



Safety Evaluation of Inactivated Polyvalent Vaccine Against Bluetongue Virus Serotypes 10, 11, and 17 in Pregnant Ewes

Girish Sarma^{1*}, Tammy Burton², Laurel Moody¹, and Dale Wallis¹

¹Hygieia Biological Laboratories, Woodland, CA 95776, USA

²Burton Sheep Ranch, Sheridan, CA 95681, USA

*Corresponding author's Email: Girishsarma1@gmail.com

ABSTRACT

Bluetongue is a noncontagious, vector-borne Orbivirus-induced disease of domestic and wild ruminants. Both live and inactivated monovalent vaccines are used to control bluetongue disease worldwide. The objective of this study was to evaluate the safety and post-vaccination seroconversion of an inactivated polyvalent bluetongue virus (BTV) vaccine containing serotypes 10, 11 and 17 in pregnant ewes. The vaccine was evaluated by vaccinating 66 pregnant ewes (33 for each study group). The ewes enrolled for this study represented 24 dairy, 30 meat, and 12 crossbreds and were aged 1-9 years. The average body weight of the animals ranged from 73 to 82 kg. Pregnant ewes representing all three trimesters of pregnancy were used in this study. Each animal was vaccinated with a 2.0 ml dose of the vaccine subcutaneously. All ewes were revaccinated similarly after one month. Local injection site reactions after both vaccinations, systemic and other adverse reactions, effects on pregnancy, fetal development, delivery of the lambs, and post-vaccination virus-neutralizing (VN) antibody titers were monitored after each vaccination. Mild to moderate injection site reactions such as swelling or lump formation were seen in 29 injection sites (22.0%). All injection site reactions were resolved completely within 2 – 7 weeks without further complications and treatment. Mild to moderate post-vaccination systemic reactions such as anorexia/lethargy/laminitis were seen only in two ewes, which lasted for four days. Bluetongue vaccination in the pregnant ewes did not affect the pregnancy, fetal development, and delivery of the lambs. There were no premature abortions or any form of teratogenic deformities in any of the lambs delivered. The vaccine in the pregnant ewes induced post-vaccination VN antibody titers against all three serotypes of BTV. Lambs born to the vaccinated ewes also showed a protective level of VN antibody titers against all three serotypes of the virus following passive transfer of maternal antibodies. The levels of VN antibody titers seen in the ewes and lambs were considered sufficient to provide protection against field virus challenge. The results of the current study indicated that inactivated polyvalent bluetongue virus vaccine had no adverse effects on pregnancy and lamb delivery.

Keywords: Bluetongue, Inactivated vaccine, Maternal antibody, Pregnant ewe, Safety, Virus-neutralizing antibody

INTRODUCTION

Bluetongue is a noncontagious, Orbivirus induced, insect-transmitted disease of domestic and wild ruminants (MacLachlan et al., 2009; McVey and MacLachlan, 2015). Biological vectors such as the hematophagous biting midges or gnats of the genus *Culicoides* transmit the virus from animal to animal (Tabachnick, 2004; 2010; MacLachlan, 2011; MacLachlan and Mayo, 2013a; Sarma et al., 2026). The disease has been recognized in the United States since the late 1940s. Since then, at least 15 serotypes of the virus have been isolated and identified from different parts of the USA (Johnson, 2007; MacLachlan, 2011; MacLachlan et al., 2013b).

The only vaccine currently available in the United States for use in sheep is a modified live virus vaccine against bluetongue virus (BTV) serotype 10 (McVey and MacLachlan, 2015). No vaccine is presently available for immunization of livestock against the other serotypes of the virus (McVey and MacLachlan, 2015). Although the modified live virus vaccines can induce a protective immune response in vaccinated animals against BTV infection, there are some disadvantages, including reduced milk production in lactating ewes, abortion, early embryonic death, and teratogenesis when used in pregnant animals (McVey and MacLachlan, 2015). Additionally, the live virus vaccines cause a milder form of the disease with viraemia (Van der Sluijs et al., 2013). Biological vectors such as midges or gnats could spread the virus with reversion to virulence and genetic reassortment (Savini et al., 2008).

