaches have been investigated to develop BT vaccines in order to solve problems of the currently marketed vaccines. The enhanced interest in BT vaccine development was driven by BT emergence, and new possibilities by use of reverse genetics for BTV. Some candidates have been tested extensively, whereas others are just recently developed, or not tested in the ruminant host yet. In general, non-replicating vaccine candidates are very safe but more expensive and less effective, whereas replicating vaccine candidates are more effective and cheaper, but could face hurdles with respect to safety and market acceptance.

Non-replicating vaccines

Serotyped inactivated vaccines

One of the problems with currently available inactivated vaccines is that they are only applicable for a few serotypes. When new serotypes would emerge, completely new vaccines should be developed. The availability of the reverse genetics system for BTV (Boyce et al., 2008), allows the use of one vaccine production platform for different serotypes by exchange of only the serotype determining proteins. This could significantly reduce the time needed for vaccine production, and might accelerate market introduction. All other aspects are similar to these of existing procedures for the production of inactivated BT vaccines.

Nunes et al. (2014) have demonstrated the broad application of a production platform based on BTV1 by exchange of VP2 and VP5 for 16 out of 26 serotypes, including heterologous combinations of VP2 and VP5. Similar to the commercially available inactivated BT vaccines, these strains were inactivated and adjuvanted, and protect against homologous challenge in sheep. BTV with chimeric VP2 proteins of serotypes 1 and 8 was neutralized by monoserotype sera for both serotypes. This suggests that inactivated BT vaccine with chimeric VP2 can induce neutralizing responses against both serotypes.

Subunit vaccines

Inactivation of BTV prevents spread in the field and reversion to virulence; however, the application of DIVA has not been demonstrated. Subunit vaccines would be a solution for this by omission of the antigen of the DIVA test. It has been demonstrated that VP2 alone is sufficient to elicit protective immune responses in sheep (Huismans et al., 1987), whereas VP7 is the target of commercial tests to detect antibodies induced after BTV infection.

In 1985, Huismans et al. isolated VP2 proteins from purified BTV using chemical methods. Vaccination with purified VP2 induced nAbs and protected against homologous BTV challenge in sheep, but purification of VP2 is not feasible at large scale (Huismans et al., 1985, 1987). In line with these promising results, the VP2 gene was, therefore, cloned using recombinant DNA technology (Huismans, 1985). Later, Roy et al. developed a VP2 subunit vaccine based on the baculovirus expression system (Inumaru & Roy, 1987; Urakawa et al., 1994). Booster vaccination of sheep with S. frugiperda cell lysates containing VP2 induced protection against homologous BTV serotypes regarding clinical signs and viremia. The protective dose was 100 μg of VP2, and addition of 20 μg VP5 lowered this to 50 μg VP2 (Roy et al., 1990). Addition of Freund’s adjuvant or other BTV proteins was not enhancing the protective immunity (Roy et al., 1990).

Recently, a baculovirus and E. coli produced subunit vaccine, containing 150 μg purified VP2, NS1 and NS2 proteins with AbISCO-300, an immunostimulating complex-based adjuvant, have been tested in cattle. Good cellular and humoral immunity were induced after booster vaccination. T-lymphocytes were mainly raised against NS1 and were cross reactive among different serotypes (Anderson et al., 2013). This vaccine protected calves against BTV8 challenge 3 weeks after booster vaccination. It is proposed that the cellular response to NS1 and NS2 can be the basis of an adaptable vaccine with varying VP2 serotypes (Anderson et al., 2014).

Another subunit vaccine is based on two domains of VP2 (aa 63–471 and 555–956), VP5 lacking the first 100 amino acids, and VP7. These have been produced in bacteria as soluble fusion-proteins with glutathione S-transferase. Immunized IFNAR(−/−) mice expressed neutralizing antibodies and survived homologous challenge without clinical signs after booster vaccination with 15 μg of the VP2 domains and 25 μg of VP5. Addition of VP5 protein enhanced the immunity but VP7 did not (Mohd Jaafar et al., 2014). This subunit vaccine has not yet been tested in ruminants.

Another approach to develop a BT subunit vaccine is based on expression of VP2, VP7, and NS1, which are incorporated in avian reovirus muNS-Mi microspheres. BTV proteins were tagged with a muNS region and are included in inclusions made by the muNS scaffolding protein (Marín-López et al., 2014). An advantage of production in these inclusions by the use of the baculovirus expression system is the easy method of purification and their potent adjuvant activity (Brandariz-Nuñez et al., 2010). IFNAR(−/−) mice
immunized with these particles without additional adjuvant induced both humoral and cellular immune responses, and mice were protected against lethal challenge.

