Study of the safety and efficacy of a recombinant vaccine for bluetongue virus serotype 2

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Summary

A total of 7 cows, 10 sheep and 10 goats were vaccinated subcutaneously with 5 ml of a recombinant vaccine consisting of synthetic virions containing the four principal proteins (VP2, VP3, VP5 and VP7) of bluetongue virus serotype 2 (BTV-2). The same number of animals and species were vaccinated with 2.5 ml (the normal vaccination dose) and 2 cows, 2 sheep and 2 goats were inoculated with a placebo and the adjuvant added to the vaccine. Animals vaccinated with the normal dose received a booster 14 days after the first injection and 8 sheep a third vaccination 4 months after the second inoculation. One month after the third vaccination, the 8 sheep and another 4 that had never come into contact with the virus were challenged with 1 ml of $10^{5.8}$ $TCID_{50}$ of a BTV-2 Italian field isolate. All animals showed competitive enzyme-linked immunosorbent assay (c-ELISA) antibodies starting 14 days following the first vaccination. Conversely, no animal demonstrated neutralising antibodies to BTV-2 after vaccination. Fever (>40°C) was observed in 6 vaccinated animals and 2 controls between 8 and 13 days post challenge. The virus was isolated from all animals from the 7th day post challenge. There was no significant difference in the blood chemical parameters tested and no significant interaction was found in the trial group.

Keywords

Bluetongue, Cattle, Goats, Italy, Sheep, Vaccine.

Introduction

The bluetongue (BT) virus (BTV) has a significant financial impact on the livestock industry, due not only to the severity of the clinical signs it can cause in sheep (8), but especially to the ban on livestock movements in infected or suspect zones, implemented immediately upon detection of infection.

When the BT epidemic affected Italy and other European countries at the end of the last century (1), the European Union decided to use vaccination to increase the immune population and contain the disease. The vaccine used was the modified live vaccine produced in South Africa by Onderstepoort Biological Products. The vaccine which had been in use in South Africa for over forty years was the only commercial product available at that time. There was immediate doubt over the use of a live vaccine strain, even if modified, due both to the possibility of side-effects in the vaccinated animal and to its potential to replicate in the organism, attaining titres capable of infecting the vector and then spreading in the environment. Consequently, there was clearly a need for a different immunising product that would avoid these problems.

A recombinant BT vaccine was developed and produced by Polly Roy, with promising results

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in an initial experimental trial in South Africa soon after its production (9, 10). The essential requisite for a vaccine is its ability to provoke a lasting immune response that protects the animal against contact with homologous field virulent strains. The aim of this study was to test this recombinant vaccine in cattle, sheep and goats in Italy, to assess its safety and evaluate the efficacy of the immune response by challenging vaccinated animals with homologous virulent virus. This enabled an evaluation of the true ability of the vaccine to prevent clinical onset of the disease and reduce the intensity and duration of viraemia following infection with virulent strains.

Materials and methods

Safety test

Vaccine preparation

The recombinant vaccine produced by Polly Roy was used to vaccinate the animals. The vaccine consisted of synthetic virions containing the four principal proteins (VP2, VP3, VP5 and VP7) of the BTV serotype 2 (BTV-2) (2, 3). The proteins were synthesised using baculovirus cell lines.

VP2 originated from the AcBTV2-2Sar strain, with a viral titre of 5×10^7 plaque-forming units (pfu)/ml, VP5 from AcBTV2-5 with a viral titre of 1.8×10^8 pfu/ml. VP3 and VP7 originated from AcVC3/7Dual, with a viral titre of 5×10^7 pfu/ml.

In vivo test

The vaccine was prepared, handled and administered by staff from Roy's research team. All the animals in the trial were treated in accordance with national and European Union animal welfare regulations. A total of 60 BTV-seronegative animals were used for the trial, namely:

- 16 crossbred cows
- 22 Sardinian sheep
- 22 crossbred goats.

Of these, 27 (7 cows, 10 sheep and 10 goats) were vaccinated subcutaneously with 5 ml of recombinant vaccine, equivalent to twice the normal inoculation dose; 27 (7 cows, 10 sheep and 10 goats) with 2.5 ml and the remaining animals (2 cows, 2 sheep and 2 goats) with placebo added to the vaccine adjuvant. The site of injection was the lateral face of the neck for cows and the axillary area for sheep and goats. In accordance with the authorisation of the Ministry of Health, the animals were kept at the Sardinian *Istituto Zooprofilattico Sperimentale* farm in Sassari in a *Culicoides* spp.-free area. The rectal temperature of all animals was recorded for two weeks and a clinical examination conducted to reveal any symptoms or particular reactions.