Conversely, inactivated vaccines are generally safe and induce complete and long-lasting immunity in vaccinated animals (Savini et al., 2008; Sarma et al., 2026). However, to be effective, inactivated vaccines need to be manufactured using higher doses of the antigens, appropriate adjuvants, and generally require a booster dose (Wilson-Welder et al., 2009). The safety of the subcutaneously administered monovalent inactivated BTV vaccines has been reported earlier

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from vaccination studies in sheep, goats, and other animals (Hammers et al., 2009a; Zientara et al., 2010; McVey and MacLachlan, 2015). These studies revealed that the monovalent inactivated vaccines were safe and caused no systemic reactions, including fever, weight loss, loss of appetite, and reproductive anomalies in the vaccinated animals. Except for some vaccine-induced transient local injection site reactions of variable severity (mild to moderate), no safety issues were observed in any of these studies with the monovalent inactivated vaccines (Hammers et al., 2009 a,b; Sarma et al., 2026).

In the United States, inactivated BTV vaccines are not yet commercially available. Autogenous vaccines are produced to meet customers' demand when needed. Due to the lack of published information on the safety of using a polyvalent inactivated vaccine containing BTV serotypes 10, 11, and 17 in pregnant ewes, this study was undertaken to evaluate a polyvalent vaccine for its suitability for use in pregnant animals as well as to determine the potential of maternal antibody transfer to the newborns to provide early protection against the disease.

MATERIALS AND METHODS

Ethical approval

The present study was conducted under field conditions in a commercial sheep ranch. The project was approved by the institutional R and D committee (Reference number: HPRD-RR24) of Hygieia Biological Laboratories (USA).

Study location and animals

This study consisted of two consecutive trials (Trial 1 and Trial 2) conducted at the same geographical location (Burton sheep ranch, Sheridan, California). In Sheridan, the average summer temperatures range from daytime highs of about 32.8°C to 35.6°C, and the average winter temperatures range from 12.8°C to 14.4°C. The rainy season starts in October and lasts until March/April. This location is 32 to 34 meters above sea level, and its latitude is approximately 38.98°N. The ranch is a commercial sheep ranch, consisting of both dairy and meat breeds. A total of 66 randomly selected pregnant ewes (33 for each trial), including both pure dairy breeds (East Friesian and Lacaune), meat breeds (Tunis and Suffolk), and crossbreds, were used to evaluate the safety of the inactivated BTV vaccine. The animals' age ranged from 1 to 9 years (Table 1). The average weight of the animals ranged from 73 to 82 kg. Pregnant ewes used for the study encompassed all three trimesters of pregnancy. The gestational status of each ewe was confirmed by ultrasound (SonoSite Edge II ultrasound system, USA) using either the 8-3 MHz transducer or the 5-2 MHz transducer. The ewes were housed in dry lot pens of 20-40 head per pen. Each pen measured approximately 23 × 61 meters. All animals had daily access to seasonal pasture consisting of native grasses. All animals had access to barns within pens for protection from environmental elements such as rain and sun. Pens had shared fence lines. Ewes were fed balanced rations as required by each stage of gestation. The dietary schedule consisted of a standard protocol adopted by the ranch owner and operator. Water and trace minerals (Wind and Rain sheep minerals, catalog number 3005607-106, Purina, USA) were provided *ad libitum*. All animals appeared healthy at the time of study enrolment when examined by a veterinarian.

Table 1. Pregnant ewes enrolled in the bluetongue virus vaccine safety study

Trial number	Number of pregnant Ewes vaccinated	Age at vaccination			Breed type			Previous BTV vaccination history*	Previous BTV field virus exposure	Environmental conditions at time of vaccinations
		1-3 (years)	4-6 (years)	7-9 (years)	Purebred dairy (East Friesian and Lacaune)	Purebred meat (Tunis, Suffolk)	Crossbreds			
1	33	3	18	12	3	27	3	Yes	Yes	Late Spring to Summer (35°C-37.8°C)
2	33	17	16	0	21	3	9	Yes	Yes	Winter (18.3°C-21.1°C)
Total	66	20	34	12	24	30	12	-	-	-