VP2 has also been fused to the antigen presenting cell homing (APCH) molecule, which is a single-chain variable fragment (scFv) that specifically recognizes an invariant epitope of the MHC II DR molecule on the surface of antigen-presenting cells (Legisa et al., 2015). APCH fusion has been demonstrated to be very efficient in improving the immune responses induced against many different antigens. Again these were produced by use of the baculovirus expression system, and were formulated with oil adjuvant Montanide ISA50. This vaccine candidate showed a good humoral immune response in cattle with a minimal dose of 0.9 µg, but a BTV challenge has not been performed. IFNAR(−/−) mice have also been vaccinated with APCH fused VP2 and specific CD4⁺ and CD8⁺ T cells producing IFNγ following virus stimulation were observed, whereas lower levels were recorded for animals immunized with VP2 only. This showed that the cellular response in mice was enhanced by fusion of VP2 to the APCH protein.

Part of the VP2 gene has also been expressed using Pichia pastoris. High level of secreted expression was achieved, and the produced proteins were immunogenic in rabbits, but have not yet been tested in the natural ruminant host of BTV (Asthmaram et al., 2007).

**Virus-like particles**

Virus-like particles (VLPs) are empty virus particles, containing the structural proteins needed to enter host cells; however, virus replication and gene expression cannot occur due to the lack of genetic material. Conformation and intracellular location of VLPs is similar to that of BTV, and, therefore, better protection compared to subunit vaccines will likely be induced. Core-like particles of BTV consisting of the inner capsid proteins VP3 and VP7 have been produced by baculovirus expression and were tested as vaccine candidate, but these did not induce protective immunity (Stewart et al., 2012; Thuenemann et al., 2013), likely due to the lack of VP2 expression.

BTV VLPs have been generated by expression of all four capsid proteins (VP2, VP5, VP3, and VP7) in insect cells using baculovirus expression (French et al., 1990). VLPs revealed an icosahedral structure of 86 nm in diameter with the same features as native BTV particles (Hewat et al., 1994). DIVA in combination with VLP vaccines should be possible, based on the absence of nonstructural proteins, or the missing structural proteins VP1, 4, or 6. At first, multiple baculovirus expression vectors were used to produce VLPs. Ten µg (containing approximately 1–2 µg VP2) in conjunction with ISA-50 adjuvant elicited nAbs and fully protected sheep against virulent homologous challenge. Assuming that VP2 plays the primary role in protection, these results imply that 25–50 fold less VP2 is sufficient for protection if presented by VLPs, compared with VP2 or VP2/VP5 subunit vaccines (Roy et al., 1992). Later, multiple gene baculovirus expression vectors have been used, which express all four BT capsid proteins by one baculovirus leading to more efficient VLP production (Belyaev and Roy, 1993; Stewart et al., 2010). VLPs have been generated for multiple serotypes and cocktails of serotypes 1, 2, 10, 13, and 17 have been tested. Booster vaccination with this cocktail vaccine protected against homologous challenge to all five serotypes after 4 and 14 months post-vaccination and partially against some heterologous serotypes (Roy et al., 1994). When testing a cocktail of VLPs of serotypes 1 and 4; however, some interference in protective response for serotype 4 was measured (Perez de Diego et al., 2011). Large animal experiments to test the efficacy of VLPs have been undertaken with 50–200 sheep per trial. Each time, VLP vaccination afforded protection against homologous challenge (Roy, 2004).

Despite all these efforts with baculovirus expressed VLPs in >20 years of time with very promising results in large animal experiments, VLPs have not been marketed. The exact reason for this has not been described in the literature, but it is likely that the production method is not cost effective to compete with the currently marketed inactivated vaccines. An alternative method for VLP production is the use of the Nicotiana benthamiana plant and the cowpea mosaic virus-based HyperTrans plant transient expression vector system. Plants have become an increasingly popular alternative host for heterologous expression of complex high-value proteins, and might become cost effective (Rybacki, 2010). VLPs for serotype 8 have been generated using this method by transfection of four plasmids expressing the capsid proteins into Agrobacterium tumefaciens, followed by inoculation of plants with this bacterium. Plant leaves were harvested after 8–9 d, and VLPs were purified using extraction, filtration, and centrifugation. Particles were purified by several density gradient centrifugation steps. Sheep were booster vaccinated with 50 µg of adjuvanted VLPs. Five weeks post-vaccination sheep were challenged with virulent BTV8 and were protected against clinical signs, but viremia was not examined (Thuenemann et al., 2013).

