Determination of the minimum protective dose for bluetongue virus serotype 2 and 8 vaccines in sheep

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Recent outbreaks of bluetongue virus (BTV) serotypes 2 and 8 in many European countries provided an opportunity to investigate the possibility of improving the safety of the modified live vaccines administered mainly in South Africa. Modified live vaccines (MLV) released at a titre of 5 x 10^4 PFU/mL, raised concerns and prompted the need to determine the minimum titre which will still be protective and also safe. The BTVserotypes 2 and 8 vaccines were produced at the following titres: 10^2 PFU/mL, 10^3 PFU/mL and 10^4 PFU/mL, and were injected into 24 sheep which were then monitored. Blood was collected on days 0, 3, 6, 9, 12, 15, 18, 21, 25, 28 and 4 months post vaccination, for seroconversion and viraemia studies. These sheep were later challenged at 4 months post vaccination using BTV infected cell culture material, they were then observed and bled and again tested for viraemia. There was no viraemia post vaccination, however, a febrile reaction did occur and seroconversion was demonstrated at low titres for both BTV 2 and 8. Although viraemia was demonstrated post challenge, sheep vaccinated with the low titre BTV 2 vaccine showed more than a 90% protection index at a lower titre of 10^3 PFU/mL, compared with BTV 8 that showed a protection index above 90% at all the titres used It is recommended that for BTV 2 vaccine, sheep should be vaccinated at a titre of 10^3 PFU/mL and at a titre of 10^2 PFU/mL with BTV 8 vaccine.

Introduction

Bluetongue (BT) is a non-contagious, insect-transmitted disease that infects certain domestic and wild ruminants that is caused by the bluetongue virus (BTV)[1]. Bluetongue viruses share a common group-specific antigen but are distinguishable on the basis of their serotype-specific antigen by using an *in vitro* virus serum neutralising assay (SNT). In the late 1960's Howell applied this test in order to antigenically group 22 strains of bluetongue virus into 12 serotypes.[2] Later other serotypes were identified and, to date, 26 known serotypes of BTV have been described worldwide.[3,4] Twenty two of these serotypes are known to occur in South Africa (SA)[5] of which 15 are considered to be pathogenic to sheep.[6,7] This disease is endemic in SA and Africa and since 1999 BTV serotypes 2, 4, 6, 8, 9 and 16 have also been prevalent in Europe.[3,8,9]

Bluetongue virus can infect a wide host range, and because of this the different BTV serotypes and the role played by *Culicoides* spp. as vector, the eradication of this disease in endemic areas such as in SA would be difficult to achieve.[6] In countries endemically infected with BTV, vaccination has proved the most effective and practical method implemented to reduce the spread of this disease.[5,6,7,10] The economic impact of using other methods of control, such as slaughter, has led most countries to consider vaccination as the best tool available to control the spread of the disease.

Different types of vaccines have been developed to prevent BTV infection of ruminants. These include: modified live vaccines (MLV), inactivated whole (killed) virus preparations[11,12] and virus like particles (VLPs). The latter allows the co-expression of three, four or five BTV genes from a single recombinant vector like the baculovirus multigene expression vector system. Other vector systems include vaccinia-, capripox- or canarypox viruses.[13,14,15] Only MLV and inactivated vaccines are commercially available and have been administered in recent years.

An attenuated BTV blood vaccine, developed after serial passage in sheep, has been administered for almost 40 years.[1,7] Subsequently, other serotypes of BTV were identified in outbreaks and, thus, a quadrivalent vaccine was developed in the early 1950's.[16,17] Further attenuation of the vaccine strains was achieved through serial passage of the BTV in embryonated chicken eggs and tissue culture aided by plaque purification of the virus.[6,18] Modified live attenuated vaccines are easy and economical to produce, administered once and have been administered successfully for 50 years.[6,14] These vaccines replicate in the host without causing significant adverse clinical effects, and provide protection against challenge with virulent virus of the same serotype. [8,18,19,20,21,22]

They are able to elicit both cellular and humoral immune responses, which are able to protect sheep against BT.[23,24]

Millions of doses of MLV are issued annually in South Africa to vaccinate sheep. Since 2000 Onderstepoort Biological Products (OBP) has also sold several million doses of monovalent BTV vaccines to different European countries.[25,26,27] Currently all the serotypes in the cell culture adapted vaccine are released at a titre of 5 x 10^4 PFU/mL. However, concerns were regularly raised about the long duration of viraemia that may sometimes occur after vaccination, especially in merino sheep.[28,29] This prompted the need to investigate the possibility of producing low titre BTV 2 and 8 vaccines which would still be protective, more economical and without severe side effects.

