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An inactivated vaccine for the control of bluetongue virus serotype 16 infection in sheep in Italy

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Abstract

Because no suitable products are at the moment available to safely control the spread of BTV-16 in Europe, an inactivated vaccine was produced from the reference field isolate of bluetongue virus serotype 16. One group of six sheep was vaccinated subcutaneously with the inactivated vaccine twice, on days 0 and 28, whereas a second group of 8 sheep was inoculated with saline solution and used as mock-vaccinated control animals. Seventy eight days after the first vaccination, all sheep were inoculated subcutaneously with a suspension containing 10^{6.3}TCID_{50} of a virulent reference BTV-16 isolate. Apart from a transient inflammatory reaction at the injection site, no adverse effects were reported following vaccination. All vaccinated animals developed high titres (7.3-9.3 Log_{2} (ED50%/50µl)) of virus-specific neutralizing antibodies and were resistant...
to challenge with BTV-16. Conversely, following challenge, control animals developed hyperthermia and long lasting high-titre viraemia.

Key words: Bluetongue virus serotype 16, inactivated vaccine, sheep, virus isolation, virus neutralisation

Introduction

Bluetongue (BT) is an infectious, non contagious disease of ruminants caused by an arthropod-borne virus belonging to the family Reoviridae, genus Orbivirus. The bluetongue virus includes twenty four distinct serotypes which are transmitted by haematophagous insects belonging to the family Ceratopogonidae, genus Culicoides (Mellor, 1992). Infection can cause severe clinical disease in sheep whereas, in other species including cattle and goats, the infection is mainly subclinical (Anderson et al., 1985). From 1998, BTV infection spread progressively all over the Mediterranean Basin, Balkan areas and more recently in Central Europe. Up till now, 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). In Italy, the first evidence of BT infection was recorded in Sardinia on August 2000 and, since then, numerous outbreaks have been described involving BTV serotypes 1, 2, 4, 9 and 16. BT was later reported in Sicily, Calabria, Basilicata, Puglia, Campania, Lazio, Tuscany, Abruzzo, Molise, Umbria, Marche, Emilia Romagna and Liguria (Calistri et al., 2004, OIE, 2006f).

In an attempt to reduce direct losses due to disease and indirect losses due to the trade embargo caused by virus circulation, the Italian government has, since February 2002, been carrying out a compulsory BT vaccination campaign of all domestic ruminants using modified-live virus vaccines (MLV) produced by Onderstepoort Biological Products (OBP), South Africa. Based on the serotype/s present in a given area, various MLV monovalent serotype formulations were used. During 2004, BTV-16 MLV was included
in the vaccination campaign and many vaccinated and unvaccinated animals became ill as a result of BTV-16 vaccine strain infection (Monaco et al., 2006). The incidents were attributed to inadequate attenuation of the vaccine strain employed and the use of monovalent MLV was discontinued a few months after the beginning of the campaign (Italian Ministry of Health, 2005). These events increased the concerns on safety issues regarding the MLV vaccine and prompted researchers to investigate new immune-stimulating products capable of preventing the dissemination of the virus. Inactivated vaccines for BTV serotypes 2 and 4 have recently been developed and successfully used during the 2005-2006 BTV vaccination campaigns.

Because no suitable products are available to safely control the spread of BTV-16, no strategies are currently applied for this serotype in Italy resulting in BTV-16 circulation. There is an urgent need for a safe and efficacious vaccine which could be used in the BTV vaccination campaign and that is capable of preventing the spread of BTV-16 in Italy. In this study a BTV-16 inactivated vaccine was developed from the BTV-16 reference serotype and its safety and efficacy was evaluated in sheep which is the species most susceptible to BTV.

**Materials and Methods**

**Cells and Virus**

Baby hamster kidney cells (BHK21, C13) were maintained in minimal essential medium containing Earle’s salts, L-glutamine and NAHCO₃ (EMEM) and supplemented with nystatin, colistin and neomycin sulphate, and 10% foetal calf serum (FCS). The BTV-16 reference strain, obtained from the Virology Department of the Onderstepoort Veterinary Institute, OIE Reference Laboratory for Bluetongue, Onderstepoort, South Africa, was used as seed virus.
Vaccine production and in process control