Subunit vaccine and VLP approaches provide opportunities to develop safe and effective vaccines, with the potential of DIVA. However, the challenge is the
production of these vaccines for an affordable price for veterinary application.

**DNA vaccines**

A DNA vaccine usually consists of a DNA plasmid expressing one or more antigens after inoculation of the animal. The endogenous production of a foreign protein with natural conformation and post-translational modification favors the induction of neutralizing antibodies and a balanced cellular immune response. Advantages of this vaccination strategy are rapid manufacturing, high stability at ambient temperatures and potent induction of Th1 responses. However, inefficient delivery often reduces the reliability and effectiveness of DNA vaccines and use on a global scale is, therefore, still limited (reviewed in Grunwald & Ulbert, 2015). This vaccination strategy has been used for BT in a prime-boost combination with vector vaccines, as described below.

**Replicating vaccines**

**Vector vaccines**

Viral vector-based vaccines use infectious viruses to express desired antigens, and different viruses have been proposed as suitable vector for this approach. An obstacle of vector vaccines is, however, the pre-existing immunity to the vector, in particular when used to successively vaccinate against different diseases, or in case booster vaccination or annual revaccination is required (reviewed in Ura et al., 2014). Priming by DNA vaccination (see above) followed by vaccination with a vector vaccine can partially overcome this disadvantage of vector vaccines. For BTV, this prime-boost strategy has been examined by several research groups. Another advantage of vector vaccines is the ability of DIVA.

Li et al. tested a BTV1 pCAGGS DNA vaccine and a recombinant fowlpox virus (rFPV) vector vaccine in different combinations for VP2, VP5, or both proteins. The strategy combining the DNA vaccine prime followed by the rFPV boost induced the highest nAb titer and T-cell response in BALB/c mice, but no challenge was performed. Sheep were vaccinated with 500 μg pCAG-VP2 + VP5 and 10⁷ PFU rFPV VP2 + VP5, and nAbs were induced, but again no challenge was performed (Li et al., 2015).

Calvo-Pinilla et al. tested a similar strategy with a combination of plasmids encoding VP2, VP5, and VP7, and modified vaccinia virus Ankara (rMVA). IFNAR(−/−) mice were primed intramuscularly with 50 μg of each plasmid, and boosted with 10⁷ PFU rMVA intraperitoneally after two weeks. Mice showed protection against a lethal dose of BTV4 2 weeks after the booster vaccination (Calvo-Pinilla et al., 2009). A similar vaccination strategy with NS1 instead of VP5 has also been tested. A better T-cell response and induction of heterologous immunity was reported. Heterologous booster vaccination with DNA and rMVA was compared with homologous rMVA booster vaccination and efficacy was similar in IFNAR(−/−) mice. VP2 expression induced similar protection to homologous challenge compared with expression of VP2, VP5, and VP7 together (Jabbar et al., 2013). This prime-boost strategy was, however, not tested in the ruminant host (Calvo-Pinilla et al., 2012).

Canarypox virus expressing both VP2 and VP5 were tested in sheep. Prime boost vaccination with 2 × 10⁶ 6.3 × 10⁸ TCID₅₀ ml⁻¹ induced sterile immunity to virulent BTB at 34 d after booster vaccination (Boone et al., 2007). An advantage of this vector is that several vaccines for other veterinary pathogens have already been marketed. Capripox virus expressing VP2, VP7, NS1, and NS3 were also tested in goat and sheep. Partial protection against virulent BTB challenge was induced by inoculation of 2 × 10⁶ TCID₅₀ of each virus at 3 weeks post-vaccination (Perrin et al., 2007).

Another poxvirus, the myxomavirus, was used to express VP2 or both VP2 and VP5 in sheep. Booster vaccination with the variant expressing VP2 only, protected partially against BT in sheep (Top et al., 2012).

Bovine herpes virus expressing VP2 targeted to the cell membrane has also been studied. Booster vaccination with 10⁶ PFU induced partial protection against virulent BTB in IFNAR(−/−) mice (Franceschi et al., 2011). Two immunizations of IFNAR(−/−) mice with 10⁶ PFU of Equine herpes virus expressing both VP2 and VP5 with a 3 weeks interval protected against mortality at 3 weeks post-booster vaccination but mild clinical signs were still observed (Ma et al., 2012).