*: For animals ≥ 6 years old, BTV: Bluetongue virus

Vaccine

Experimental serials of BTV inactivated vaccine (Serial # BTV001, BTV002 and BTV003) manufactured by Hygieia Biological Laboratories, Woodland, California 95776, USA were used for vaccination and revaccination of the pregnant ewes. Since BTV serotypes 10, 11, and 17 are the most prevalent serotypes in the USA, the vaccines used in

this study were manufactured using inactivated viruses of these three serotypes. The vaccine was adjuvanted with a water-in-oil emulsion. Binary ethyleneimine was used as an inactivating agent for the virus, and the emulsion was prepared by mixing the inactivated antigens with mineral oil (Univar Solutions, Sacramento, California 95838, USA). The vaccines (Ref. # PC1075.10) contained the same quantity of inactivated antigens and adjuvant per dose and were manufactured following the identical vaccine manufacturing protocol (PO1075.10). Prior to use, the vaccines were tested for sterility, purity, laboratory animal safety, and virus inactivation following the applicable guidelines as stated in the Code of Federal Regulations, Title 9, Sections 113.200 and 113.303. The vaccines passed all required tests and were stored refrigerated at 2-7°C until used.

Method of vaccination

The vaccination was done in two consecutive trials to verify the repeatability of the results as well as to find out the effect of environmental conditions (Summer vs Winter) on the performance of the vaccine. Prior to vaccination, the bottles containing the vaccine were shaken vigorously for uniform mixing. Due to the slightly thicker viscosity of the mineral oil-adjuvanted vaccine, as well as to get rid of the bubbles created by the mixing process, the vaccine bottles after thorough mixing were allowed to sit at room temperature for about an hour to make the process of vaccine administration easier and more accurate. Consistent with industry practice, injection sites were not clipped or sanitized prior to vaccination but were marked with paint to enable subsequent evaluation of injection site reactions. For initial vaccination, each pregnant ewe received 2.0 ml of the vaccine subcutaneously in the middle of the left side of the neck. An identical dose was administered one month later subcutaneously on the right side of the neck. Separate needles were used for vaccinating each animal. A one-month interval between the first and second vaccination was chosen in order to allow sufficient time to heal the injection site reactions that may have developed following administration of the first dose of the vaccine.

Vaccination trial 1

Trial 1 consisted of 33 ewes, including 27 meat breed ewes, three dairy ewes, and three crossbreed ewes. With the exception of one animal, the ewes in both trials were in their second or third trimester of pregnancy when vaccines were administered. All pregnant ewes were selected for the study based on availability. Pregnant ewes in trial 1 were vaccinated with the experimental vaccines from late spring to summer during exceptionally high and uncomfortable environmental temperatures (35°C-37.8°C). In this trial, all pregnant ewes received several additional treatments simultaneously with the first vaccination with the experimental bluetongue vaccine. Ewes were dewormed with an Ivermectin drench, treated for external parasites with a permethrin topical pour-on, vaccinated against Clostridial diseases utilizing Covexin 8 (Merck Animal Health, catalog 067696, Nebraska 68103 USA), and injected with selenium (Intervet/Merck Animal Health, New Jersey 07065 USA). To reduce stress for gestating ewes from frequent handling during extremely inclement weather, the vaccinated ewes were not bled pre- or post-vaccination for serological assessment of BTV antibodies in trial 1.

Vaccination Trial 2

Trial 2 consisted of 33 vaccinated pregnant ewes, including three meat breed ewes, 21 dairy ewes, and nine crossbred ewes. Unlike trial 1, the pregnant ewes of trial 2 were vaccinated with the BTV vaccine during mid-winter, with more comfortable environmental temperatures (18.3°C-21.1°C). Moreover, in trial 2, fewer interventions coincided with the BTV vaccinations. The animals were handled only for the BTV vaccinations, collection of pre-and post-vaccination blood samples for assessment of virus-neutralizing antibodies against BTV serotypes 10, 11 and 17, ultrasound examinations for pregnancy, evaluation of the injection site reactions and prenatal administration of Covexin 8, *E. coli*-J-5 (Hygeia Biological Laboratories, Woodland, California USA), vitamins A/D and selenium (one month prior to parturition) following manufacturers' directions.

