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ASSESSMENT OF EFFICACY OF A BIVALENT BTV-2 AND BTV-4 INACTIVATED VACCINE
BY VACCINATION AND CHALLENGE IN CATTLE


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ABSTRACT

The efficacy of a bivalent inactivated vaccine against bluetongue virus (BTV) serotypes 2 (BTV-2) and 4 (BTV-4) was evaluated in cattle by general and local examination, serological follow-up, and challenge.

Thirty-two 4 month-old calves were randomly allocated into 2 groups of 16 animals each. One group was vaccinated subcutaneously (s/c) with two injections of bivalent inactivated vaccine at a 28-day interval, and the second group was left unvaccinated and used as control. Sixty-five days after first vaccination, 8 vaccinated and 8 unvaccinated calves were s/c challenged with 1 mL of 6.2 Log10 TCID50/mL of an Italian field isolate of BTV serotype 2, while the remaining 8 vaccinated and 8 unvaccinated animals were challenged by 1 mL of 6.2 Log10
TCID$_{50}$/mL of an Italian field isolate of BTV serotype 4. Three additional calves were included in the study and used as sentinels to confirm that no BTV was circulating locally.

At the time of the challenge, only one vaccinated animal did not have neutralizing antibodies against BTV-4, while the remaining 15 showed titres of at least 1:10 for either BTV-2 or BTV-4. However, the BTV-2 component of the inactivated vaccine elicited a stronger immune response in terms of both the number of virus neutralization (VN) positive animals and antibody titres. After challenge, no animal showed signs of disease. Similarly, none of the vaccinated animals developed detectable viraemia while bluetongue virus serotype 2 and 4 titres were detected in the circulating blood of all unvaccinated animals, commencing on day 3 post challenge and lasting 16 days. It is concluded that administration of the bivalent BTV-2 and 4 inactivated vaccine resulted in a complete prevention of detectable viraemia in all calves when challenged with high doses of BTV-2 or BTV-4.

KEY WORDS: Bluetongue virus serotype 2, Bluetongue virus serotype 4, Cattle, Inactivated vaccine

INTRODUCTION

Bluetongue is an infectious, non-contagious disease of wild and domestic ruminants caused by an RNA virus belonging to the family Reoviridae, genus Orbivirus. Biting midges of the Culicoides genus are the biological vector of the virus, and their distribution affects the spreading of the infection in the temperate and tropical regions of the world (Gibbs et al., 1994; Tabachnick, 2004). The disease is typically evident in sheep and only recently clinical cases have unexpectedly been observed in cattle (Verwoerd and Erasmus, 2004; Toussaint et al., 2006). Since 1998, BTV infection has spread progressively all over the Mediterranean Basin, Balkan areas and more recently in Northern Europe. To date, 7 serotypes have been detected in the Mediterranean Basin: BTV-1, BTV-2, BTV-4, BTV-8, BTV-9, BTV-15 and BTV-16 (Mellor and Wittmann, 2002; OIE, 2006a,b,c,d,e,f,g,h; Saegerman et al., 2008). In Italy, the first evidence of BTV infection was recorded in Sardinia in August 2000, and since then, numerous outbreaks of BTV serotypes 1, 2, 4, 9 and 16 have been reported impacting most of
the central and southern regions of Italy (Calistri et al., 2004, OIE, 2006f). The incursion of BTV into Europe is having a considerable negative economic impact, partly due to direct losses from death and reduced production in affected livestock but, more importantly, because of the total ban of ruminant trade between BTV-infected and non-infected areas (Calistri et al., 2004). Despite the low occurrence of clinical cases in bovines, BTV is one of the 16 diseases formerly ranked as list ‘A’ by the Office International des Epizooties (OIE). As a consequence, Bluetongue affected countries are banned from trading livestock and livestock products. To reduce direct losses due to disease and indirect losses due to the trade embargo caused by virus circulation, European authorities have been undertaking vaccination campaigns according to their individual national policies, the geographic distribution of the incurring BTV serotype(s), and the availability of appropriate vaccines. Prior to 2003, only live vaccines were used. They were the only product available in the market. They are inexpensive and have proven highly effective in preventing bluetongue disease in the areas where they have been used (Savini et al. 2007). However, they could be inadequately attenuated, depress milk production in lactating sheep; and be teratogenic if used in pregnant animals (Savini et al. 2004; MacLachlan et al. 1985). Because of their potential to replicate in the organism attaining titres capable of infecting vectors, vaccine viruses have been demonstrated to spread into the environment (Ferrari et al. 2005, Savini et al. 2007) with the potential for reversion to virulence and re-assortment of their genes with those of wild type BTV (Murray et al. 1996, Venter et al. 2004, Ferrari et al. 2005, Monaco et al. 2006).