**Serotyped modified-live vaccines**

The availability of the reverse genetics system for BTV (Boyce et al., 2008; van Gennip et al., 2012b) has opened endless possibilities for fundamental and applied BTV research. This includes the use of one vaccine backbone for different serotypes by exchange of the serotype determining proteins. A backbone of a safety proven MLV reduces the risk on emergence of more virulent variants by reassortment with field virus. More importantly, reassortment between “serotyped” vaccine viruses cannot lead to new, more virulent variants. Similar to inactivated serotyped vaccines as described above, the time needed for launching vaccines for emerging serotypes can be significantly reduced and might accelerate market introduction.
van Gennip et al. (2012a) applied this strategy for the modified-live BTV6/net08 backbone (Maan et al., 2010), with VP2 and VP5 of serotype 1 or 8. NAbs against the respective serotypes were specifically raised. Three weeks post-vaccination, sheep were protected against virulent BTV8, irrespective of the used serotyped vaccine. Feenstra et al. (2015b) have investigated BTV expressing chimeric VP2 proteins to generate serotyped vaccines for which exchange of entire VP2 was not possible. These “serotyped” vaccine viruses with chimeric VP2 raised nAbs against both serotypes, suggesting protection against both serotypes.

**Disabled infectious single-cycle (DISC) vaccine**

DISC virus particles are infectious virus particles that are able to infect cells only once, due to deletion of an essential gene. DISC BTV does not express the viral helicase VP6 and must be rescued on a complementary cell line expressing this essential protein (Matsuo et al., 2011). DISC BTV induces an aborted infection resulting in expression of viral proteins at the natural sites of infection. This vaccine candidate, therefore, combines the efficacy of MLVs with the safety of inactivated vaccines.

DISC vaccine based on BTV1 has been serotyped by exchange of VP2 and VP5 of serotype 8. Sheep were vaccinated twice with a three weeks interval with crude cell lysate containing 10^8 plague forming units (PFU). No viremia of vaccine virus was detected. Three weeks after booster vaccination, sheep were protected against BVT8 and did not show clinical signs or viremia. Single vaccination with 10^8 PFU/sheep and booster vaccination with 5 x 10^7 PFU also protected against challenge virus (Matsuo et al., 2011).

These DISC vaccines have also been tested in cattle. Six weeks after booster vaccination with unknown dose, cows were protected against both clinical signs and viremia (Celma et al., 2013).

DISC vaccines have been generated for serotypes 2, 4, 8, 10, 13, 21, and 24 and were tested as cocktail vaccine in sheep. The dose was not described, but DISC vaccine was detected by PCR in blood after vaccination. After booster vaccination, all animals had nAbs against all included serotypes and no interference between serotypes was detected. Vaccinated sheep were protected against clinical signs after challenge with homologous viruses and no viremia of challenge virus was detected (Celma et al., 2013).

BT DISC vaccines are very promising and efficacy and safety has been experimentally proven. Since all viral proteins are present in the DISC vaccine, the ability of DIVA is unlikely, in particular after revaccinations. The need of a complementary cell line for culturing of DISC vaccine is disadvantageous for large scale vaccine production. Finally, the protective dose seems to be high, although single vaccination was protective against challenge and the minimal protective dose has not been determined yet.

**Disabled infectious single-animal (DISA) vaccine**

A novel approach for vaccine development using reverse genetics is the DISA vaccine, generated by deletion of NS3/NS3a expression (Feenstra et al., 2014b; van Gennip et al., 2014). NS3/NS3a is involved in virus release, in particular from Culicoides cells (French et al., 1989; Guirakhoo et al., 1995; Wechsler & McHolland, 1988; Wechsler et al., 1989), and is also an IFN antagonist (Chauveau et al., 2013). van Gennip et al. (2014) showed that NS3/NS3a expression is not essential for BTV replication in vitro. This mean that DISA vaccine can be produced on the same cell lines as used for MLV production. The titer of DISA vaccine virus is, however, slightly lower, which would increase the costs for production. DISA vaccines are based on the backbone of BTV6/net08, a well-known BTV strain related to MLV for serotype 6 (Maan et al., 2010; van Rijn et al., 2012). Further, it contains a deletion in Seg-10 abolishing NS3/NS3a expression (Feenstra et al., 2014c). DISA vaccines for different serotypes can be generated by exchange of VP2 of these BTV serotypes.

