Effect of levamisole administration on bluetongue vaccination in sheep

C. Stelletta[(1)], V. Cuteri[(2)], L. Bonizzi[(3)], A. Frangipane di Regalbono[(4)], F. Orsi[(5)], L. Nisoli[(6)], D. Lulla[(6)] & M. Morgante[(1)]

(1) Dipartimento di Scienze Cliniche Veterinarie, Facoltà di Medicina Veterinaria, Università di Padova, via Romea 16 Agripolis, 35120 Legnaro (PD), Italy
(2) Dipartimento Scienze Veterinarie,Università di Camerino, Via Circonvallazione, 93/95 - 62024 Matelica (MC), Italy
(3) Dipartimento di Sanità pubblica Patologia Comparata e Igiene Veterinaria, Università di Padova, via Romea 16 Agripolis, 35120 Legnaro (PD), Italy
(4) Dipartimento di Scienze Sperimentali Veterinarie, Università di Padova, via Romea 16 Agripolis, 35120 Legnaro (PD), Italy
(5) DVM, Viterbo, Loc.Campopadella 1, 01012 Capranica (VT), Italy
(6) Bayer HealthCare – Animal Health Division, Viale Certosa 130, 20156 (MI), Italy

Summary

Levamisole is an anthelmintic drug with immunostimulant properties when administered at repeated doses of 2.5 mg/kg prior to a vaccine being administered. In order to assess the effect of levamisole administration on bluetongue (BT) vaccination in sheep, four groups of unvaccinated pregnant sheep (8 sheep per group) were used. Group A received vaccine only; Group B received levamisole + vaccine; Group C received Levamisole only; Group D was a non-treated control. Levamisole (Citarin L – 10%) was administered three times weekly at an initial dose of 5.0 mg/kg of body weight and subsequently at 2.5 mg/kg of body weight. There was a significant decrease in faecal egg count of gastrointestinal strongyles in Groups B and C. At the beginning of the trial, all animals were serologically negative for BT antibodies; after vaccination, there was a difference in antibody response in animals in the treated groups. Significantly, more animals in Group B developed BT antibodies following vaccination than those in Group A. In conclusion, levamisole appeared to have an immunostimulating effect on the response of sheep to BT vaccination.

Keywords

Anthelmintic – Bluetongue – Levamisole – Sheep – Vaccination.

Introduction

Levamisole is an anthelmintic drug that stimulates the parasympathetic and sympathetic ganglia in susceptible worms. It is also an immunomodulator and exerts an immunostimulant action in different animal species when administered at repeated doses of 2.5 mg/kg prior to vaccine being administered. Immunostimulating effects are not well understood. It is believed that an immunomodulator restores cell-mediated immune function in peripheral T-lymphocytes and phagocytosis by monocytes (7, 8). Furthermore, an immunomodulator appears to stimulate the production of interleukin-2 (IL-2) and lysozyme, to enhance lymphocyte blastogenesis and to increase the level of specific immunoglobulin in the colostrum of vaccinated animals (1, 2, 3). Bluetongue (BT) is an arthropod-borne disease of domestic and wild ruminants. Its causative agent is an *Orbivirus* in the family *Reoviridae*. Clinical disease is usually mild or absent in cattle, camelids and goats, but sheep can be severely affected with mortality rates varying from 1% to 30% (5). To date, 24 different bluetongue virus serotypes have been identified in several countries in tropical and temperate areas that support the survival of biting midges (*Culicoides* spp.), the vectors responsible for transmission of the disease. One of the control methods for the disease is the use of a vaccine containing live-attenuated BT virus (BTV). The response to vaccination is directly linked to the immunological condition of the vaccinated animals. The aim of the present clinical trial was to determine if levamisole administration can exert an immunostimulating effect on sheep when vaccinated against BT.

Materials and methods

Animals

Four groups of sheep in the last month of gestation (8 Sardinian ewes in each group) were used: Group A received only vaccine, Group B received levamisole and vaccine, Group C received only levamisole and Group D was the untreated control group. Before the trial was initiated, the animals were examined and found to be clinically healthy and had not previously received BT vaccine. The ewes came from a typical, reasonably productive sheep farm and had all been subjected to the same environmental and nutritional conditions.