Materials and methods

Animals

The experiment was conducted at OBP Ltd in SA (25°29'S, 28°.11 E, 1219 m a.s.1.). A total of 28 merino sheep that tested negative for antibodies to BTV (Indirect ELISA, Agriculture Research Council, Onderstepoort Research Institute [ARC-OVI]) were selected and ranged in age from 9–12 months. The sheep were kept in an insect free isolation stable, were fed daily according to a random schedule and had continuous access to clean water.

Vaccine development

Vaccines for BTV serotypes 2 and 8 were developed from field isolates obtained in SA and isolated at the ARC-OVI.

Production of the vaccines was implemented according to the current Standard operating procedure (SOP) of OBP (proprietary information). Prior to production, the identity of the working seed virus was firstly confirmed at the Biochemistry Section of the ARC-OVI using the SNT and a reverse transcriptase polymerase chain reaction (RT-PCR). Viruses were grown on baby hamster kidney cells and titres post freeze drying were 1.17 x10^5 PFU/mL for BTV 2 and 1.17 x 10^6 PFU/mL for BTV 8. Prior to injecting sheep, the freeze dried material was reconstituted with sterile water and diluted further to obtain 10^2, 10^3 and 10^4 PFU/mL titres for both serotypes.

Inoculation of sheep

Vaccines were injected into 12 sheep per serotype and subdivided to 4 sheep per titre. A total of 24 sheep were vaccinated using 1 mL of vaccine and injected subcutaneously on the inner side of the thigh. A positive control, which was not vaccinated but was challenged and a negative control, which was not vaccinated and not challenged for each BTV serotype, was included. The sheep were clinically monitored post vaccination, and temperature readings were recorded for 14 days. The sheep were post bled using 10 mL vacu-tubes containing lithium heparin as an anticoagulant and 10 mL vacu-tube for serum, without an anticoagulant. Blood was collected on days 0, 3, 6, 9, 12, 15, 18, 21, 25 and 4 weeks and also 4 months post vaccination. Heparin blood was tested for viraemia and the serum for neutralising antibodies indicating seroconversion.

Four months post vaccination, both heparin blood and serum were collected from the controls and vaccinated sheep, and, following this, the sheep were challenged (except the negative control sheep) with the homologous BTV serotype. They were then clinically monitored, temperature reactions were recorded for 14 days post challenge and were scored using the method of Huismans, Van der Walt, Cloete and Erasmus (1987).[30] Blood was collected in heparin tubes on days 0, 3, 6, 9, 12, 15, 18, 21, 25 and 28 post challenge.

Challenge material preparation

The bluetongue virus serotype 2 challenge material was obtained from the ARC-OVI collection whilst serotype 8 was an isolate, BTV 8(G) 5/10A 2006/01KC 3BHK, that was obtained from the Institute for Animal Health (IAH) Pirbright, UK. The challenge material was prepared from cell cultures in which Vero cells were infected with infected sheep blood, observed for cytopathic effect (CPE) and the titre determined by viral plague assay and adjusted to a titre of 1x10^6 PFU/mL.

Viraemia testing

Every 3rd day post vaccination and post challenge, 5 mL – 10 mL heparin blood was collected for virus isolation. The method applied was according to the SOP of OBP, as described in the OIE Terrestrial Manual 2008[31] and also as described by Clavijo, Hecket, Dulac and Afshar (2000).[32] Blood was considered negative for viraemia when no CPE was evident, even after the blind passage of the supernatant. Samples that showed CPE were further titrated on confluent Vero cells in 6–well plates to confirm results.

Serological assays

Pre-vaccination serum was collected and tested for the presence of antibodies to BTV. Post vaccination serum was collected and tested for seroconversion and to quantify the level of antibodies post vaccination. Serum samples were collected on days 0, 3, 6, 9, 12, 15, 18, 21, 28 and four months post vaccination and tested using a commercially available competitive ELISA (VMRD, Inc.). Serum samples collected 4 weeks and also 4 months post vaccination were tested by introducing the SNT on Vero cells in 96–well plates.[31]

Quality control and percentage protection index determination

Tests were conducted to confirm the titres of the freeze dried vaccine, the safety in laboratory animals and for sterility to confirm the absence of other organisms. All were performed as described in the OIE Terrestrial Manual.[31,33] The determination of the clinical reaction index (CRI) and the percentage protection index were calculated using the

method described by Huismans, Van der Walt, Cloete and Erasmus (1987).[30]

Ethical considerations

Vaccinated sheep showed a rise in temperature but no clinical disease. Some of the challenged sheep showed a rise in temperature but no clinical disease. One of the positive control sheep (challenged but not vaccinated) died 8 dpi.

