Vaccines against bluetongue in Europe

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Abstract

After the incursion of bluetongue virus (BTV) into European Mediterranean countries in 1998, vaccination was used in an effort to minimize direct economic losses to animal production, reduce virus circulation and allow safe movements of animals from endemic areas. Vaccination strategies in different countries were developed according to their individual policies, the geographic distribution of the incurring serotypes of BTV and the availability of appropriate vaccines. Four monovalent modified live virus (MLV) vaccines were imported from South Africa and subsequently used extensively in both cattle and sheep. MLVs were found to be immunogenic and capable of generating strong protective immunity in vaccinated ruminants. Adverse side effects were principally evident in sheep. Specifically, some vaccinated sheep developed signs of clinical bluetongue with fever, facial oedema and lameness. Lactating sheep that developed fever also had reduced milk production. More severe clinical signs occurred in large numbers of sheep that were vaccinated with vaccine combinations containing the BTV-16 MLV, and the use of the monovalent BTV-16 MLV was discontinued as a consequence. Abortion occurred in <0.5% of vaccinated animals. The length of viraemia in sheep and cattle that received MLVs did not exceed 35 days, with the single notable exception

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of a cow vaccinated with a multivalent BTV-2, -4, -9 and -16 vaccine in which viraemia persisted at least 78 days. Viraemia of sufficient titre to infect Culicoides insects was observed transiently in MLV-vaccinated ruminants, and natural transmission of MLV strains has been confirmed. An inactivated vaccine was first developed against BTV-2 and used in the field. An inactivated vaccine against BTV-4 as well as a bivalent vaccine against serotypes 2 and 4 were subsequently developed and used in Corsica, Spain, Portugal and Italy. These inactivated vaccines were generally safe although on few occasions reactions occurred at the site of inoculation. Two doses of these BTV inactivated vaccines provided complete, long-lasting immunity against both clinical signs and viraemia, whereas a single immunization with the BTV-4 inactivated vaccine gave only partial reduction of viraemia in vaccinated cattle when challenged with the homologous BTV serotype. Additional BTV inactivated vaccines are currently under development, as well as new generation vaccines including recombinant vaccines.

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Keywords: Arbovirus; Orbivirus; Bluetongue; Vaccines

Résumé

Après l’incursion du virus de la fièvre catarrhale ovine (FCO ou Bluetongue, BT) dans le bassin méditerranéen en 1998, les autorités italienne, française, portugaise et espagnole ont utilisé la vaccination pour réduire les pertes économiques directes dans le domaine des productions animales et pour réduire la circulation virale. Les stratégies de vaccination suivies dans les différents pays ont été effectuées selon leurs propres politiques vaccinales, les sérotypes du virus de la FCO introduits et leurs distributions géographiques et les capacités d’accès aux vaccins. Quatre différents vaccins atténués monovalents ont été importés d’Afrique du sud. Plusieurs associations de ces vaccins entre eux ont été utilisées à large échelle chez les bovins et les moutons. Les études expérimentales et les données de terrain ont montré que ces vaccins atténués sont immunogènes et capables d’induire une immunité solide chez les ruminants vaccinés. Des effets secondaires ont toutefois été mis en évidence chez les ovis. En particulier, des moutons ont présenté de la fièvre, des oedèmes de la face et des boiteries qui apparaissaient en général dans la seconde semaine suivant la vaccination et disparaissaient 7 à 10 jours après. Les brebis qui ont manifesté des syndromes fébriles ont présenté des chutes de production laitière. Des avortements ont été observés chez moins de 0,5% des animaux vaccinés. Des signes cliniques plus sévères ont été observés chez les moutons vaccinés avec des combinaisons de vaccins qui contenaient le sérotype 16; en conséquence, l’utilisation du vaccin monovalent 16 a été arrêtée. La durée de la viremie chez les moutons et les bovins qui avaient reçu un vaccin vivant n’a pas dépassé 35 jours, à l’exception notable d’un bovin vacciné avec un vaccin polyvalent 2, 4, 9 et 16 chez lequel la viremie dépassait au moins 78 jours. Le titre de ces viremies transitoires était suffisant pour infecter des vecteurs Culicoides et des transmissions naturelles des souches vaccinales ont été rapportées sur le terrain. Un vaccin inactivé contre le sérotype 2 puis un vaccin inactivé contre le sérotype 4 ainsi qu’un vaccin bivalent 2 et 4 ont été développés et utilisés en Corse, en Espagne, au Portugal et en Italie. Ces vaccins inactivés ont une innocuité satisfaisante même si parfois des réactions locales aux sites d’inoculation peuvent s’observer chez les ruminants vaccinés. Deux doses de vaccin inactivé induisent une immunité de longue durée et protègent les ruminants à la fois contre les signes cliniques et la viremie alors qu’une seule dose avec le vaccin de sérotype 4 n’induit qu’une réduction partielle de la viremie chez les bovins infectés après vaccination. De nouveaux
1. Introduction

Bluetongue (BT) is a non-contagious, insect-transmitted disease of certain breeds of sheep and some species of wild ruminants that is caused by bluetongue virus (BTV) [1]. BTV infection of ruminants occurs throughout much of the temperate and tropical regions of the world, coincident with the distribution of specific species of *Culicoides* biting midges that are biological vectors of the virus [2,3]. BT typically occurs when susceptible sheep are introduced into areas where virulent strains of BTV circulate, or when virulent strains of BTV extend their range into previously unexposed populations of ruminants. The global distribution of BTV has historically been between latitudes of approximately 40–50°N and 35°S. However, during the recent northern European epidemic, the virus spread far beyond its prior known upper northern limits [4].