Injection site reactions

The injection sites of the vaccinated ewes after first and second vaccinations were palpated weekly through eight weeks post-vaccination for development of firm or fluctuant lumps or masses, abscess formation, ulceration, and healing. The post-vaccination observations for injection site reactions continued until eight weeks after administering the second dose of the vaccine. For palpating the injection sites and to measure the size of the lumps as well as for ultrasound examinations, the animals were handled using chutes and headgate. The size of the masses was measured roughly using digital palpation, where one finger width was considered approximately equal to one-half inch. Based on size measurement, the lumps or masses at the injection sites were identified as mild reaction (< 1.0 to 1.5 inches in diameter), moderate reaction (1.6 to 2.0 inches in diameter), and severe reaction (> 2.0 inches in diameter).

Post-vaccination observation

All vaccinated ewes were observed daily for the development of any post-vaccination adverse or systemic reactions such as anaphylaxis, anorexia, lethargy, laminitis, abortion, and mortality for up to 30 days following each of the two doses administered subcutaneously. Based on the duration of disappearance of the post-vaccination systemic reactions, the observations were recorded as mild (lasting < 4 days) and moderate to severe (lasting > 4 days). In trials 1 and 2, ewe and lamb mortality was examined by a veterinarian, and samples were sent to the California Animal Health and Food Safety (CAHFS) laboratory, Davis, California, for complete necropsy.

Pregnancy and lamb delivery

Pregnant ewes were monitored daily during the entirety of gestation until the cessation of lambing season. Ewes were observed for premature abortions, stillbirths, mastitis, uterine and/or vaginal prolapses, and other health issues by a veterinarian. Ewes were also observed at the time of delivery to assess normal delivery and/or dystocia. Assisted deliveries were performed whenever necessary. Ewes were treated with antibiotics (Oxytetracycline 300 mg/ml, 4.5 ml per 45 kg) following assisted invasive deliveries and the treatment followed the attending veterinarian's standard practice. Data collected during lambing season included the number of lambs born to each ewe, any weak, stillborn lambs, or lambs with teratogenic deformity. Aborted lambs and dead lambs born with or without assisted deliveries were categorized as dead on arrival (DOA). The lambs that died prior to weaning were transported directly to CAHFS for necropsy. Lambs were identified with USDA scrapie tags, paint branded with their mother's flock tag number, and injected with selenium (subcutaneous BoSe injectable 2.19 mg sodium selenite per mL, equivalent of 1 mg selenium; used 1 mL per 18 kg body weight) within the first three days postpartum. No other additives or medications were used in the newborns.

Antibody screening of pre- and post-vaccination serum samples

At least 5.0 ml of blood was collected by jugular venipuncture from all vaccinated ewes in trial 2 immediately prior to first vaccination and one month following administration of the first and second doses of the vaccine. The pre-vaccination blood collection was done to screen the animals for residual antibodies to BTV acquired either through previous vaccination, active infection due to field exposure to the virus, or passive transfer through colostrum from the antibody-positive dams. The post-vaccination bleeding was done to test the ewe sera for BTV serotype-specific virus-neutralizing (VN) antibody titer. The pre-and-post-vaccination serum samples collected from the pregnant ewes in trial 2 were screened for VN antibodies against BTV serotypes 10, 11, and 17 using a virus neutralization assay. The virus neutralization assay was performed following the previously described procedure ([Sarma et al., 2026](#)).

Briefly, using a 96-well plate, the serum samples were serially diluted 2-fold in serum free cell maintenance medium containing Dulbecco's modified Eagle's medium (DMEM, Ref. # 10566-016, Source: Gibco, Grand Island, New York 14072, USA) with 100 IU/ml penicillin and 30 µg/ml streptomycin (Pen Strep, Ref. # 15140-122, Source: Gibco, Grand Island, New York 14072, USA). For each dilution, 50 µl of the diluted serum was mixed with 50 µl of cell maintenance medium containing 100 to 200 TCID₅₀ of the BTV serotype 10, 11, or 17. The plate containing the serum-virus mixtures was incubated at 37°C for one hour. Following incubation, the serum-virus mixtures from the plate were transferred to the corresponding wells of a flat-bottomed 96-well cell culture plate with near-confluent monolayers of Vero cells and incubated for three days at 37°C in a humidified CO₂ incubator. Appropriate cell controls without virus and virus controls without BTV antibody-positive serum were included in each assay for accurate interpretation of the test results. The same procedure was followed for screening each test serum sample separately against each of the three serotypes of the virus (BTV-10, BTV-11, and BTV-17) used in the vaccine.