Animals vaccinated with the normal dose received a booster 14 days after the first vaccination and 8 sheep were inoculated for the third time four months after the second vaccination.

Efficacy test

Serological tests

A serum sample was taken from each animal prior to vaccination and 14, 33, 58 and 110 days after inoculation. Serum samples were examined for BTV antibodies using the competitive enzyme-linked immunosorbent assay (c-ELISA) and serum neutralisation test (SN).

Competitive enzyme-linked immunosorbent assay

The c-ELISA was conducted using a kit developed by the *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise* (IZS A&M) (6). The purified antigen was diluted in pH 9.6 carbonate/bicarbonate buffer solution and dispensed into microplate wells, where it was left to adsorb overnight at 4°C. After washing, the sera and monoclonal antibody, labelled with peroxidase, were added. The antigen-antibody reaction was revealed by the addition of substrate. Samples presenting an optical density of less than 35% of that found in the monoclonal control wells were considered as positive.

Serum neutralisation test

The SN (12) revealed the serotype and antibody titre. In a 96-well plate, serum samples were diluted from an initial dilution of 1:10 by doubling and placed in contact with 100 $TCID_{50}$ of previously titrated BTV-2 virus.

After incubation for 1 h at 37°C with 5% CO_2 to enable viral neutralisation, 3×10^5/ml of Vero (African green monkey kidney) cells, suspended in minimum essentials medium (MEM) (Eurobio, France) containing the following antibiotics: penicillin 100 IU/ml) (Sigma, Germany), streptomycin 100 µg/ml (Sigma), gentamicin 5 µg/ml (Sigma), nystatin 50 IU/ml (Sigma) and 10% foetal calf serum (FCS) (Sigma), were added to each well. After 3 days, the cytopathic effect (CPE) in the wells was evaluated and the antibody titre was defined as the highest serum dilution able to inhibit at least 50% of the virus' CPE. The positive and negative reference sera, cell and virus controls were included in each plate. Positive and negative control sera were kindly supplied by Onderstepoort Veterinary Institute (OVI) in South Africa, the reference laboratory of the World Organisation for Animal Health (OIE: *Office International des Épizooties*) for BT.

Challenge test

Preparation of strain used for challenge test

A wild BTV strain isolated from the spleen of a sheep which died due to BTV infection during the 2000 epidemic in Sardinia (12) was used for the challenge test. The inoculum was prepared according to the method described by Savini *et al.* (11): the spleen was fragmented using sterile quartz powder, suspended in a lactose peptone buffer containing antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml and nystatin 100 µg/ml 50 IU/ml), sonicated and centrifuged. After centrifugation, 100 µl of the suspension was inoculated intravenously into embryonated chicken eggs and then passaged on a confluent monolayer of Vero cells. At maximum CPE, the material was collected, divided into aliquots and stored at –80°C. The strain was then titrated and typed by using the virus neutralisation test.

Virus titre

Eight ten-fold dilutions (from 10^{-1} to 10^{-8}) were tested for each positive sample. Six replicates for each dilution were dispensed into a 96 flat bottomed microtitre plate wells; Vero cells at a concentration of approx. 10^4 cells/ml in MEM with antibiotics and 10% FCS were added to each well as the detection system. The test was read after six days' incubation at 37°C with 5% CO_2. The content, calculated using the Reed and Muench formula, is defined as the highest virus dilution producing a CPE in 50% of the inoculated Vero cells ($TCID_{50}$).

Serological typing

Starting from 1:10, four ten-fold viral dilutions were placed in contact with each of 24 serum-specific antisera diluted 1:20, as described in the OIE *Manual of diagnostic tests and vaccines for terrestrial animals* (14). Guinea-pig-specific antisera, kindly provided by the OVI, were used for this test. The viral serotype was identified on the basis of the specific antiserum able to neutralise viral growth on a cell monolayer, detectable through 50% inhibition of the CPE.