The seed virus was checked for the presence of contaminating bacteria, viruses, fungi and mycoplasmas (European Pharmacopoeia, 2001). Its identity was confirmed by serum neutralisation test. BHK$_{21}$ confluent monolayers in 175 cm$^2$ culture flasks were used for virus amplification. When 80% of the monolayers showed CPE, the viral suspension was harvested, centrifuged at 203 g for 10 min at 4°C, the supernatant collected, dispensed in 1ml aliquots and stored at –80°C. The virus stock was checked for purity, infectivity titre and identity. For the preparation of a batch of experimental vaccine, BTV-16 at the third passage level was used to infect BHK$_{21}$ monolayers in 850 cm$^2$ roller bottles. The viral suspension (VS) was aseptically collected and clarified by centrifugation at 9000 RPM (centrifuge Heraeus mod. Stratos 76000083) in continuous flow (rotor Heraeus Titanium 3049) and tested for purity, infectivity titre and protein concentration (BCATM Protein Assay Reagent Kit, Pierce, Rockford, IL). After passing the tests, the suspension was purified and ten fold concentrated through a 300kD molecular cut cassette membrane. During the purification-concentration phase, the VS was kept on ice. The final product was again checked for purity (European Pharmacopoeia, 2001), virus identity and infectivity titre. For inactivation, Tris-Hydroxymethyl-Aminomethane (Tris) 1M pH 8.0 was added to the VS and stirred for 10 min at room temperature. Beta-propiolactone diluted in PBS pH 7.4 was subsequently added to the VS to have a final dilution of the inactivant equal to 0.2% (v/v) (Parker et al., 1975). The suspension was stirred for 3 hours at 37 °C and then for 18 hours at 5°C. The inactivated VS was subsequently checked in vitro for inactivation as follows. Three 175 cm$^2$ tissue culture flasks containing confluent monolayers of BHK$_{21}$ cells were each inoculated with 10 ml of inactivated VS. Three passages were carried out. If no CPE was observed at the end of the third passage, the cells were scraped, fixed onto a slide and...
checked for the presence of BTV by using VP7 monoclonal BTV antibodies, conjugated with fluorescein-5-isothiocyanate (FITC) (Istituto Zooprofilattico Sperimentale dell’Abruzzo e Molise, Teramo, Italy (IZSAM)). Once the inactivation was verified, the VS and an equal volume of adjuvant MONTANIDE® ISA 206, (Seppic) each previously incubated 12 hours at 27°C, were mixed by magnetic stirring for 2 hours at room temperature and for 12 hours at 4°C. The vaccine was then tested for safety in accordance with European Union legislation (European Council, 1981; European Commission, 1991, 2001) and European Pharmacopoeia guidelines (2001).

Safety tests

Two guinea pigs weighing 350g were injected subcutaneously with 2 ml doses each and 5 mice weighing 18 g with 0.5 ml each. The animals were monitored 7 days for any clinical signs or local reaction at the injection site.

Efficacy test

Fourteen adult local cross bred sheep which tested negative for BTV- antibodies by competitive enzyme linked immunosorbent assay (c-ELISA) were selected and housed in insect-proof isolation facilities throughout these studies.

Six sheep were each inoculated subcutaneously with 2 ml of inactivated vaccine and revaccinated 28 days later with the same dose. Similarly on the same days, the remaining 8 sheep were inoculated subcutaneously with 2 ml of saline solution and used as mock-vaccinated control animals. Rectal temperatures and injection sites of all animals were monitored daily for 14 days. Blood samples were collected by jugular puncture from each animals once a week from the day of vaccination and for the following 8 weeks. Sera were separated, tested by c-ELISA and titrated for antibodies against BTV-2, BTV-4, BTV-9 and BTV-16 by virus neutralisation (VN).
Seventy eight days after the first vaccination, all 14 sheep were challenged by subcutaneous injection of 1 ml of viral suspension containing $10^{6.3}$ TCDI$_{50}$ of BTV serotype 16 reference isolate. Rectal temperature and clinical signs were recorded daily for 14 days. Ethylene-diaminetetra-acetic acid (EDTA) blood samples were taken from all animals three times a week for two months and tested for viraemia. An additional serum sample was taken 60 days after challenge to determine the presence of BTV specific antibodies using the c-ELISA and VN test.

The c-ELISA kit manufactured by IZSAM (Lelli et al., 2003) was used to test the sheep sera according to the method described by the manufacturer. The neutralising titre was determined as described by Savini et al. (2005). EDTA blood samples were screened for the presence of BTV and if virus was detected, the virus titre was determined for each isolated virus as described previously (Savini et al., 2004). Virus typing assay was also employed to verify the serotype (Savini et al., 2004).