Levamisole and vaccine administration

Both Groups B and C were given three subcutaneous injections of levamisole (Citarin® L 10%, Bayer) at seven-day intervals, at an initial dose of 5 mg/kg of body weight and subsequently at 2.5 mg/kg of body weight. In Group B, the last dose was administered in conjunction with vaccination. Groups A and B received vaccine containing live-attenuated BTV-2 virus (Onderstepoort Biological Products, South Africa).

Sampling and laboratory analysis

Blood and faecal samplings were performed four times on all sheep: at the first administration of levamisole (sample 1); at vaccination (sample 2), 48 h after vaccination (sample 3) and three weeks after vaccination (sample 4). Faecal samples were analysed using the McMaster technique with a minimum sensitivity of 50 epg/opg (eggs per gram/oocysts per gram). Blood samples were taken in duplicate (clot tubes and tubes containing ethylene-diaminetetra-acetic acid [EDTA]). Serum samples were screened using a competitive enzyme-linked imunosorbent assay (c-ELISA) to detect antibodies against BTV (BTV antibody test kit, c-ELISA, VMRD, Inc.). EDTA tubes were placed in refrigerated containers and used for haematological parameter determinations. A complete blood count was performed: red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT), mean red blood cell volume (MCV), mean cellular haemoglobin content by mass (MCH), mean cellular haemoglobin concentration (MCHC) and the white blood cell, neutrophil, lymphocyte, monocyte, eosinophil and basophil cell count using an automated haematology analyser (Advia 120 Haematology System).

Statistical analysis

Anova double factors analysis (time of sampling and group) was used comparing groups and the response to the treatment by each group using procedure GLM of software SigmaStat 2.03.

Results

White blood cells and leukogram data are presented in Table I; all animals had leukocyte counts higher than the reference range for sheep (4-12×10^3/μl) (6). Neutrophilia were not present (Table I) and all values were normal (reference range 0.7-6×10^3/μl); therefore, the other leukogram data influenced the total number of leucocytes (e.g. lymphocytes or eosinophils with reference ranges of 2-9×10^3/μl and 0-1×10^3/μl, respectively). In response to the treatments, the absolute number of monocytes significantly increased ($P<0.05$) after vaccination from 0.57 ± 0.1×10^3/ μl to 0.94 ± 0.3×10^3/ μl in Group B. The absolute number of eosinophils increased in all groups in the fourth sample with a significant difference ($P<0.05$) for Groups B and C (Table I). The erythron data (Table II) showed slight anaemia with RBC, HGB and HCT lower than the reference range (9-15×10^6/μl, 9-15 g/dl and 27%-45%, respectively). The MCHC value decreased significantly for all groups. Anaemia could have been caused by heavy helminthic infestation. Treatment with levamisole (Groups B and C) demonstrated a significant decrease in the helminthic populations that are usually sensitive to the action of this anthelmintic drug (Table III).

Regarding vaccination, it was clear that the administration of levamisole affected seroconversion (Table IV); the mean antibody response, measured by optical density, of Group B was higher than that of Group A (0.323 ± 0.0320 vs 0.346 ± 0.0339). Moreover, with the serological technique used (c-ELISA) a value lower than 0.325 is considered positive. Therefore, the mean value of Group A could be indicative of vaccination failure; in addition, it was important to evaluate the total number of animals within each group that had an optical density lower than the positive cut-off. In fact only four animals (50%) from Group A seroconverted (<0.325) while seven animals (87.5%) from Group B showed a strong seroconvertion.