Results

Temperature reactions post vaccination

Bluetongue virus serotype 2

Sheep vaccinated with the BTV 2 vaccine titre of 10^2 PFU/mL had pyrexia from day 7 to 9. All sheep vaccinated with BTV 2 vaccine titre of 10^3 PFU/mL had pyrexia from day 5 to 7 and also on days 11 to 13. Two sheep had high temperature reactions of 41 °C and 40.7 °C, respectively. Three sheep vaccinated with the high titre BTV 2 vaccine (10^4 PFU/mL) had pyrexia from day 6 to 8 on different days.

Bluetongue virus serotype 8

All sheep vaccinated with the low titre BTV 8 (10^2 PFU/mL) vaccine had pyrexia on different days (5, 7 and 8). Additionally, those vaccinated with 10^3 PFU/mL had pyrexia on different days from day 5 to 8, however, one sheep reacted only on day 6. The same reaction was seen in sheep vaccinated with high titre vaccine pyrexia, on days 6, 7 and 9. The temperature of the negative control was normal.

Viraemia post vaccination

No viraemia was detected in any of the sheep post vaccination.

Temperature reactions post challenge

Bluetongue virus serotype 2

Three sheep vaccinated with the BTV 2 vaccine titre of 10^2 PFU/mL, and challenged with the BTV 2 cell culture material, had pyrexia from day 1 and again on day 3 to 4 post challenge. Only one sheep, vaccinated with BTV 2 vaccine (10^3 PFU/mL), had pyrexia from day 2 to 6 post challenge. There was no pyrexia post challenge in sheep vaccinated with the high titer vaccine (10^4 PFU/mL), except the positive control sheep which had pyrexia from day 3 to 5 before it died on day 8.

Bluetongue virus serotype 8

Two sheep vaccinated with low titre BTV 8 vaccines (10^2 PFU/mL and 10^2 PFU/mL) had pyrexia on day 1 and 4 after challenge. The sheep vaccinated with the high titre vaccine (10^4 PFU/mL) did not demonstrate any abnormal reactions, except the positive control sheep that had pyrexia from day 6–10.

Viraemia post challenge

Viraemia was detected in two sheep post challenge, at 4 months post vaccination with both low titre BTV 2 vaccines (10^2 PFU/mL and 10^3 PFU/mL). Only one sheep showed viraemia, of those vaccinated with the high titre vaccine (10^4 PFU/mL). Viraemia was not detected in sheep challenged with BTV 8 in all three vaccinated groups.

Seroconversion

Bluetongue virus serotype 2

The sheep vaccinated with the low titre vaccine (10^2 PFU/mL and 10^3 PFU/mL) were seropositive on ELISA by day 21 post vaccination, and showed varied neutralisation titres of 1:16 to 1:64 within 4 weeks post vaccination. Similar titres were observed at 4 months post vaccination. Those vaccinated with high titre vaccine also had neutralising titres within 4 weeks and also 4 months post vaccination (Table 1).

Bluetongue virus serotype 8

Two sheep seroconverted on day 9 post vaccination, whilst one sheep did not demonstrate detectable antibodies, even 4 weeks post vaccination. However, all sheep had neutralising antibody titres above 1:16 at 4 months post vaccination (Table 2).

TABLE 1: Seroconversion of sheep vaccinated with different titres of bluetongue virus serotype 2 vaccines.

Animal number	Titres (PFU/mL)	ELISA results (days post vaccination)					SNT titres	
		0	3	9	15	21	4 weeks	4 months
30	10^2	-	-	-	-	-	< 1:4	0.08611
149	10^2	-	-	-	+	+	0.08611	0.08611
126	10^2	-	-	-	-	+	01:16	01:04
122	10^2	-	-	-	+	+	01:04	01:04
17	10^3	-	-		+	+	01:32	01:32
125	10^3	-	-	-	+	+	0.08611	0.08611
38	10^3	-	-		-	+	01:32	01:16
140	10^3	-	-	-	+	+	01:32	01:16
104	10^4	-	-	+	-	+	01:16	01:04
57	10^4	-	-	-	-	+	01:04	01:16
56	10^4	-	-	+	+	+	01:04	01:16
52	10^4	-	-	-	+	+	01:04	01:32
132	Negative control	-	-	-	-	-	< 1:4	< 1:4

SNT, serum neutralisation test.
-, negative ; +, positive.

TABLE 2: Seroconversion of sheep vaccinated with different titres of bluetongue virus serotype 8 vaccines.