BTV is the prototype member of the genus *Orbivirus*, family *Reoviridae* [5]. All reoviruses share distinctive common properties including segmented genomes of double-stranded RNA (dsRNA) and characteristic virion morphology and structure. There are currently 12 genera within the family *Reoviridae*, which include pathogens of plants, crustaceans, fish, insects, reptiles and mammals including humans. To date, 24 distinct serotypes of BTV have been described that all share common group antigens but which are distinguished on the basis of serotype-specific virus neutralization assays (VNTs). Importantly, there is considerable variation amongst field strains of BTV, even those of the same serotype, which reflects differences in the nucleotide sequence of each of the 10 distinct dsRNA segments of the BTV genome [6,7]. Genetic heterogeneity of field strains of BTV occurs as a consequence both of genetic drift and genetic shift, the latter as a result of reassortment of viral genes during mixed infections of either the vertebrate or invertebrate hosts of the virus [8]. Variation in the sequence of individual genes occurs through a complex process of genetic drift and founder effect during alternating passage of BTV in its ruminant and insect hosts [9].

Five different BTV serotypes (1, 2, 4, 9 and 16) have recently spread throughout extensive portions of Mediterranean Europe, and BTV serotype 8 emerged in northern Europe in 2006 [4]. BT epidemics had been reported, although irregularly, from 1979 to 1999 in Greece, in the Eastern Islands (Lesbos, Leros and Kos) and the Dodecanese archipelago. In 1999, BT was reported in mainland Greece and southeastern Bulgaria, close to the Turkish and Greek borders. From August to September 2000, BTV-9 spread progressively through the Balkan region to Albania, Bosnia Herzegovina, Croatia, Kosovo, Macedonia Republic, Republic of Serbia and Montenegro [10]. BTV-2 emerged in 2000 in France (Corsica), Italy (Sardinia) and
Spain (Balearic Islands) [11–13] and BTV-9 appeared later during the same year in Calabria (Italy). In 2001–02, BTV-2 and -9 spread throughout Sicily and southern and central Italy. In 2002, serotypes 4 and 16 were detected in the southern regions of Italy, and in 2003 serotype 4 emerged in Sardinia, Corsica and Menorca; in 2003, serotype 16 was reported in Sardinia and then in 2004 in Corsica [14]. In the same year, a new epizootic of BTV-4 affected the mainland of Spain and neighboring regions of Portugal. In 2005, the infection spread to central Spain but no further outbreaks were reported in 2006 [15], in contrast with Portugal where the epidemic continued through 2006. In the same year, BTV serotype 8 emerged very unexpectedly in the North of Europe involving Belgium, France, Germany, Luxembourg and the Netherlands [4].

The incursion of BTV into Mediterranean Europe is having a considerable negative economic impact, partly due to direct losses from mortality and reduced production in affected livestock but, more importantly, because of the total ban of ruminant trade between BTV-infected and non-infected areas [10]. To limit direct losses and in an effort to minimize the circulation of BTV, as well as to allow the safe movement of animals, the Italian, French, Portuguese and Spanish authorities all undertook vaccination of livestock according to their individual national policies, the geographic distribution of the incurring BTV serotype(s), and the availability of appropriate vaccines. In France, only sheep were vaccinated whereas in Italy, all susceptible domestic ruminant species were vaccinated, i.e. sheep, goats, cattle and water buffalos [16]. In Spain, initially only sheep were vaccinated (2001–2003 in Balearic Islands) but in later outbreaks (southwest Spain) both cattle and sheep were subjected to vaccination [17]. The Italian vaccination campaign was based on a risk assessment that demonstrated that such a vaccination strategy would prevent direct economic losses, significantly reduce virus circulation [18] and minimize risks linked to the movement of animals from infected to free areas.

This paper will describe the different vaccines against BT that have been used or which have recently been developed in Europe. These include inactivated whole [killed] virus preparations, virus-like particles (VLPs) produced from recombinant baculoviruses, live attenuated vaccines (modified live viruses, MLVs) and live recombinant vaccinia or canarypox virus-vectored vaccines [19,20]. All have inherent potential advantages and disadvantages, but only MLVs and some inactivated vaccines are currently available under European Community approved national disease control programs. VLPs are also safe and have been shown to be efficacious in laboratory trials [23–26], but their efficacy in the field is still under evaluation.