Discussion

Vaccination is a means to reduce losses caused by a disease and so maintain the normal profit margins of the farmer. These clinical trials demonstrated that animals considered immunologically competent and under favourable environmental conditions could experience a vaccination failure. Anaemia found in

Table I
Values of leukogram data (mean ± standard error means) obtained in the clinical trial

Groups	Sample	White blood cells (× 10^3/µl)	Neutrophils (× 10^3/µl)	Lymphocytes (× 10^3/µl)	Monocytes (× 10^3/µl)	Eosinophils (× 10^3/µl)	Basophils (× 10^3/µl)
A	1	19.48 ± 1.6	5.75 ± 0.8	11.95 ± 1.2	0.78 ± 0.1	0.69 ± 0.2	0.20 ± 0.03
	2	16.54 ± 1.6	3.97 ± 0.8	10.77 ± 1.2	0.58 ± 0.1	0.84 ± 0.2	0.23 ± 0.03
	3	15.93 ± 0.9	3.66 ± 0.2	10.63 ± 0.7	0.48 ± 0.1	0.76 ± 0.2	0.20 ± 0.02
	4	17.93 ± 1.6	3.85 ± 0.8	11.45 ± 1.2	0.58 ± 0.1	1.65 ± 0.2	0.26 ± 0.03
B	1	16.47 ± 1.5	3.73 ± 0.8	11.09 ± 1.1	0.48 ± 0.1[a]	0.91 ± 0.2[a]	0.16 ± 0.03
	2	17.04 ± 1.6	4.48 ± 0.8	11.16 ± 1.2	0.57 ± 0.1[a]	0.46 ± 0.2[a]	0.23 ± 0.03
	3	15.14 ± 1.6	3.69 ± 0.4	9.81 ± 1.3	0.94 ± 0.1[b]	0.43 ± 0.05[a]	0.17 ± 0.03
	4	17.87 ± 1.5	4.01 ± 0.8	10.95 ± 1.1	0.40 ± 0.1[b]	2.19 ± 0.2[b]	0.20 ± 0.03
C	1	16.98 ± 1.5	5.38 ± 0.8	9.87 ± 1.1	0.49 ± 0.1	0.92 ± 0.2[a]	0.19 ± 0.03
	2	14.99 ± 1.5	4.85 ± 0.8	8.76 ± 1.1	0.47 ± 0.1	0.62 ± 0.2[a]	0.23 ± 0.03
	3	13.68 ± 1.5	4.97 ± 0.8	7.98 ± 1.1	0.49 ± 0.1	0.79 ± 0.2[a]	0.20 ± 0.03
	4	15.05 ± 1.5	3.61 ± 0.8	8.78 ± 1.1	0.42 ± 0.1	1.93 ± 0.2[b]	0.20 ± 0.03
D	1	14.29 ± 1.7	3.87 ± 0.9	8.49 ± 1.3	0.78 ± 0.1	0.83 ± 0.3	0.14 ± 0.03
	2	15.39 ± 1.6	3.7 ± 0.8	10.04 ± 1.2	0.42 ± 0.1	0.93 ± 0.2	0.23 ± 0.03
	3	13.79 ± 2.5	4.47 ± 0.4	7.66 ± 1.9	0.51 ± 0.2	0.86 ± 0.3	0.19 ± 0.05
	4	16.27 ± 1.6	4.23 ± 0.8	9.42 ± 1.1	0.53 ± 0.1	1.83 ± 0.2	0.21 ± 0.03

Different superscript letters (a, b) within each group show a significant difference among samples: P<0.05

Table II
Values of erythron data (mean ± standard error means) obtained in the clinical trial