Animal number	Titres (PFU/mL)	ELISA titres (days post vaccination)					SNT titres	
		0	3	9	15	21	4 weeks	4 months
114	10^2	-	-	-	+	+	nd	1:2048
118	10^2	-	-	+	+	+	nd	1:16
64	10^2	-	-	+	-	+	nd	1:512
45	10^2	-	-	+	+	+	nd	1:4096
44	10^3	-	-	-	+	+	nd	1:1024
35	10^3	-	-	+	+	+	nd	1:32
76	10^3	-	-	-	+	+	nd	1:572
1	10^3	-	-	-	-	-	nd	1:128
5	10^4	-	-	+	+	+	nd	1:512
41	10^4	-	-	-	+	+	nd	1:256
82	10^4	-	-	+	+	+	nd	1:64
127	10^4	-	-	+	+	+	nd	1:64
147	Control	-	-	-	-	-	< 1:4	< 1:4

nd, not done; SNT, serum neutralisation test.
-, negative ; +, positive.

TABLE 3: Clinical reaction and percentage protection index for bluetongue virus serotype 2 vaccine at different titres challenged at 4 months post vaccination.

Animal number	Titres (PFU/mL)	CRI	Average (%)
30	10^2	0	72
149	10^2	2	
126	10^2	9	
122	10^2	0	
17	10^3	0	82.5
125	10^3	0	
38	10^3	7	
140	10^3	0	
104	10^4	0	100
57	10^4	0	
56	10^4	0	
52	10^4	0	
59	Positive control	10	-

CRI, clinical reaction index.

TABLE 4: Clinical reaction and percentage protection index of sheep vaccinated with bluetongue virus serotype 8 and challenged at 4 months post vaccination.

Animal number	Titres PFU /mL	CRI	Protection (%)	Average (%)
114	10^2	0	100	100
118	10^2	0	100	
64	10^2	0	100	
45	10^2	0	100	
44	10^3	0	100	90.75
35	10^3	0	100	
76	10^3	0	100	
1	10^3	2	100	
5	10^4	0	100	100
41	10^4	0	100	
82	10^4	0	100	
127	10^4	0	100	
147	Positive control	5.5	-	-

CRI, clinical reaction index.

Percentage protection index

Bluetongue virus serotype 2

At four months post challenge the sheep vaccinated with the low titre vaccine (10^2 PFU/mL) had 72% protection and two sheep had very high clinical reaction indexes. The positive control sheep had a high CRI and died on day 8 post challenge (Table 3). The presence of BTV 2 was confirmed by viral isolation.

Bluetongue virus serotype 8

All sheep vaccinated with low titre vaccine (10^2 PFU/mL and 10^3 PFU/mL), and challenged with BTV 8, showed protection of 100% and 90.75%, respectively. The sheep vaccinated with the high titre vaccine had a 100% protection level (Table 4).

Trustworthiness

The relevant controls were used during the vaccination and challenge of the sheep as well as in all laboratory tests performed. Validated standard operating procedures were followed in the laboratory. Results obtained are trustworthy.

Discussion

In BT endemic areas, such as SA, the BTV MLV is predominantly applied to control BT disease in sheep,[8,10] and this vaccine has successfully played a major role in controlling the spread of the virus in Europe.[5,10,25] Virus titres induced by BTV MLV should be kept to an absolute minimum especially if transmission by vectors of viruses, included in the vaccine, is a concern.[8] This is because the complex interaction of the different serotypes of BTV, *Culicoides* vectors and animal hosts in the life cycle of the virus.

The existence of at least 22 serotypes of BTV's in SA and the presence of vectors that can transmit the disease, to different susceptible hosts, make the administration of BTV MLV economical for sheep farmers. However, the European Union has decided not to administer BTV MLV on the grounds of various concerns. These include the possible reassortment of vaccine viruses with circulating field strains and the possible reversion to virulence,[34,35,36] the transmission of these viruses by vectors and the lack of a DIVA test for these vaccines.[3,27] Another concern is the presence of a long duration viraemia when BTV MLV, with titres of or above 10^4 PFU/mL, are administered. It is also at this titre that susceptible sheep show clinical signs and suffer abortions.[6,22] The administration of BTV MLV in Europe has, therefore, created concern and the need has arisen to investigate the use of reduced titres of 10^2 PFU/mL and 10^3 PFU/mL in the vaccine for administration in sheep.