With this respect, whole inactivated virus vaccines would represent a safer alternative. Since 2005 BTV inactivated vaccines have been in the market and used in vaccination campaigns in Italy, France, Spain and Portugal. Even though most of the animal movements are associated to cattle, the inactivated vaccine launched in the market was for sheep use only.

This paper describes the clinical evaluation of a commercial inactivated vaccine containing purified BTV serotypes 2 and 4 in cattle. An experiment was conducted to determine the level of protection induced by the vaccine against a challenge with homologous serotypes.

MATERIALS AND METHODS
1. **Vaccine**

A bivalent BTV-2/BTV-4 inactivated vaccine produced at an industrial scale by Merial (France) was used in this study. The vaccine is inactivated and adjuvanted with Saponin/Aluminium hydroxide.

Both BTV-2 and BTV-4 vaccine strains originated from France (Corsica).

Sixteen cattle were vaccinated with 1 mL of the vaccine on day 0 and on day 28. The vaccine was administered s/c on the lateral face of the neck, height centred on the left (day 0) or right (day 28) side. Rectal temperatures of all animals (including controls) were recorded prior to vaccinations.

2. **Challenge strain**

Field isolates recovered from Sardinian infected animals during the BTV outbreaks of 2000-2003 were used as challenge strains: the 8341/00 strain for BTV-2 and 10353/03 for BTV-4.

Before challenging, each strain was amplified by one passage in VERO (African green monkey kidney) cell cultures. The strains were titrated after cell passage and then used as challenge strain. Viral suspensions were diluted in phosphate buffer saline (PBS, pH 7.2) to adjust the viral titre to $10^{6.2} \text{TCID}_{50}/\text{mL}$. Each animal was challenged s/c on the left face of the neck on day 65 with 1 mL of its respective viral suspension.

3. **Animals**

This study was conducted between November 2005 and April 2006 in the province of Teramo at an altitude of 1000 m in an insect-proof facility.

Thirty-five 4 month-old calves that were free of respiratory, digestive, umbilical and osteo-articular disease were included in the study. All animals originated from BTV-free herd, located in a BTV-free area (France) and tested negative for BTV-antibodies (c-ELISA) before entering the study.

Thirty-two calves were randomly allocated into 2 groups of 16 animals each. One group was vaccinated and the second group was left unvaccinated and used as control.
Sixty-five days after first vaccination, 8 vaccinated and 8 unvaccinated calves were challenged by BTV serotype 2, while the remaining 8 vaccinated and 8 unvaccinated animals were challenged by BTV serotype 4.

Thus, animals were allocated to four groups: (1) "Controls BTV-2", (2) "Controls BTV-4", (3) "Vaccinates BTV-2", (4) "Vaccinates BTV-4".

The three remaining animals were kept as environmental controls (not vaccinated nor challenged), and used as sentinels to confirm that no BTV was circulating locally.

On days 2, 14, 28 (before vaccination), 42, 65 (before challenge), and 86 all cattle were blood sampled by jugular puncture with plain tubes; serum samples were tested by c-ELISA and titrated for antibodies against BTV-2, BTV-4, BTV-9 and BTV-16 by virus neutralisation test (VN).

Prior to challenge, all bovines were blood sampled for detection of viraemia by jugular puncture with ethylene-diaminetetra-acetic acid (EDTA) tubes. After challenge, blood samples were taken 3 times a week for 42 days (day 65 -> day 107).

To exclude viral circulation in the stable, the environmental control animals were bled once a week for the entire trial length. Serum samples were tested by ELISA. In case of positive reaction they were subsequently typed by VN.

4. Virological and serological tests

The presence and titre of the virus in the blood were assessed by titration on VERO cells, according to the methods described by Savini et al., 2004 and OIE Manual of diagnostic tests and vaccines for terrestrial animals (2004). The presence of BTV in EDTA-blood samples was also assessed by using the RT-PCR which amplifies a portion of S5 as described by Katz et al. (1993). Its sensitivity for BTV-2 and BTV-4 of either field or vaccine origin was evaluated by testing viral suspensions whose titer was previously determined.

On day 7, in the absence of cytopathic effect (CPE), cultures were scraped, centrifuged, and the supernatant was re-passaged into a 24 microplate flat bottomed wells containing a confluent monolayer of VERO cells and the plates handled as described before. In the presence of CPE as well as at the end of the second passage, cells were scraped and collected from each
well. Cells and medium were then centrifuged at 812 g for 10 min. and the pellet was re-
suspended in approximately 1 mL of PBS. Ten µL of the cell suspension were subsequently put
onto a well of a multiwell slide, fixed for 20 min. in acetone at −20° C and checked for the
presence of BTV by immunofluorescence (IF) using BTV monoclonal antibodies. Virus
characterised as BTV was consequently typed by virus microneutralisation assays using type-
specific antisera. The virus titer was determined using the Reed and Munch formula applied on
the four replicates after the first passage. Those samples which showed CPE after the second
passage or were positive to IF were considered positive for BT with a titer of less than $10^{2.3}$
TCID$_{50}$/mL. (Savini et al. 2007)

As for the virus neutralisation assays, the method described by Savini et al. 2007 was applied
to both challenge inocula to demonstrate their serotype. The positive and negative controls as
well as OIE standard reference BTV serotypes 2, 4, 9 and 16 were kindly provided by the OIE
reference laboratory, Onderstepoort Veterinary Institute (OVI) in South Africa.