Groups	Sample	RBC (× 10^6/l)	HGB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)
A	1	7.57 ± 0.4	8.91 ± 0.4	27.45 ± 1.5	36.27 ± 0.8	11.76 ± 0.3	32.47 ± 0.4[a]
	2	7.35 ± 0.4	8.76 ± 0.4	26.71 ± 1.5	36.34 ± 0.8	11.94 ± 0.3	32.87 ± 0.4[a]
	3	7.13 ± 0.2	8.51 ± 0.3	25.81 ± 0.8	36.24 ± 0.7	11.98 ± 0.2	33.07 ± 0.4[a]
	4	7.06 ± 0.4	8.14 ± 0.4	26.11 ± 1.5	37.04 ± 0.8	11.57 ± 0.3	31.22 ± 0.4[b]
B	1	7.26 ± 0.4	8.80 ± 0.4	27.32 ± 1.4	37.73 ± 0.7	12.11 ± 0.2	32.14 ± 0.3[a b]
	2	7.04 ± 0.4	8.76 ± 0.4	26.66 ± 1.5	37.95 ± 0.8	12.45 ± 0.3	32.85 ± 0.4[a]
	3	7.41 ± 0.3	9.21 ± 0.4	27.97 ± 1.3	37.72 ± 0.6	12.42 ± 0.2	32.96 ± 0.3[b]
	4	6.97 ± 0.4	8.22 ± 0.4	26.30 ± 1.4	37.89 ± 0.7	11.86 ± 0.2	31.29 ± 0.3[b]
C	1	7.65 ± 0.4	9.20 ± 0.4	28.66 ± 1.4	37.74 ± 0.7	12.13 ± 0.2	32.14 ± 0.3[a]
	2	7.30 ± 0.4	8.99 ± 0.4	27.34 ± 1.4	37.70 ± 0.7	12.41 ± 0.2	32.92 ± 0.3[a]
	3	7.36 ± 0.4	8.76 ± 0.4	27.56 ± 1.4	37.90 ± 0.8	12.23 ± 0.2	32.59 ± 0.3[a]
	4	7.40 ± 0.4	8.74 ± 0.4	27.98 ± 1.4	38.02 ± 0.8	11.88 ± 0.2	31.27 ± 0.3[b]
D	1	6.83 ± 0.5	8.30 ± 0.5	25.63 ± 1.6	37.81 ± 0.8	12.31 ± 0.3	32.56 ± 0.4
	2	6.54 ± 0.4	7.86 ± 0.4	24.07 ± 1.5	36.99 ± 0.8	12.07 ± 0.3	32.72 ± 0.4
	3	6.58 ± 0.5	7.98 ± 0.4	24.66 ± 1.7	37.80 ± 1.6	12.34 ± 0.5	32.62 ± 0.5
	4	6.60 ± 0.4	7.87 ± 0.4	24.84 ± 1.5	37.95 ± 0.8	12.07 ± 0.3	31.76 ± 0.4

Different superscript letters (a, b) within each group show a significant difference among samples: P<0.05

RBC red blood cels
HGB haemoglobin
HCT hematocrit
MCV mean red blood cell volume
MCH mean cellular haemoglobin content by mass
MCHC mean cellular haemoglobin concentration

Table III
Faecal egg/oocyst counts in treated (B and C) and untreated (A and D) groups with levamisole

Groups	Treatment	Gastrointestinal strongyles[a]			*Trichuris* sp.[a]	Tapeworms[a]	*Eimeria* sp.[b]
		Nematodirus sp.	*Strongyloides papillosus*	Other species			
A	Before	0	450 ± 96.8	352.5 ± 111.1	6.2 ± 6.9	125 ± 29.2	1050 ± 464.1
	After	6.2 ± 7.1	468.7 ± 96.8^{c}	518.7 ± 111.1^{c}	18.7 ± 6.9	31.2 ± 29.2	1643.7 ± 464.1
B	Before	11.1 ± 6.7	122.2 ± 91.3	130 ± 104.8	0	0	727.8 ± 437.6
	After	0	22.2 ± 91.3^{d}	0^{b}	0	22.2 ± 27.5	972.2 ± 437.6
C	Before	5.5 ± 6.7	100 ± 91.3	238.9 ± 104.8	0	0	555.5 ± 437.6
	After	0	0 b	22.2 ± 104.8^{d}	5.5 ± 6.5	5.5 ± 27.5	716.7 ± 437.6
D	Before	12.5 ± 7.1	218.7 ± 96.8	425 ± 111.1	0	18.7 ± 29.2	987.5 ± 464.1
	After	6.2 ± 7.1	318.7 ± 96.8^{c}	393.7 ± 111.1^{c}	12.5 ± 6.9	43.7 ± 29.2	2287.5 ± 464.1

a) eggs per gram of faeces (mean ± standard error)

b) oocysts per gram of faeces (mean ± standard error)

Different superscript letters (c, d) among groups in the treatment rows show a significant difference: P<0.05

Table IV
Bluetongue virus antibody response as measured by optical density (mean ± s.e.m.), c-ELISA method
Optical densities of less than 0.325 are considered positive