Statistical analysis

Statistical significance of the tests was based on p values equal or lower than 0.05. Differences between the viraemic titres of the vaccinated and unvaccinated groups of sheep after challenge were analysed using the non parametric Mann-Whitney test for independent groups (Siegel and Castellan, 1988). For the daily rectal temperatures, the mean values at the day of challenge were calculated for the vaccinated and unvaccinated group and the statistical analysis was performed on the variations of the daily temperature values from the group mean value at time 0. Statistical differences between the variations were also determined using the Mann-Whitney test.

Results

Control of materials
Materials used for the vaccine production fulfilled all the EU and European Pharmacopoeia guidelines; controls for culture purity were found to be satisfactory. The virus proved to be BTV-16 with a titre of $10^{7.3}$ TCID/ml.

Vaccine production and in process control

Forty eight hours after infection, the BHK$_{21}$ monolayer in the 175 cm$^2$ roller bottles showed 100% CPE. The viral suspension had a titre of $10^{7.6}$ TCID$_{50}$/ml and a protein concentration equal to 1.631 mg/ml. Viral identity, sterility and absence of viral contaminants were confirmed. Following the purification process, the BTV titre of the VS was $10^{7.7}$ TCID$_{50}$/ml while the protein concentration was 2.577 mg/ml. The final product was also found to be free of contaminants. The *in vitro* inactivation test was satisfactory and no fever or adverse effects either in mice or guinea pigs were observed after inoculation.

Vaccination with BTV-16 inactivated vaccine

Prior to vaccination, all sheep were serologically negative by both c-ELISA and micro-neutralisation assays and controls remained negative until challenge. Following immunisation, rectal temperatures of the animals in the trial remained within the normal range and none of them developed clinical signs. However, both injections produced an inflammatory reaction at the inoculation site which often developed in a small nodule fully resolving over a period of a few weeks. Seroconversion was observed in one sheep as early as day 7 after vaccination (pv) and only two animals showed neutralising titres on day 28 pv. Following the booster vaccination, all animals seroconverted with neutralising antibody titres peaking on day 47 pv (mean VN titre $8.4 \text{ Log}_2$ (ED50%/50µl)) and dropping to 5.64 just before challenge (Fig. 1).

Protection of sheep immunized with BTV-16 inactivated vaccine
After challenge, vaccinated sheep did not show clinical signs of BT disease and their temperature remained normal. Conversely, seven of the 8 unvaccinated control animals developed 2 to 6 days of hyperthermia within the first week of challenge. The increase was significantly higher (p < 0.05) on days 5, 6 and 7 post challenge (pc) as compared to the animals in the vaccinated group. Average evolution of rectal temperatures of the groups are presented in Fig. 2. An unvaccinated animal also showed nasal discharge on days 4 and 5 pc. BTV-16 virus was isolated in the blood of all unvaccinated control animals from day 3 to day 35 pc, with peak titres observed on day 5 pc (Fig. 3). In none of the vaccinated animals was BTV ever detected. They were significantly protected from viraemia up to day 21 pc as compared to control animals. No animal developed BTV-2, 4 and 9 antibody titres. On day 60 pc, the BTV-16 challenged group showed high homologous neutralising antibody titres.