Animal inoculation

One month after the third vaccination (5 months after the first), the 8 sheep plus another 4 that had never come into contact with the virus, were infected with 1 ml containing $10^{5.8}$ $TCID_{50}$ of BTV-2 Italian field isolate. After challenge, the following were performed: daily clinical examination, temperature readings, blood samples taken with and without anticoagulants for determination of blood virus levels and biochemical, physical and complete blood count (CBC) tests. A blood sample was taken from each animal with and without anticoagulants before and 7 days after infection (PI). From the seventh day PI, samples were taken twice a week for the entire duration of the trial.

Biochemical and physical tests and complete blood count

The following parameters were measured for the serum: alkaline phosphatase, amylase, uric acid, total bilirubin, calcium, creatine, gamma glutamyl transferase, glutamic oxalacetic transferase, glutamic pyruvic transferase, lactic dehydrogenase, phosphor, total proteins, triglycerides, urea. CBC was conducted on blood and ethylenediamine tetra-acetic acid (EDTA) with the following determinations: white blood cells, red blood cells, haemoglobin, total haemoglobin, mean

corpuscular volume, mean haemoglobin content, mean haemoglobin concentration, platelets, platelet volume, lymphocytes, monocytes, red blood cells, eosinophils, basophils. The results of the biochemical analysis and CBC were analysed using the mixed procedure of the Statistical Analysis Software (SAS/STAT®), in which days and groups were considered as fixed factors, the animal in a random group and the first control as covariate (in order to take account of the initial difference in variable values among the three groups); a day*group interaction factor was also introduced, to establish if quantities varied in a different way in the two groups as time passed.

Viral isolation

To detect BTV, blood samples with anticoagulant were examined using the technique described by Savini *et al.* (11): 1 ml of each EDTA blood sample was centrifuged at 812 g for 10 min to separate the plasma from the red blood cells; the cell sediment was washed twice by suspension in phosphate buffer (PBS) with antibiotics and centrifugation at 812 g for 10 min. The pellet was then resuspended in 9 ml of MEM with antibiotics. The suspensions were sonicated to release the virus from the red blood cells, centrifuged and then the supernatant inoculated intravenously in embryonated chicken eggs on the 12th day of incubation.

Positive samples caused an intense, with widespread and often fatal haemorrhaging of embryos. Dead embryos were examined between the second and seventh day PI: the heart and brain were homogenised in PBS and antibiotics and centrifuged at 1 827 g for 15 min; 0.8 ml of the supernatant was used to infect a confluent monolayer of Vero cells in 25 cm^2 flasks. After one hour of contact at 35°C, approximately 8 ml of MEM with antibiotics and 3% FCS was added to each flask, which were then placed in an incubator at 37°C with 5% CO_2. Cells were checked daily for the appearance of the typical BTV CPE: presence of highly granulated round cells, initially limited to a few foci but tending to spread over the entire monolayer in a short time. In the absence of a clear CPE after incubation for one week, cells were detached mechanically, harvested with the culture medium, centrifuged at 812 g for 10 min and the supernatant was used to infect a new monolayer. The infected flasks were checked daily for the appearance of any CPE or signs of cell toxicity. After the second passage or following the appearance of a CPE, each sample was assayed with a direct immunofluorescence test: the monolayer cells were harvested, washed twice by dilution in PBS and centrifugation at 812 g for 10 min, placed onto a multi-well glass slide, adding positive and negative controls, left to dry at room temperature and fixed in acetone at –20°C for 20 min. The virus was identified using a monoclonal antibody produced by ISZ A&M (6). Positive samples showed the presence of fluorescent intracytoplasmatic granules, a typical sign of BTV. All positive samples were titrated and typed using the methods described above.

Results

Safety test

Five days after vaccination, local reactions (oedema and redness) were seen at the injection site. These reactions were seen in all animals, whether vaccinated or not. No clinical symptoms attributable to BT were seen in either vaccinated or control animals.

Efficacy test

Serological tests

On day 14 PI, all animals presented an antibody response to c-ELISA. In contrast, no BTV-2 neutralising antibodies were seen in any animal following vaccination.

Challenge test

Between 8 and 13 days PI, 6 vaccinated (75.5%) and 2 control (50%) animals showed high temperatures (>40°C) (Fig. 1).

Bluetongue virus was recovered from all animals from the 7th day (1st sample) until the 16th day PI. Figure 2 shows the blood virus titres in the vaccinated and control groups after infection with the field strain of BTV-2.