Sample	A (vaccine)	B (vaccine + levamisole)	C (levamisole)	D (control)
1	0.720 ± 0.0339	0.730 ± 0.0320	0.716 ± 0.0320	0.670 ± 0.0363
2	0.677 ± 0.0339	0.700 ± 0.0339	0.675 ± 0.0320	0.660 ± 0.0339
3	0.587 ± 0.0297	0.681 ± 0.0274	0.656 ± 0.0320	0.611 ± 0.051
4	0.346 ± 0.0339^{a}	0.323 ± 0.0320^{a}	0.449 ± 0.0320^{b}	0.467 ± 0.0339^{b}

Different superscript letters (a, b) within each sample show a significant difference among groups: P<0.05

all animals was probably due to excessive parasitism caused by the absence of pasture rotation. The decrease of MCHC in all animals, but more especially in Groups B and C, could be indicative of reticulocytosis (erythroid regeneration). Therefore, improvement in health was due to a decrease of parasitosis following levamisole treatment. Eosinophilia present in the fourth sample in all groups could be ascribed to the activation of hypobiotic larvae after parturition.

The blood monocytes, together with tissue macrophages, constitute the mononuclear phagocyte system which has a microbiocidal action against bacteria, viruses, fungi and protozoa. Moreover, the function of monocytes (macrophages) includes regulation of the immune response when exposed to bacteria, antigens or tissue injury; they produce cytokines (IL-1 and tumour-necrosis factor). In Group B (vaccination combined with levamisole), there was a clear increase in the absolute number of monocytes 48 h post vaccination, probably due to the action of levamisole treatment. Indeed, the anthelmintic drug seems to stimulate the production of IL-2 and lysozyme. It has been hypothesised that levamisole restores the cell-mediated immune function in peripheral T-lymphocytes and phagocytosis by monocytes (4, 8). Furthermore, Group B showed a higher seroconversion rate when compared to Group A. This result is similar to other trials demonstrating an increase in specific immunoglobulin levels after treatment (3). In conclusion, this clinical trial demonstrated the immunostimulating effect of levamisole on BT vaccination in sheep due to an improvement in their general condition with a decrease of helminthic infestation and a direct effect on immunocompetent cells.

References

1. Bozic F., Bilic V. & Valpotic I. (2002). – Modulation by levamisole of CD45RA and CD45RC isoform expression in the gut of weaned pigs vaccinated against colibacillosis. *J. Vet. Pharmacol. Therapy*, **25,** 69-72.
2. Findlay V.L. & Munday B.L. (2000). – The immunomodulatory effects of levamisole on the nonspecific immune system of Atlantic salmon, *Salmo salar* L. *J. Fish Dis.*, **23,** 369-378.
3. Krakowski L., Krzyzanowski J., Wrona Z. & Siwicki A.K. (1999). – The effect of nonspecific immunostimulation of pregnant mares with 1,3/1,6 glucan and levamisole on the immunoglobulin levels

in colostrum, selected indices of nonspecific cellular and humoral immunity in foals in neonatal and postnatal periods. *Vet. Immunol. Immunopathol.*, **68**, 1-11.

4. Lewinski U., Mavligib G. & Hersh E. (1980). – Cellular immune modulation after a single high dose of levamisole in patients with carcinoma. *Cancer*, **46**, 2185-2194.
5. MacLachlan N.J. (1994). – The pathogenesis and immunology of bluetongue: virus infection of ruminants. *Comp. Immunol. Microbiol. Infect. Dis.*, **17**, 197-206.
6. Morris D.D. (1996). – Alteration in the leukogram, Chapter 25. *In* Large animal internal medicine, 2nd Ed. Mosby, St Louis, Missouri, 480-488.
7. Mulcahy G. & Quinn P.J. (1986). – A review of immunomodulators and their application in veterinary medicine. *J. Vet. Pharmacol. Therapy*, **9**, 119-139.
8. Naylor P.H. & Hadden J.W. (2003). – T-cell targeted immune enhancement yields effective T-cell adjuvants. *Int. Immunopharmacol.*, **3** (8), 1205-1215.