2. Inactivated vaccines

The first inactivated vaccine that was developed and used in the field after the emergence of BT in Europe was the vaccine against BTV-2. Subsequently, a monovalent BTV-4 and a bivalent BTV-2 and -4 vaccines have been developed and used in Corsica, Spain, Portugal and Italy. Other inactivated vaccines have recently been developed or are under development (Table 1).
<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Year of vaccination</th>
<th>France (Corsica)</th>
<th>Italy(^a)</th>
<th>Spain</th>
<th>Portugal</th>
<th>Bulgaria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sheep</td>
<td>Sheep</td>
<td>Sheep</td>
<td>Sheep</td>
<td>Sheep</td>
</tr>
<tr>
<td><em>MLV</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BTV-2, -4, -9, -16</td>
<td>2004(^c)</td>
<td>2004(^c)</td>
<td>2004(^c)</td>
<td>2004(^c)</td>
<td>2004(^c)</td>
<td>2004(^c)</td>
</tr>
<tr>
<td>Inactivated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)In Italy various monovalent MLV combinations have been used in different regions.

\(^b\)The 2000 vaccination campaigns started in November.

\(^c\)The use of the BTV-16 MLV was discontinued.
Inactivated whole virus vaccines are very safe if properly produced. They can be highly efficacious [21,22] and although not yet available, strategies for differentiating infected from vaccinated animals (DIVA) are theoretically possible with these type of vaccines. Their inherent potential disadvantages include: (1) their high costs of production, as vaccination requires large amounts of antigen; and (2) the need for booster immunizations, as inactivated vaccines generally induce a relatively transient immunity.

2.1. Quality control

For quality control testing, no data were given regarding the control tests performed during the different stages of production of the inactivated vaccine; however, all companies must follow the current guidelines described in the EU legislation concerning the manufacture of veterinary vaccines.

2.2. Safety

Several studies have been conducted on sheep to evaluate the safety of the subcutaneous injection of inactivated prototype vaccines against BTV-2, BTV-4 and BTV-2&4 in either simple, repeated or overdose trials. In all conditions, the inactivated BTV prototype vaccines were very well tolerated as demonstrated by the absence of systemic reaction (fever, weight loss, reproductive dysfunction, etc.) related to vaccination. Some vaccines induced transient local reactions of variable severity (mild to moderate) with different frequency (unusual to common). These usually disappeared within 3 days but, in a single case, a moderate local reaction persisted for 2 weeks [27]. Anaphylactic shock was also reported in 0.02% sheep following vaccination with a BTV-4 inactivated vaccine. This event was observed only in areas where BTV-4 MLV had previously been used.

BTV-4 and BTV-2&4 inactivated vaccines have also been tested for safety in cattle [17,28,29]. The vaccines were very well tolerated, and no side effects or local reactions were observed even when five doses of the BTV-4 inactivated vaccine were administered to the same animal [17].

No data documenting systemic or local reactions in vaccinated animals are available from the field use of these vaccines, as no complaints were reported from farmers. In addition, as no significant antigenic variation was yet documented amongst the BTV-2 and -4 strains currently circulating in Europe, the strains used to produce the inactivated vaccine are still suitable for the production of effective vaccines against these serotypes.

2.3. Efficacy

Most companies producing BTV inactivated vaccines follow the guidelines of the European Pharmacopea and Committee for Veterinary Medicines Products for quality and safety control and efficacy. Assessment of efficacy is based on clinical and virological data as well as on immunogenicity. Immunogenicity is assessed by
the analysis of the antibody response induced by each immunization, as measured by ELISA and by titration in a VNT against the same serotype (BTV-2 or BTV-4). The efficacy of the vaccine is evaluated in vaccinated animals by inoculation of an infective dose of live virulent BTV. The level of viremia after virus challenge is considered the most objective way to assess the efficacy of the vaccine-induced immunity. The level of viremia is analyzed by either a BTV-specific quantitative real-time RT-PCR assay [30] or by virus isolation. In addition, clinical signs (fever, general congestion of the skin, edema and lameness) are evaluated after challenge.

The inactivated prototype BTV vaccines induced significant titres of neutralising antibodies after either one or two injections in sheep. A booster effect was observed after the second immunization [27,31]. In cattle, one dose of BTV-4 or BTV-2&4 inactivated vaccines induced a weak humoral response which rapidly declined to be undetectable 21 days following vaccination. However, the second dose of vaccine elicited high and stable titers of neutralizing antibodies [17,28].

Table 2 shows the inactivated vaccines that have been tested to date for efficacy. The number, age and breed of animals, as well as the challenge dose and virus strain vary for the different vaccines. A preliminary study was carried out to establish and standardize the optimal conditions for BTV vaccine trials on sheep and cattle. In this study, the virus strain chosen for the challenge experiment was the isolate BTV-4/SPA-1/2004. The effect of factors like the number of cell culture passages of the virus inoculum, the virus dose, type of inoculum and route of inoculation have all been evaluated. The infectivity of the field isolate was confirmed after four passages in cell culture, and the route of inoculation (subcutaneous or intravenous) did not affect either the occurrence of clinical signs or the duration and titer of viremia. Appropriate titers of viremia were also obtained with different inocula (infected blood or cell culture-propagated virus) at different doses ($2 \times 10^7$ and $2 \times 10^6$ TCID$_{50}$ (50% tissue culture infective dose)/dose or threshold cycle (TC): 25–27 and TC: 30–33). The inoculation of cell culture-propagated virus-induced detectable viremia 3 days earlier (day 4) than that of blood from highly viremic sheep.