The antibody response was monitored using both the c-ELISA (Lelli et al., 2003) and VN test
(Savini et al., 2004).

5. Statistical analysis

Differences between the neutralising titres against BTV-2 and 4 per sampling day in the
vaccinated group after immunisation, were analysed using the non-parametric Wilcoxon test
for paired groups (Siegel and Castellani, 1998), while differences between BTV-2 and 4
viraemic titres per sampling day in the control groups were analysed using the non-parametric
Mann-Whitney test for independent groups. The probability of the various possible sensitivity
values of the C-ELISA in the vaccinated animals were estimated through a Bayesian approach
using the Beta ($s+1$, $n-s+1$) distribution (Sivia, 1996) where $s$ is the total number of positives
and $n$ is the total number of tested animals. Different Beta distributions were calculated on the
basis of different periods from vaccination. The probability distribution of the percentage of
positive animals shows not only the most probable value of sensitivity, but also the level of
uncertainty due to sample size. From an epidemiological point of view, it is far more interesting
to know the percentage of vaccinated animals that are protected after being challenged with
homologous BTV serotypes. The probability that more than a certain percentage of animals would be protected after challenge was calculated by using 1-Beta (s+1, n-s+1).

RESULTS

1. Virological results
After challenge, none of the vaccinated animals developed detectable viraemia by either cell culture or RT-PCR. Conversely bluetongue virus nucleic acid and serotype 2 and 4 titres were detected in the circulating blood of all unvaccinated animals. In these animals the RT-PCR detected BTV starting on day 5 post infection (pi) and remained positive up to the end of the experimental period (42 days pi). The cell culture virus isolation detected BTV-2 and BTV-4 titers commencing on day 3 pi and lasting 16 days. In both infections, maximum viral titre was detected on day 10 pi with average titres of $10^{4.02}$ TCID$_{50}$/mL and $10^{3.54}$ TCID$_{50}$/mL for BTV-2 and BTV-4, respectively (Fig. 1). The difference between BTV-2 and BTV-4 viraemic titres was statistically significant (p<0.05), with BTV-2 titres much higher than those with BTV-4. Figure 2 shows the curve of the probability that vaccinated animals are protected, not showing any detectable viraemia after challenge infection with homologous BTV.

2. Serological results
None of the vaccinated animals showed detectable c-ELISA antibodies after the first vaccine injection. Conversely, commencing on day 42 (14 days after the second shot), the immuno-enzymatic assay detected antibodies in all vaccinated calves. Figure 3 shows the probability distributions of the percentage of calves showing c-ELISA antibodies after being vaccinated with the inactivated bivalent BTV-2 and 4 vaccine. Apart from those used as environmental controls, all animals were positive to c-ELISA on day 86. For BTV-2, 28 days after first vaccination, 13 had sero-converted, but at low titres. By day 42, all had BTV-2 neutralizing antibodies. For BTV-4, none of the calves had detectable BTV-4 neutralizing antibodies 28 days after the first vaccination. By day 42, fifteen out of sixteen had BTV-4 neutralizing antibodies. On day 28, the number of BTV-2 positive calves was significantly higher (p<0.05) than that of BTV-4. In one animal BTV-4 antibodies were not
detected. The difference between BTV-2 and BTV-4 neutralising titres was statistically significant ($p<0.05$) at all time points between day 14 and day 65, with BTV-2 titres being much higher than those of BTV-4. However, for both serotypes, the highest peak of antibody response was observed on day 42 (Fig. 4). No animals developed BTV-9 and BTV-16 antibody titres. On day 86, BTV-2 and BTV-4 challenged groups developed homologous neutralising antibodies while no BTV antibody titres were observed in the animals used as environmental controls. Neither BTV nor antibodies were detected in the blood samples taken from the environmental control group.