Discussion

As the only product commercially available, BTV-16 MLV vaccine produced by Onderstepoort Biological Products, South Africa was used in the 2004 BT Italian vaccination campaign with the purpose of controlling the BTV-16 spreading. According to the manufacturer, it was attenuated by adapting in vitro a BTV-16 Pakistan isolate through serial passages in embryonating chicken eggs and mammal cells. The attenuation process meant to select viruses with a reduced capacity of growing in vivo and, consequently, of causing disease. However, after the use of BTV-16 vaccine, high viraemic titres and clinical signs were observed in vaccinated animals and, few months later, BTV-16 vaccine strain was isolated from animals in areas previously found free of this serotype. Innovative sophisticated and high tech vaccines have been developed to control BTV infection in ruminants, including virus-like particles (VLPs) produced from recombinant baculoviruses, and recombinant vector vaccines (Murray and Eaton, 1996;
Roy, 2004). Even though 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., 2006). Similarly, recombinant vector vaccines expressing both VP2 and VP5 although revealing some potential in terms of safety and protection, still require further trials before being ready for the field use (Lobato et al., 1997). Concerning the inactivated virus vaccines, during the last few decades several attempts have been made to develop an efficacious inactivated vaccine for bluetongue. The antigenic stimulus of this type of vaccine depends exclusively on the dose of virus, resulting significantly higher than that of MLV. As a consequence, two doses, in the presence of adjuvant, are often required for inactivated vaccines. The need of higher and repeated doses of virus administered considerably increases the cost of production and vaccination. However, because of the potential risk associated with MLV and the lack of certainty about commercial availability of recombinant vaccines, the value of inactivated vaccines have been recently re-evaluated. New products for BTV-2 and BTV-4 have been developed, commercialised and successfully employed in the 2005-2006 Italian BTV vaccination campaigns. However, no BTV-16 vaccines are presently used in the Italian vaccination campaign. To face the current epidemiological scenario in Italy due to BTV-16 viral circulation, a BTV-16 inactivated vaccine with a reference serotype was produced according to the requirements of the European Pharmacopoeia. When administered in adult local cross breed sheep, the vaccine resulted in complete prevention of clinical signs and detectable viraemia after challenge using virulent homologous virus. A single dose induced detectable levels of neutralising antibodies in two animals only. However, antibody titres were observed in all animals a week following the booster injection. Because the presence of type-specific neutralising antibodies is indicative of protective
immunity to BT (Verwoerd and Erasmus, 1994), it could be inferred that two inoculations of the BTV-16 inactivated vaccine are required to protect all animals against challenge.

Following immunisation, all vaccinated animals developed a local inflammatory reaction consisting of a transient small nodule at the site of injection. To increase the immune-stimulation of the inactivated virus, the Montanide ISA 206 was used as adjuvant for the BTV-16 inactivated vaccine. Montanide is an emulsion based on manide-oleate compound that has been shown to produce antibody levels equivalent to Freund's complete adjuvant (Martinez et al., 1996). Several studies indicated the consistent immune-enhancing effect of Montanide (Cook et al., 1990). This effect is mediated by the creation of a depot from where the antigen is slowly released, ensuring continuous stimulation of the immune system and the induction of a local inflammatory reaction consisting mainly of oil-ingesting macrophages and lymphocytes (Altman and Dixon, 1989). However, as observed in this trial, Montanide, being a water-in-oil-in-water emulsion, produces nodules much smaller and with a shorter period of resolution when compared to other oil adjuvants (Barnett et al., 1996). Furthermore the Montanide offers the advantage of being less viscose, easy to administer and stable, making it an ideal adjuvant candidate for virus vaccines (Barnett et al., 1996).

In conclusion, although further studies involving larger number of animals and different species will be required, this investigation has shown that a two-dose schedule Montanide-adjuvanted BTV-16 inactivated vaccine appears to safely and effectively induce protective immunity in sheep. It possesses the attributes which would make it suitable for a BTV vaccination campaign, is preferable to the existing MLV BTV-16 vaccines and ideal for use in BTV-free countries where emergency ring vaccination may be necessary. However, it is important to note that an effective vaccine although crucial is only one factor in a multi-faceted control programme. To achieve complete control of
BTV-16 field and vaccine strains, the vaccination should be combined with extensive surveillance and an effective monitoring programme.

References


laws of the Member States relating to analytical, pharmaco-toxicological and clinical
standards and protocols in respect of the testing of veterinary medicinal products. Off. J.,


provvedimenti ed impiego del vaccino sierotipo 16 nella IV campagna di vaccinazione

Production of a competitive ELISA kit for the serological diagnosis of bluetongue

responses and protective immunity to recombinant vaccinia expressed-expressed

Martinez, D., Perez, P.M., Sheikboudou, C., Debus, A., Bensaid, A., 1996. Comparative
efficacy of Freunds and Montanide ISA 50 adjuvants for the immunization of goats

Osburn, B.I. (Eds), Bluetongue, African Horse Sickness and Related Orbiviruses. CRC


Monaco, F., Cammà, C., Serini, S., Savini, G., 2006. Differentiation between field and


Figure 1: Neutralising antibody titres (±SD) in sheep after vaccination with bluetongue virus serotype 16 inactivated vaccine (first dose on day 0, second dose on day 28)
Figure 2: Rectal temperatures (±SD) in sheep vaccinated with bluetongue virus serotype 16 inactivated vaccine and challenged with the homologous reference field isolate.
Figure 3: Virus titres (±SD) in sheep vaccinated with bluetongue virus serotype 16 inactivated vaccine after challenge with the homologous reference field isolate.