One or two doses of inactivated BTV-2, BTV-4 and BTV-2&4 vaccine at 3–4 week intervals gave full and significant protection against clinical signs and viremia in sheep that were intradermally challenged with virulent BTV-2 and/or BTV-4 a week or a month after last vaccination. For BTV-2, it was also shown that a single dose protected sheep against both clinical signs and viremia for at least 6 months [27,31]. For BTV-2&4, two doses fully protect sheep for up to 12 months (Table 2).

In cattle, efficacy studies have been performed on BTV-4 and BTV-2&4 vaccines. Two doses of the inactivated BTV-4 vaccine administered at a 24-day interval prevented viremia in vaccinated animals challenged with the homologous virulent serotype. Similarly, none of the animals vaccinated with two doses of BTV-2&4 inactivated vaccine developed detectable viremia following challenge with virulent field strains of BTV-2 and/or BTV-4 that were performed up to 1 month after the second vaccination [28]. However, although a single dose of BTV-4 inactivated vaccine prevented viremia in vaccinated animals challenged 2 weeks after
### Table 2
Summary of the results of efficacy studies of different inactivated bluetongue vaccines (experimental studies)

<table>
<thead>
<tr>
<th>BTV vaccine serotype</th>
<th>BTV-2</th>
<th>BTV-4</th>
<th>BTV-2&amp;4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Product code</strong></td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td><strong>Species</strong></td>
<td>Sheep</td>
<td>Sheep</td>
<td>Sheep</td>
</tr>
<tr>
<td><strong>Breed</strong></td>
<td>ns</td>
<td>ns</td>
<td>Assaf, Merino, Churra and Ripollesa</td>
</tr>
<tr>
<td><strong>Age of vaccination</strong></td>
<td>ns</td>
<td>3–6 months</td>
<td>Adult 3 months</td>
</tr>
<tr>
<td><strong>No. of shoots</strong></td>
<td>1 and 2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Interval</strong></td>
<td>1 month</td>
<td>21 days</td>
<td>21 days</td>
</tr>
<tr>
<td><strong>Dose (ml)</strong></td>
<td>1</td>
<td>SC</td>
<td>SC</td>
</tr>
<tr>
<td><strong>Route</strong></td>
<td>1</td>
<td>SC</td>
<td>SC</td>
</tr>
<tr>
<td><strong>Challenge after first vaccination (route)</strong></td>
<td>35 days</td>
<td>23 days</td>
<td>6 months</td>
</tr>
<tr>
<td><strong>Virus challenge serotype</strong></td>
<td>BTV-2</td>
<td>BTV-2</td>
<td>BTV-4-ESPA-1/2005</td>
</tr>
<tr>
<td><strong>Dose (TCID&lt;sub&gt;50&lt;/sub&gt;/ml)</strong></td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Trials and no. of vaccinated</strong></td>
<td>5+5</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td><strong>Controls Samples</strong></td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

(a) Trial B, 5 + 6

7 vaccine batch 1 + 5 vaccine batch 2

8 + 8

10 + trial B, 8

16
<table>
<thead>
<tr>
<th>Viremia results in vaccinated group</th>
<th>5, 7, 9, 12, 14 dpc</th>
<th>3, 5, 7, 10, 18, 21, 24 and 27 dpc</th>
<th>3, 5, 7, 10, 18, 21, 24 and 27 dpc</th>
<th>Three times a week for 6 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viremia results in control group</td>
<td>0/5 0/5 0/7 ns</td>
<td>0/10 A: 0/6+6 B: 0/5+6 C: 4/4+4</td>
<td>5/5 6/6 7/7 ns 2/2 A: 2/2 B: 2/2 C: 2/2</td>
<td>ns 0/5 4/4 0/4 0/7 0/7 0/7 0/8 0/8</td>
</tr>
<tr>
<td>Method used</td>
<td>Real time RT-PCR</td>
<td>Real time RT-PCR</td>
<td>Real time RT-PCR</td>
<td>Real time RT-PCR</td>
</tr>
<tr>
<td></td>
<td>0/5 0/5</td>
<td>5/5 2/2</td>
<td>6/7</td>
<td>BTV-2: 8/8 BTV-4: 8/8</td>
</tr>
<tr>
<td></td>
<td>0/7 0/7 ns</td>
<td>ns</td>
<td></td>
<td>Virus isolation</td>
</tr>
</tbody>
</table>

Trial C: two or four animals vaccinated with half-dose had slight viremia when challenged after 14 months post-vaccination.

ID, intradermally; SC, subcutaneously; IV, intravenously; ns: data not provided.

Hammers et al. [27,31]; Savini et al. [28]; MAPA [17].

Real time RT-PCR: Jimenez-Clavero et al. [30]; Toussaint et al. [4].
vaccination, a single vaccination did not fully prevent viremia in animals challenged 7 months after vaccination [17].