Titres of 10^4 PFU/mL, 10^3 PFU/mL and 10^2 PFU/mL were chosen to be tested as a dosage to be included in vaccines of BTV MLV. In this study there was no local or systemic reaction post vaccination and this confirms the results obtained by Hammoumi, Bréard and Sailleau (2003).[37] However, transient fever was seen in this study within the first 14 days post vaccination which lasted only one to two days. Although viraemia could not be detected post vaccination, temperature reactions could be demonstrated and this corresponds with what has been indicated by Hammoumi et al., (2003) and Hunter and Modumo (2000),[20,37]and this also confirms the results obtained by Dungu, Gerdes and Smit (2004).[5]

Cell culture material, and not blood, was selected as challenge material. From previous experience it was noted that the reaction to this in sheep is either late or poor when blood was used, as compared with cell culture material.[8,38] The reason for this might be that BTV is a cell-associated virus and binds mainly to red blood cells, platelets and mononuclear cells.[29,39,40] It is, however, important to realise that cell culture generated material might lead to the attenuation of the virus, which includes changes in virulence and antigenicity.[8,36] Blood-cultured material should, therefore, rather be applied as challenge material.

Three sheep vaccinated with low titre 10^2 PFU/mL BTV 2 had higher temperature reactions, above 40.5 °C – 41.5 °C, within the first 14 days post challenge, for 3–4 days. The same result was also seen in sheep vaccinated with low titre BTV 8 vaccine.

Viraemia was detected post challenge with BTV 2, except with the high titre 10^4 PFU/mL BTV 2 vaccine, and no viraemia was detected using the three different vaccines for BTV 8. The reason for this might be that the cell culture material for BTV 2 was highly virulent, as the positive control died. It was also interesting to note that four sheep demonstrated both viraemia and seroconversion within the same period of 21 days. This might be attributed to the close association of BTV to cells which protect the virus from circulating antibodies.[27,40] It was noted previously that a strong antibody response correlates with the virus circulating and replicating in the host body and, thus, with the possibility of viraemia.[8]

Although the BTV 2 low titre vaccine (10^2 PFU/mL) gave a protection index lower than 90%, the vaccine was able to protect the sheep from this clinical disease, especially when it is noted that the positive control sheep died. This confirms what was previously described, that BTV MLV offers 90.5% protection against clinical disease.[10,18,20,25] There was also a good correlation between seroconversion and the protection index of sheep vaccinated with BTV 8 vaccines. The protection index of sheep vaccinated with BTV 8 was above 90% at all titres. It should also be noted that the positive control of sheep challenged with BTV 2 had a far higher CRI (10) compared with the vaccinated group. The results also confirm that BTV 2 challenge material was more pathogenic than BTV 8 challenge material if comparison is made of the two viruses (Tables 3 and 4).

It was clearly shown in this study that sheep vaccinated with the BTV 2 and 8 vaccines reacted differently. This confirms the results obtained by Howell (1969),[18] that each BTV serotype reacts differently and their immunogenic potential differs from serotype to serotype.

Although at a low titre (10^2 PFU/mL) BTV 2 vaccine offers protection against severe clinical disease, it is recommended to release the vaccine at a titre of 10^3 PFU/mL. The bluetongue virus serotype 8 can, however, be administered at the low titre of 10^2 PFU/mL. This study confirms that low titre BTV 2 and 8 vaccines are both immunogenic, and it is recommended that during production and release of multi serotype BTV vaccines, specific serotype titres should be considered rather than the average of all titres in a batch, as is currently practiced.

More work remains to be undertaken to quantify the duration and level of viraemia post vaccination and post challenge, especially when low titres of virus are administered. Low titre vaccines should also be tested when included in a polyvalent format, as opposed to monovalent vaccines, as was done in this study. The response of indigenous European sheep breeds, different from those in SA, to low titre BTV vaccines should be assessed. To evaluate this, other diagnostic tools, like PCR, should also be used. It will additionally be important to investigate and assess the cost benefit of using both polyvalent and movalent low titre modified live attenuated BTV vaccines, and compare the risk factors associated with their use in sheep.

Conclusion

Although viraemia was demonstrated post challenge, sheep vaccinated with the low titre BTV 2 vaccine showed more than a 90% protection index at a lower titre of 10^3 PFU/mL, compared with BTV 8 that showed a protection index above 90% at all the titres used. It is therefore recommended that for BTV 2 vaccine, sheep should be vaccinated at a titre of 10^3 PFU/mL and at a titre of 10^2 PFU/mL with BTV 8 vaccine.

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Competing interests

No competing interests that the authors are aware of.

Authors' contributions

The research was done as part of a masters dissertation by J.M. (Onderstepoort Biological Products). The practical work was done by J.M. (Onderstepoort Biological Products) and supervised by E.V. (University of Pretoria). E.V. (University of Pretoria) wrote and submitted the manuscript.

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