At the end of the incubation period, the cell monolayer in each well of the plate was examined under 100x magnification of an inverted microscope (Nikon Eclipse TS2-S-SM, Nikon Corporation, Japan) to check for development of BTV-induced cytopathic effect (CPE) in the cells. The CPE-positive cells become enlarged, rounded, and eventually die and detach from the surface. The VN antibody titers for each serum sample against BTV serotypes 10, 11, and 17 were calculated from the reciprocal of the highest serum dilution that completely (100%) inhibited CPE development in the Vero cell monolayer ([Sarma et al., 2026](#)).

Screening for maternal antibody in the lambs

In trial 2, serum samples collected from the lambs were subsequently screened for maternal antibody status against BTV serotypes 10, 11, and 17 using the previously described virus neutralization assay ([Sarma et al., 2026](#)). The lambs were bled for serum collection within 26 days of birth.

Handling of the animals

Handling of the pregnant ewes for vaccination, blood collection, post-vaccination observations for local and systemic reactions, monitoring of the pregnant animals throughout the pregnancy until lambing, and monitoring of the newborns for their health status and blood collection were all done by the trained personnel under the supervision of a veterinarian and following the standard protocols practiced by the ranch owner.

Statistical analysis

The VN antibody titer data of the pregnant ewes were analyzed for statistical significance by using a t-test, and the maternal antibody titer data of the lambs were analyzed by using a correlation coefficient test. Chi-square statistics were applied to analyze the post-vaccination injection site reactions data. All statistical tests were performed with Microsoft Excel 365.

RESULTS

Injection site reactions

The results of injection site examination following the first and second vaccinations are presented in Table 2. Post-vaccination site reactions in the pregnant ewes were observed after the first and second vaccinations in both trials 1 and 2. In trial 1, 10 injection sites had positive site reactions following the first vaccination, and five injection sites had positive site reactions following the second vaccination. A total of 15 injection sites out of 66 (22.7%) manifested positive site reactions in trial 1. In trial 2, eight injection sites revealed positive site reactions following the first vaccination and six injection sites exhibited positive site reactions following the second vaccination. A total of 14 injection sites out of 66 (21.2%) manifested positive site reactions in trial 2. The combination of trials 1 and 2 yielded a total of 29 injection sites out of 132 (22.0%) that were positive for post-vaccination reactions.

Table 2. Post-vaccination injection site reactions after administering the initial and booster doses of the bluetongue virus vaccine

Trial number	Number of pregnant Ewes vaccinated	Number of post-first vaccination injection sites with reactions					Number of post-second vaccination injection sites with reactions					Total number of positive sites	(%) total positive*
		Mild	Moderate	Severe	Abscess**	Total positive	Mild	Moderate	Severe	Abscess**	Total positive		
1	33	7	3	0	0	10	5	0	0	0	5	15***	22.7
2	33	6	2	0	0	8	6	0	0	0	6	14***	21.2
Total	66	13	5	0	0	18	11	0	0	0	11	29 ^a	22.0

*: The difference in percentages of total positive injection site reactions between trial 1 and 2 were not statistically significant ($p > 0.05$), **: Abscess at the injection site due to BTV vaccination, Mild reaction < 1.0 to 1.5 inch in diameter, Moderate reaction 1.6 to 2.0 inch in diameter, Severe reaction > 2.0 inch in diameter, Pos: positive, ***: Out of 66 sites from two vaccinations of 33 ewes; ^a: Out of 132 sites from two trials combined.

Breed and age susceptibility

The relationship of development of post-vaccination injection site reactions to the age and breed of the vaccinated animals and time required to resolve the site reactions revealed that the development of post-vaccination injection site reactions was not correlated to either of those factors. Positive site reactions were observed across the board irrespective of the age and breed of the vaccinated ewes. Most of the injection site reactions in both trials 1 and 2 were mild and resolved completely in 2-7 weeks without any further complications or treatment.

Systemic reactions

The results of evaluating post-vaccination adverse/systemic reactions in the pregnant ewes are shown in Table 3. None of the vaccinated ewes of trials 1 and 2 had any anaphylactic reactions following administration of both doses of the vaccine through the subcutaneous route. Only one vaccinated ewe showed mild symptoms, and another ewe developed moderate to severe anorexia/lethargy/laminitis in the first trial. Another ewe from the first trial also died of environmental stress unrelated to vaccinations. None of the ewes in trial 2 developed anorexia/lethargy/laminitis after vaccinations. However, two vaccinated ewes in the second trial died (one due to cardiac contraction band necrosis and the other due to *Pseudomonas* mastitis). All mortalities in these two trials were unrelated to BTV vaccination as determined by CAHFS.