The efficacy of the inactivated BTV vaccine was indirectly confirmed in the field when all but 2 of more than 40,000 seasonally migrating vaccinated Spanish cattle remained negative for BTV by RT-PCR after staying in a restricted area in the presence of BTV circulation [30].

3. Modified live virus (MLV) vaccines

Outside of the European Union (EU), including the USA, Turkey, Republic of South Africa and India, BTV MLVs are available for many BTV serotypes. MLVs are produced by adapting BTV field isolates to growth in vitro through serial passages in tissue culture or in embryonated chicken eggs. Stimulation of a strong antibody response by these vaccines directly is correlated with their ability to replicate in the vaccinated host. MLVs are cheap to produce in large quantities, they generate protective immunity after a single inoculation and have proven effective in preventing clinical BT disease in the areas where they are used [16,32,33]. However, BTV MLVs suffer from a variety of documented or potential drawbacks including under-attenuation, whose impact may vary with sheep of different breeds. Potential adverse consequences are depressed milk production in lactating sheep, and abortion/embryonic death and teratogenesis in offspring when used on pregnant females [34–39,44]. Another risk associated with the use of MLVs is that of their potential for spread by vectors, with eventual reversion to virulence and/or reassortment of MLV genes with those of wild-type virus strains. The frequency and significance of these events remain poorly defined but natural and local dissemination of BTV-2 and -16 MLV vaccine strains has already been documented in Europe. Natural dissemination of MLV strains of BTV likely also is responsible for the sporadic incidence of teratogenic defects in unvaccinated cattle in South Africa and North America. Finally, the intrinsic inability to serologically distinguish naturally infected from MLV vaccinated animals precludes the possibility of developing a DIVA strategy with the MLV vaccines.

After the incursion of BTV into Mediterranean Europe, the Spanish, French, Italian and Portuguese authorities have all carried out compulsory vaccination campaigns since 2000 using MLVs produced by Onderstepoort Biological Products in an attempt to reduce direct losses due to disease and indirect losses due to trade embargoes caused by the presence of BTV (Table 1). At that time, these were the only commercially available BTV vaccines. Based on the serotype(s) present in a given country/area, various MLV monovalent serotype formulations have been used.

3.1. Quality control

Four monovalent MLV vaccines have been imported from South Africa and used in the EU (Table 3). Before use, these vaccines were confirmed by the various EU
National Laboratories to be free of bacterial, fungal and viral contaminants. Titres and serotype of the MLV batches also have been verified with no major discrepancies with information provided by the manufacturer. For the BTV-2 MLV, it was observed that the titer strongly decreased at temperatures above 35°C [41], but that it was retained for at least 36–48 h if sterility conditions were ensured during rehydration and the reconstituted vaccine was stored at a temperature below 19°C [42].

### 3.2. Safety

Modified live viruses have different potential adverse impacts according to the specific formulation used, the specific serotypes and the number of serotypes included in the vaccine.

#### 3.2.1. Fever and sickness

Apart from a study performed by the Institute of Animal Health at Pirbright using either BTV-2 or BTV-9 and Polled Dorset sheep, in which moderate to severe clinical signs of BT, albeit short lived, were observed following vaccination [40], only mild symptoms were observed in most experimental MLV vaccination studies. These were characterized by transient fever starting from the fifth day after vaccination (p.v.) and mild hyperemia of the oral cavity during the second week.

Reports of adverse events in the field greatly vary with the strain of BT MLV used for vaccination of the animals. The monovalent BTV-2 MLV vaccine was used in Corsica (from 2001 to 2004) and Italy (from 2002) on approximately 130,000 and 4,000,000 sheep and goats, respectively. In both locations, there were no or negligible adverse reactions reported after vaccination [14,43–45]. However, when the same vaccine was used in 2000–01 in Menorca and Mallorca on 320,000 sheep, adverse events were observed in 0.13% and abortion in 0.16% of the vaccinated animals [17].

<table>
<thead>
<tr>
<th>Modified live vaccine</th>
<th>Strain</th>
<th>Date, place of isolation</th>
<th>History</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTV-2</td>
<td>Vryheid/5036</td>
<td>1958, Republic of South Africa</td>
<td>Virus passaged 50 times in embryonated chicken eggs and plaque selected three times and passaged twice in BHK&lt;sub&gt;21&lt;/sub&gt; cells.</td>
</tr>
<tr>
<td>BTV-4</td>
<td>Theiler/79043</td>
<td>1900, Republic of South Africa</td>
<td>Virus passaged 60 times in embryonated chicken eggs, followed by three small plaque selections and nine passages in BHK&lt;sub&gt;21&lt;/sub&gt; cells.</td>
</tr>
<tr>
<td>BTV-9</td>
<td></td>
<td>1900, Republic of South Africa</td>
<td>Virus passaged 70 times in embryonated chicken eggs, followed by three small plaque selections and six further passages in BHK&lt;sub&gt;21&lt;/sub&gt; cells.</td>
</tr>
<tr>
<td>BTV-16</td>
<td>Pakistan/7766</td>
<td></td>
<td>Virus passaged 37 times in eggs, followed by three large plaque selections, two passages in BHK&lt;sub&gt;21&lt;/sub&gt; cells and one in VERO cells.</td>
</tr>
</tbody>
</table>
The BTV-2 MLV vaccine was also used on more than 400,000 cattle during the 2002 Italian vaccination campaign with no adverse reactions reported.