**DISCUSSION**

Vaccination against BTV is a very important tool, not only for the control of the disease but more importantly, for ‘safe’ trade of live ruminants in accordance to OIE standards and EU legislation. To prevent BTV infection of ruminants, different types of vaccines, including inactivated and modified live virus (MLV) vaccines, Virus-like particles (VLP) produced from recombinant baculoviruses, and recombinant vaccinia virus vectored vaccines have been manufactured (Roy and Erasmus, 1992, Boone et al., 2007). Of these, the inactivated and MLV vaccines only are now available in the market and used in the official vaccination campaigns. Although VLPs are safe and neat, their inconsistent efficacy when used in field trials (Roy et al., 1990, 1992, 1994; Roy, 2004) and difficulties with commercial production, cost, and long-term stability make them ineligible for field use (Savini et al., 2007). Similarly, recombinant vector vaccines expressing both VP2 and VP5, even though revealing some potential in terms of safety and protection, still require further development before being ready for field use (Lobato et al., 1997). Conversely, MLV are cheap, easy to produce in large quantities, able to elicit protective immunity after a single inoculation, and have been proven effective in preventing clinical bluetongue (BT) disease in the areas where they have been used (Patta et al., 2004; Dungu et al., 2004). Nevertheless BTV MLV vaccines suffer from a variety of documented potential drawbacks including side effects due to under-attenuation of the modified strains and their capacity of passing the
the placental barrier, and the spread of vaccine strain in the environment with the potential for reversion to virulence and re-assortment with field isolates.

Inactivated vaccines have been recently developed and marketed. The efficacy of an inactivated vaccine is fully dependent on the dose of virus, resulting in significantly higher virus mass than that of MLV. Two doses, in the presence of adjuvant, may often be required for inactivated vaccines considerably increasing the cost of vaccination. Inactivated vaccines for BTV-2 and/or BTV-4 have been developed, commercialised and successfully employed in the 2005-2006 BTV vaccination campaigns. Despite this success, the real costs of bluetongue infection came from the ban on trading live animals in general and cattle in particular. The inactivated vaccines available in the market are primarily registered for sheep and very few information were available on the use of these products in cattle, at the time of this study. In this study, the administration of the bivalent BTV-2 and BTV-4 inactivated vaccine was safe and resulted in a complete prevention of detectable viraemia in all calves when infected with high doses of virulent BTV-2 or BTV-4. It occurred when using either the cell culture method or the RT-PCR assay. It has to be said however that, amongst the two assays, the cell culture isolation is the method capable of giving information on the presence of infectious virus. Two doses of the immunological product completely prevented vaccinated animals from developing viraemia, which statistically signify a virological protection of at least 83.8% (95% confidence interval) of vaccinated animals. It means that the product is not only safe but also effective in protecting all vaccinated animals from viraemia, including the calf which did not develop neutralising antibodies against BTV-4. At the time of the challenge, that calf was the only vaccinated animal which did not develop neutralizing antibodies, while the remaining 15 showed titres of at least 1:10 for either BTV-2 or BTV-4. The study showed that the BTV-2 component of the inactivated vaccine elicited a stronger immune response in terms of both the number of VN positive animals and antibody titres. However, the differences observed might also relate to the VN technique and further studies are required to demonstrate higher immunological stimulation of certain serotypes as compared to others. Concerning the diagnostic tests, it was surprising to note that 13 animals, even if at low titres, had sero-converted for BTV-2 28 days after first vaccination, whereas no c-ELISA antibodies were
detected in vaccinated animals following the first injection. These data were unexpected as c-ELISA normally detects antibodies earlier than VN. It is known that neutralising antibodies are stimulated by the structural proteins of the outer capsid, VP2 and partially VP5, while antibodies to the inside capsid, VP7, are detected by the c-ELISA (Huismans and Erasmus, 1981, Jeggo et al., 1991). An ineffective antigen stimulation by the VP7 following vaccination could be an explanation of this discrepancy, or alternatively, it could be due to a poor performance of the c-ELISA in detecting antibodies in vaccinated animals.

It is concluded that the BTV-2 & BTV-4 bivalent inactivated vaccine tested in the experiment safely and effectively induces protective immunity in cattle, making it suitable for BTV vaccination campaigns and particularly ideal for use in BTV-free countries where emergency ring vaccination may be necessary. Moreover, because it is an inactivated vaccine, its use does not prevent the possibility of using DIVA strategy based on the detection of antibodies versus BTV non structural proteins to distinguish vaccinated from infected animals.

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Figure 1: Titers of infectious BTV in blood samples of vaccinated and control groups following challenge infection with BTV-2 and BTV-4 field isolates.
Figure 2: Probability that calves vaccinated with an inactivated bivalent BTV-2 and BTV-4 vaccine are protected against homologous challenge. The P value is equal or bigger to the x-axis percentages. According to the trial, at least 83.8% (blue circle) of vaccinated animals with 95% confidence level would be protected (no viraemia) when challenged with BTV-2 or BTV-4 field isolates.
Figure 3: Probability distributions of the percentage of c-ELISA positive calves after vaccination with inactivated bivalent BTV-2 and BTV-4 vaccine
Figure 4: Average evolution of BTV-2 and BTV-4 serum neutralising titres in the vaccinated and unvaccinated groups.