Table 3. Post-vaccination adverse/systemic reactions in the pregnant ewes

Trial number	Number of pregnant ewes vaccinated	Number of pregnant ewes showing post-vaccination adverse/systemic reactions					Cause of mortality
		Anaphylaxis	Anorexia / Lethargy / Laminitis		Total *	Mortality	
			Mild (< 4 days)	Moderate to severe (> 4 days)			
1	33	0	1	1	2	1	Environmental stress (heat shock)
2	33	0	0	0	0	2	Cardiac contraction band necrosis (1 ewe) <i>Pseudomonas</i> mastitis (1 ewe)
Total	66	0	1	1	2	3	Mortalities not related to BTV vaccination

BTV: Bluetongue virus, *: Total number of ewes showing reactions.

Effects on pregnancy, fetal development, and lamb delivery

Bluetongue virus vaccination in the pregnant ewes did not negatively affect the pregnancy, fetal development, or parturition of the lambs (Table 4). There were no abortions or any form of teratogenic deformities in any of the lambs delivered. Out of 66 pregnant ewes, 57 ewes delivered 106 normal healthy lambs, and nine ewes delivered 12 mature stillborn lambs due to prolonged obstructive dystocia. Stillborn lambs were part of multiple births and had one or more surviving siblings.

Table 4. Effects of bluetongue virus vaccination on pregnancy, fetal development and delivery of the newborn lambs

Trial number	Number of pregnant ewes vaccinated	Total number of ewes delivering lambs					Total number of healthy lambs delivered	Total number of stillborn lambs delivered*
		Normal healthy	Mature stillborn due to dystocia	Teratogenic deformities	Abnormal/weak	Premature abortion		
1	33	29	4	0	0	0	43	4
2	33	28	5	0	0	0	63	8
Total	66	57	9	0	0	0	106	12

*: All stillborn were due to prolonged obstructive dystocia and not related to bluetongue virus vaccination

Pre- and post-vaccination virus-neutralizing antibody titers in the pregnant ewes

The pre- and post-vaccination VN antibody titers in the pregnant ewes against BTV serotype 10, 11, and 17 are shown in Table 5. The present results showed that the post-first and second vaccination mean antibody titers of all the serum samples tested from the pregnant ewes were significantly higher than the corresponding pre-vaccination titers against all three serotypes of the virus ($p < 0.05$). The mean pre-vaccination VN antibody titers against BTV serotypes 10, 11, and 17 were 65, 57, and 115 respectively. After the first vaccination, the titers significantly increased to a mean value of 142, 108, and 189 against each of the three serotypes of the virus, respectively ($p < 0.05$). The second vaccination significantly boosted these titers further to a mean value of 258, 148, and 333 against BTV serotypes 10, 11, and 17, respectively ($p < 0.05$). In general, all serum samples assessed showed more than a 2-fold increase in VN antibody titers from pre-vaccination to post-vaccination. Although administering the second dose of the vaccine considerably increased the titers from the pre-vaccination levels, this increase in antibody titer was not statistically significant when post-second vaccination titers were compared to post-first vaccination titers ($p > 0.05$).

Maternal virus-neutralizing antibody status in the lambs

The serum samples from all the lambs tested were found positive for maternally derived antibodies against all three serotypes of the virus (Table 6). The mean titers of all the serum samples tested were 28, 14, and 25 against BTV serotype 10, 11, and 17, respectively. More than 60% of the lamb serum samples tested had maternal antibody titers ≥ 8 against all three virus serotypes.