A single dose of 2 x 10^5 TCID_50 DISA vaccine for serotype 8 completely protected sheep against virulent BTV8 at 3 weeks post-vaccination (Feenstra et al., 2014a,b). Booster vaccination after 3 weeks using the same dose induced serotype-specific sterile immunity at 9 weeks post-vaccination in sheep (Feenstra et al., 2014a,b). DISA vaccine candidates have been made available for all European serotypes (1, 2, 4, 8, and 9), except for serotype 16. For this serotype, DISA vaccine candidates expressing chimeric VP2 of serotype 1 and 16 have been generated (Feenstra et al., 2015b). This chimeric DISA vaccine induced neutralizing antibodies against both serotypes in sheep but protection has not been studied. DISA vaccines are proposed to run multiple rounds of infection in the animal, but no viremia of vaccine virus was detected after vaccination. Likely, replication of DISA vaccine occurs locally at the site of injection. At present, DISA vaccines have not been tested in cattle or in cocktail formulations.

The major risk of replicating vaccines is uncontrolled spread of vaccine virus, and consequently the risk of reversion to virulence by genetic drift or by reassortment with field virus known as genetic shift. However, the absence of viremia of DISC and DISA vaccines...
prevents spread by bites of blood feeding Culicoides midges. Further, DISA vaccine virus is not released from insect cells in vitro (van Gennip et al., 2014), and the NS3/NS3a knockout mutation appeared to block in vivo propagation of DISA vaccine virus after injection in competent midges (Feenstra et al., 2015a). Knockout of NS3/NS3a by a deletion leads to complete attenuation of virulent BTV (Feenstra et al., 2014b). Reversion to virulence of DISA vaccine by restoring of NS3/NS3a expression through small mutations during genetic drift is excluded by this significant deletion in Seg-10. Regarding reassortment between DISA vaccine and field virus, the BTV6 vaccine backbone with wild type Seg-10 is still non-pathogenic (van Rijn et al., 2012). Therefore, genetic shifting by Seg-10 exchange through reassortment is of no risk. The outcome of exchange of other genome segments is unknown, although exchange of VP2 and VPS of virulent serotype 8 in the BTV6 vaccine backbone does not enhance virulence (van Gennip et al., 2012a). Reassortment between virulent and avirulent BTV have shown that virulence is related to multiple segments and raise of higher virulent variants than the ancestor viruses have not be found (Coetzee et al., 2014; Janowicz et al., 2015). Taken together, the chance on reassortment events between DISA vaccines as well as between DISA vaccine and field BTV, resulting in virulent reassortants is negligible.

BT DISA vaccines are not expressing immunogenic NS3/NS3a, and an indirect NS3 ELISA could be used for serological DIVA (Barros et al., 2009; Feenstra et al., 2014a). Recently, a competitive NS3 ELISA has also been developed (Tacken et al., 2015).

Concluding remarks

Drawbacks of currently available BT vaccines, BT emergence with outbreaks in former BT-free northern European countries, and the development of a BTV reverse genetics system has boosted the research field of BT vaccine development. A whole range of different vaccine candidates in different stages of development have been described (Table 2). Promising levels of efficacy and safety have been shown; however, none are near to market introduction. This is likely caused by marketing issues, the voluntary-based vaccination policy in Europe and the acceptance of endemics in many parts of the world versus the costs of vaccination. However, BTV will likely emerge again sooner or later and since vaccination is the best way to control outbreaks, suitable vaccines should be developed in advance in order to be instantly available.

For different field situations, vaccine candidates with suitable vaccine profiles are now available at lab scale. In case of preventive vaccination in a BT-free area at risk, cheap, safe, and broad protective vaccines that enable DIVA should be used. A good option for this would be a vector vaccine or a DISA vaccine, when used in cocktail formulation. In an outbreak situation in a former BT-free area, vaccines should be instantly available against the introduced serotype, inducing immunity as quick as possible. However, the vaccine should not spread in order to be able to regain the BT-free status. A lab scale vaccine for such a situation could be a reverse genetics based, serotyped inactivated vaccine, since such a vaccine could be quickly available. However, a vector vaccine or DISA vaccine could also be used, with the advantage of DIVA. In endemic multiple serotype situations in which eradication is not the primary goal, long-lasting protection against all involved serotypes with the lowest costs could be reached using tailor made cocktails of “serotyped” MLVs (Figure 1). However, more research on all vaccine candidates is needed to adequately proof efficacy and safety in livestock in these field situations.

At present, there are ample examples of novel lab-scale BT vaccines that could meet vaccine profiles required for different field situations. Therefore, there is no need for new vaccine types. However, further development and licensing of these vaccine candidates for many serotypes is needed in order to be prepared for future BT outbreaks.

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