The monovalent BTV-4 MLV vaccine has been only used in sheep in Corsica in 2004. No adverse reactions were reported [14].

In 2003 a new outbreak of BT occurred in Menorca that was due to BTV-4. A combined BTV-2 and -4 MLV vaccine was used to vaccinate sheep and goats, and no side effects were observed. The same vaccine combination was used in 2004 in Tuscany, and the following year in Tuscany and Sardinia, involving some 4,000,000 sheep and goats. Again, no or negligible adverse reactions were reported. Similarly, no adverse side effects were recorded among approximately 400,000 cattle vaccinated with the combined BTV-2 and -4 MLVs in 2004 and among a similar number of animals in 2005.

A bivalent BTV-2 and -9 MLV vaccine was used to vaccinate sheep and goats in some regions of Italy since 2002. Of the more than 1,700,000 animals vaccinated, only a very small percentage (<0.1%) developed fever and facial edema at 7–14 days p.v. [45]. In the same vaccination campaign, BTV-2/BTV-9 MLVs were also administered to more than 600,000 cattle and no adverse reactions were reported. Similarly, a trivalent MLV vaccine containing BTV serotypes 2, 4 and 9 was used since 2005 on more than 1,000,000 sheep, goat and cattle, and no or negligible adverse reaction were reported.

In 2004, BTV-16 was isolated in Corsica by the AFSSA Maisons-Alfort laboratory [46]. Vaccination of sheep with BTV-16 MLV was performed in the southern part of the island. Typical signs of BT were reported in vaccinated sheep several days after vaccination. The vaccination program was therefore immediately terminated. The nucleotide sequence of segments 2, 7, 8, 9 and 10 from the virus isolated from ill animals was determined and found to be exactly identical to that of the vaccine MLV strain (Zientara, unpublished).

Also in 2004, a trivalent MLV vaccine containing serotypes 2, 4 and 16 was used in Sardinia. However, a few weeks after vaccination, many vaccinated and unvaccinated sheep and goats became ill because of infection with the BTV-16 vaccine strain [47]. These incidents were attributed to inadequate attenuation of the BTV-16 MLV that was in the vaccine, and for this reason the use of monovalent BTV-16 MLV vaccine was discontinued [48]. In contrast to what occurred in sheep and goats; however, the BTV-2, -4 and -16 MLV combination did not cause significant adverse reactions in cattle (approximately 600,000 vaccinated animals).

A polyvalent MLV containing BTV serotypes 2, 4, 9 and 16 was used in southern regions of Italy in 2004, and approximately 1,700,000 sheep and goats were vaccinated. No adverse reactions were reported. The same BTV-2, -4, -9 and -16 MLV combination did not cause adverse reactions in cattle (approximately 600,000 vaccinated animals).

Finally, the safety of a pentavalent BTV serotypes 1, 2, 4, 9 and 16 MLV vaccine was evaluated in sheep maintained in isolation facilities at the Afssa Sophia Antipolis laboratory. Significant fever (41–42°C) was reported in many animals (unpublished data). Based on these observations, it was decided that this vaccine should not be used in the field.
3.2.2. Effect on pregnancy

Experimental infection studies using MLV strains of BTV-2 or BTV-2 and -9 were conducted on cattle to evaluate potentially deleterious effects on reproduction. In none of these studies was any adverse effect on pregnancy observed [38,49,50].

Abortions and/or stillbirth have however been reported in the various vaccination campaigns that used BTV MLVs. During the 2000–01 Balearic BTV-2 campaign, approximately 0.16% of the 320,000 vaccinated sheep aborted. Similarly, during the Italian BTV-2 vaccination campaign, abortion was reported on 0.42% and 0.18% of vaccinated sheep and cattle, respectively. However the virus was detected in only 0.06% and 0.01% of their respective aborted fetuses. In 2002, the combined BTV-2 and -9 MLV resulted in abortion of 0.53% and 0.14% of vaccinated sheep and cattle, respectively, although BTV was detected only in a small percentage of abortions (0.09% and 0.01% of the sheep and cattle fetuses, respectively) [49].

3.2.3. Effect on semen

The effects of the BTV-2 MLV on the quality of semen were investigated [51] in 23 rams vaccinated at a 47-day interval. Although BTV was not detected in any of the semen samples, a decrease of the semen quality (volume, sperm concentration, motility, abnormal and dead spermatozoa) was demonstrated after the first vaccination. A decrease in semen quality was also observed after the second vaccination; however, at day 69, the semen quality of the vaccinated animals was not significantly different from those of the 23 controls.

3.2.4. Milk production

Given the economic importance of milk production, numerous studies have been conducted to determine the effects of several MLV combinations on milk production in both sheep and cattle.