There was no significant difference in the blood chemical parameters tested, and no

significant interaction was found in the trial groups.

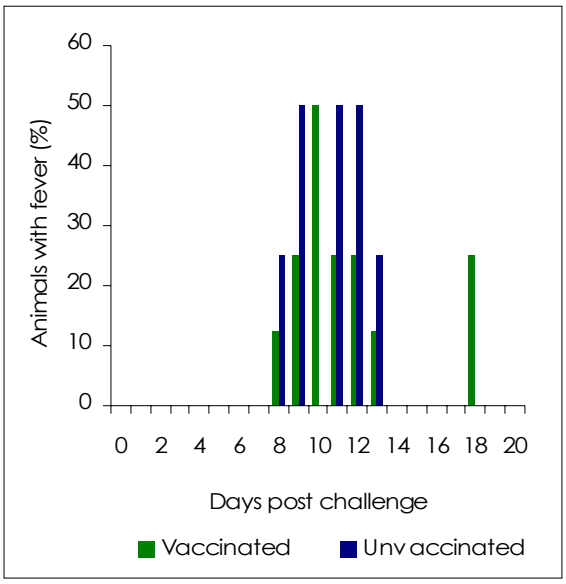

Figure 1
Fever reaction in sheep vaccinated with recombinant vaccine after challenge with a virulent strain of bluetongue serotype 2 virus

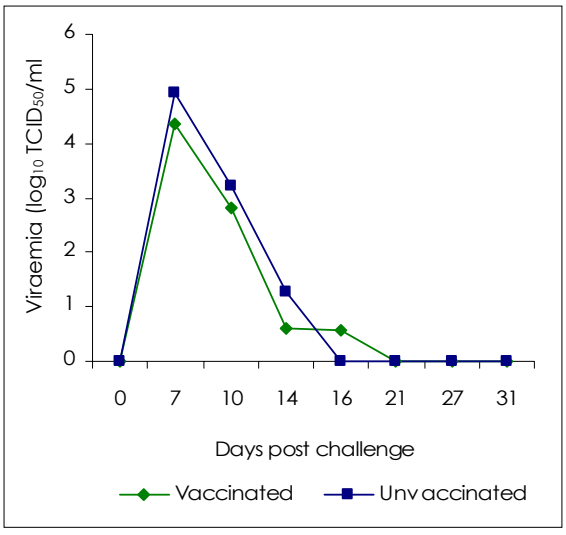

Figure 2
Viral titres in sheep vaccinated with a recombinant vaccine and challenged with a virulent strain of bluetongue virus serotype 2

Discussion

Recent developments in biotechnology have enabled the synthesis of vaccines consisting of synthetically produced virions, the main feature of which is their morphological similarity to the original virus particle while lacking the entire genetic structure necessary for the virus to replicate (2, 9). The product used in this study consists of synthetic virions containing the four principal BTV-2 viral proteins: VP2, VP5, VP7 and VP3 (3). When tested in South Africa, the same vaccine type was able to stimulate a neutralising antibody reaction in sheep, preventing the clinical form and impeding viraemia in vaccinated animals subsequently infected with the wild virus (9, 10). In this trial, the vaccine not only caused a mild reaction at the site of injection, probably attributable to the adjuvant, but was unable to inhibit viraemia and prevent the clinical onset after challenge with the homologous field strain.

The negative outcome of the trial was partly expected, following the inadequate neutralising immune response observed in all animals vaccinated twice, to the extent that a third vaccination (on Roy's instructions) – completely impractical in any vaccine campaign – was necessary before conducting the challenge test. 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 (4, 5). The protective role of the neutralising antibodies against BTV and the lack of protective action of c-ELISA antibodies have been described a number of times (7, 13). It was therefore no surprise in this study to observe raised temperatures and viraemia in c-ELISA-positive sheep after infection with the field strain. Consequently, it can reasonably be postulated that the inadequate neutralising immune response observed in vaccinated animals is likely to be due to ineffective antigen stimulation by the VP2 (and VP5) in the synthetic virion.

In line with the clinical and virological studies, blood chemical tests also failed to reveal any significant difference between the vaccinated and unvaccinated group.

It can therefore be concluded that the recombinant vaccine synthesised to protect animals from BTV-2 infection was not completely harmless, was unable to protect

animals from the clinical form and did not reduce the length or severity of viral titres in vaccinated animals after infection with the homologous virulent strain.

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