Vaccination of sheep with either BTV-2 or BTV-2 &4 MLVs did not affect the quantity and quality of milk produced by the vaccinated animals (Zientara, unpublished; and [52–54]).

Quite different were the observations made with BTV-2 and -9, BTV-2 and -16, BTV-2, -4 and -9 or BTV-2, -4, -9 and -16 MLVs. Vaccination with each of these combinations had a marked negative impact on total milk production with production decreases of 20–30% as compared to normal production levels. The decrease, evident in the second week following vaccination, was transient and not accompanied by significant changes in milk quality (cell count, pH, fat, protein and lactose) [36,53]. It was suggested that the effects of vaccination on milk production were primarily due to the transient perturbation of health induced by the vaccine and not to a direct virus effect on the mammary tissue [36].

In contrast, vaccination of 30 cows with combined BTV-2 and -9 MLV vaccines had no effect on the production and quality (somatic cell count, pH, milk fat, protein and lactose content) of their milk [38].
In the field, data collected between 1999 and 2002 on the quantity and quality of milk of 18,000 cows demonstrated that BTV-2 MLV vaccination had no effect on milk production [54].

3.2.5. Duration and titer of viremia

After immunization with MLVs, the attenuated virus circulates in the blood stream and so potentially can infect competent vectors and be transmitted to other susceptible hosts. Therefore, MLV vaccination should be performed in the cooler months when the Culicoides population and its activity typically are at the lowest level. This will limit the possibility of transmission of the vaccine strains by biting midges while immunizing susceptible animal populations before the next epidemic season.

Transmission of MLV strains of BTV to insects most likely would occur from viremic animals that are introduced into infection-free areas where competent Culicoides species are present and highly active. In this scenario, the magnitude and duration of viremia in vaccinated animals would be clearly important in determining whether or not MLV strains of BTV could be acquired and transmitted by local vectors. Although virus titers in blood less than $10^3\text{TCID}_{50}/ml$ have traditionally been considered a “safe” threshold, authentic instances of insects acquiring BTV from animals with viremic titers less than $10^3\text{TCID}_{50}/ml$ have been reported. Given the complex interaction of BTV, Culicoides vectors and animal hosts in the life cycle of infection, virus titers induced by MLV should be kept to an absolute minimum specially if field transmission of MLV strains is a concern.

Studies on the duration and titers of viremia have been performed on sheep and cattle following vaccination with different MLV combinations. Viremia following vaccination with BTV-2, -4, -9 and -16 MLV strains (including multivalent combinations) was found to persist for up to 24 days in sheep and 78 days in cattle [37,38,47,55–59]. Information pertaining to the MLV strains used in Europe is however limited; available data suggests that cattle vaccinated with BTV-2 and BTV-9 MLVs can be moved safely 32 days after vaccination [37], whereas sheep vaccinated with the same strains can be moved 28 days following immunization [36]. From the viremia data obtained in cattle following BTV-2, -4, -9 and -16 MLV vaccination, it was determined that cattle could be moved safely (risk of infection < 0.01%) at 60 days after vaccination [29]. The latter result, however, is most likely related to the inadequate attenuation of the BTV-16 MLV strain and cannot be extrapolated to MLV vaccines that do not include this serotype.

Apart from some BTV-2 MLV vaccination studies on sheep and cows where virus titers were never found to be higher than $10^3\text{TCID}_{50}/ml$ [41,49], all other MLV combinations which have been studied in sheep (BTV-2, BTV-9, BTV-16, BTV-2 and -9, BTV-2 and -4, BTV-2, -4 and -16, BTV-2, -4, -9 and -16) and cattle (BTV-2 and -9, BTV-2, -4, -9 and -16) gave rise, for a brief period of 2–4 days, to viremic titers above the infecting threshold at least in some of the vaccinated animals [28,29,36,37,40,47,59].

No data have been reported, however, on the duration and titers of viremia in animals vaccinated with these MLV in the field, but local transmission of BTV-2 and BTV-16 vaccine strains in the field has been demonstrated [39,47].
3.3. Efficacy

An important factor in confirming the efficacy of MLV vaccines is their ability to elicit neutralizing antibodies in vaccinated animals. Neutralizing antibodies play a key role in protecting animals from disease and viremia following infection with the homologous wild-type BTV. Knowing the duration of the immune status derived from vaccination is of paramount importance for both planning the frequency of vaccine booster immunizations to adequately protect the animals against disease, and to facilitate the safe movement of vaccinated animals [60].

Experimental challenge studies have demonstrated that vaccination with the BTV-2 MLV strain prevented viremia in at least 90.5% of vaccinated cattle that were challenged at 7 months after vaccination with a dose of $2 \times 10^{5.8} \text{TCID}_{50}$ of virulent homologous field isolate [57]. In this trial, the control animals had viremas that persisted through the 35th day with titers above $10^{5.8} \text{TCID}_{50}/\text{ml}$. Serological studies performed on cattle and sheep that were vaccinated with several MLV combinations have shown that more than 80% of the vaccinated animals had specific BTV antibodies [41,43,55,58]. Colostral antibodies were found in calves born from vaccinated dams until 39 days of age [58].

The efficacy of MLV vaccination has widely been demonstrated in the field. Following the 2000–01 and 2003 BT vaccination campaigns in the Balearic Islands, no outbreaks have been detected since December 2003 in the area. With regard to the vaccination strategy in Italy, several points warrant attention. First, on the basis of a risk assessment [18] and considering the encouraging results of preliminary studies, the Italian Authorities decided to vaccinate all susceptible domestic ruminant species (i.e. sheep, goats, cattle and water buffalo) in the infected and at risk areas, with the aim of limiting direct losses and reducing virus BTV circulation [16]. Mass vaccination of susceptible populations started in January 2002, although the starting dates and the percentages of vaccinated population achieved varied greatly among regions [16]. In those areas where more than 80% of the target population was properly vaccinated before the new epidemic peak, clinical disease in sheep disappeared almost completely and virus circulation was significantly reduced [16], with substantial benefit to internal animal trade/movement. The results obtained in some Italian regions with mass vaccination of all susceptible domestic ruminants and the experience gained during the vaccination campaigns contributed to the modifications of BT international standards. Specifically, risk analysis can be used as an alternative to individual testing to assess immunity level in the population of origin and determine the risk of spreading infection to free areas by movement of vaccinated animals from infected territories [61].

In the absence of effective inactivated vaccines and in an emergency, MLVs still represent a valid option for vaccination, provided that the quality, safety and efficacy of the MLV strains match EU standards. These vaccines can be an
alternative also in a non-emergency situation when local conditions (e.g. in case a large amount of animal must be immunized in a very short period of time) indicate their use.

4. Recombinant vaccines

Several experimental recombinant vaccines have been described and they clearly have numerous inherent potential benefits, including rapid onset of immunity, lack of transmissibility and even a polyvalent strategy.

A recombinant vaccinia virus that expressed both VP2 and VP5 of Australian BTV serotype 1 induced variable titers of neutralizing antibody in sheep and afforded protection against homologous challenge [62], but this approach has not been pursued further.

A recombinant capripoxvirus expressing VP7 was shown to provide partial protection against heterologous BTV challenge [63], but like the recombinant vaccinia BTV vaccine, its development was not continued.

Finally, a recombinant canarypox virus-VP2/VP5 vaccine was recently described that induced highly effective protective immunity in sheep [20]. This vaccine has a major inherent advantage in that the existing VP7 competitive ELISA assay would distinguish vaccinated from naturally infected animals (DIVA), and it utilizes an expression vector that is incorporated in several vaccines already in use in the EU and elsewhere. The vaccine still is at a development stage.

5. Conclusions

Bluetongue vaccines may be used for different purposes or strategies, depending on the epidemiological situation of the affected area and strategy desired. The main purposes of BT vaccination strategies are (i) to prevent clinical disease, (ii) to limit the regional extension of BTV infection through reduction of the spread of the virus, (iii) to allow regional or country eradication of the disease based on the reduction of virus circulation and (iv) to authorize the safe movement of susceptible animals between affected and free zones.

In BT endemic regions, vaccines have been used to prevent clinical disease and death losses in sheep. In these regions, vector Culicoides spp. vectors may be present year-round with continuous circulation of different BTV serotypes. This has led to the design of multivalent vaccines containing different MLV serotypes, as done in South Africa, where BTV infection is endemic. The South African MLVs were developed only to control clinical BT disease in sheep, as cattle and other ruminants, although susceptible to BTV infection, usually do not suffer clinical disease.

Since the incursion of BTV into previously non-endemic zones, as in some Mediterranean countries, BTV vaccines are used as an aid to prevent further extension of the infection to border zones, for local/regional reduction of virus circulation and for safe movements of animals, which play an important role in the
European livestock industry. Depending on their availability, MLV or inactivated serotype-specific vaccines can be used. Climatic and geographic factors as well as abundance of suitable BTV insect vectors are probably all important for the outcome and persistence (reemergence) of BTV infection in an area. It is therefore commonly accepted that vaccines can help limit the spread of the disease. Ideally, for the purpose of eradication, a successful vaccination campaign should cover all susceptible ruminant species, attain a high degree of herd immunity and encompass extensive areas surrounding any active BT outbreak. Successful control also requires restriction of animal movements between BT-affected and BT-free zones.

Both MLV (outbreaks in Balearic Islands and Corsica in 2000 and 2001 and in Italy in 2003–04) and inactivated vaccines (outbreak in southwest Spain in 2004 and 2005) have been successfully used after emergence of BTV in non-endemic areas. Although a risk model integrating factors determinant for BTV persistence (including vaccinations) needs to be developed, it is likely that eradication of BTV with vaccines may be achievable only under concurrent favorable geo-climatic conditions. These conditions limit or prevent vector activity, and as a result, prevent BTV circulation (e.g. cold winter impeding overwintering, geographical barriers, low vector densities, low probability of BTV incursion from neighboring zones), but this relies on the assumption that vertical BTV transmission does not occur in insects.

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