Table 5. Virus-neutralizing antibody titer against bluetongue virus in the serum samples collected from the pregnant ewes from trial 2

BTV serotype	Number of pre-vaccination serum samples showing VN antibody titer					Number of post-first vaccination serum samples showing VN antibody titer					Number of post-second vaccination serum samples showing VN antibody titer				
	2 - 8	16 - 32	64 -128	256 -1024	Mean titer ^a	5 -10	20 -40	80 -160	320 -1280	Mean titer ^b	5 -10	20 -40	80 -160	320 -1280	Mean titer ^c
10	14	8	7	4	65 (n = 33)	2	17	5	7	142 (n = 31)	1	7	13	8	258 (n = 29)
11	14	13	5	1	57 (n = 33)	1	6	19	4	108 (n = 30)	0	4	21	4	148 (n = 29)
17	11	11	7	4	115 (n = 33)	1	15	8	7	189 (n = 31)	1	2	19	7	333 (n = 29)

^{a,b} Post-1st vaccination mean VN antibody titers were significantly higher ($p < 0.05$) than the pre-vaccination mean VN antibody titers, ^{a,c} Post-2nd vaccination mean VN antibody titers were significantly higher ($p < 0.05$) than the pre-vaccination mean VN antibody titers, ^{b,c} Post-2nd vaccination mean VN antibody titers were not significantly higher ($p > 0.05$) than the post-1st vaccination mean VN antibody titers. BTV: Bluetongue virus.

Table 6. Maternal virus-neutralizing antibody titer in the serum samples collected from the lambs delivered by the pregnant ewes from Trial 2

BTV serotype	Mean VN antibody titers in the ewes before lambing ^b	Number of lamb serum samples showing VN maternal antibody titer								Total number of lamb serum samples with maternal antibody titer $\geq 8^a$	Mean VN maternal antibody titer in the lambs ^c
		2	4	8	16	32	64	128	256		
10	258	7	7	16	7	8	4	1	2	38 (73.0%)	28 (n = 52)
11	148	6	9	16	12	7	1	0	0	36 (70.6%)	14 (n = 51)
17	333	10	9	8	5	8	6	3	0	30 (61.2%)	25 (n = 49)

^a: VN antibody titer of ≥ 8 is likely to provide protection against clinical signs of the disease, ^{b,c}: A correlation coefficient analysis of the data under these two columns indicated a positive correlation between the mean VN antibody titers in the ewes and corresponding maternal antibody titers in the lambs ($r = 0.814$, $p < 0.05$), BTV: Bluetongue virus

DISCUSSION

Bluetongue in sheep and other ruminants is quite prevalent in the USA and in several other parts of the world (Sarma et al., 2026). Immunization with a safe and efficacious vaccine is one of the preferred methods to control the disease (Savini et al., 2008). Vaccination results in the induction of VN antibodies, which may prevent viraemia and protect the animals against the development of clinical signs of the disease. Prior to this study, Hygieia Biological Laboratories' polyvalent bluetongue virus vaccine using serotypes 10, 11, and 17 was tested for its post-vaccination safety and seroconversion in other ruminants (Sarma et al., 2026). However, it was not tested in pregnant animals.

The post-vaccination injection site reactions in the pregnant ewes were mostly mild, resolving completely within 2 - 7 weeks (Table 2). The reasons for slightly higher injection site reactions in trial 1 could be due to unpleasantly high environmental temperature at the time of initial vaccination and revaccination. Development of mild to moderate tissue reactions resulting in the formation of lumps or masses at the sites of vaccine administration are common sequelae with the use of mineral oil-adjuvanted inactivated vaccines. The mineral oil-adjuvanted vaccines usually form a depot at the site of vaccine administration. The antigen is released slowly from this depot under the skin for a better and prolonged immune response. Therefore, formation of sterile lumps or masses at the vaccine injection site is expected. However, severe tissue reaction leading to formation of large sterile tissue masses at the vaccine injection sites could be due to accidental injection of the vaccine into the underlying musculature.

The vaccine also did not induce any adverse systemic reactions leading to death of the vaccinated animals, premature abortion, anaphylaxis, teratogenic deformities in the lambs, weak lambs, and stillbirths (Table 3). Twelve stillborn lambs delivered by nine of 66 pregnant ewes were due to prolonged obstructive dystocia and were not associated with vaccination. Only two out of 33 pregnant ewes in trial 1 developed mild to moderate anorexia/lethargy/laminitis lasting for about four days. This was most likely due to adverse and uncomfortable environmental conditions and the multitude of simultaneous other treatments given. All vaccinations in trial 1 were done in late spring to summer when the environmental temperature was unusually high (35°C-37.8°C) and uncomfortable. None of the pregnant ewes in trial 2 showed any sign of anorexia/lethargy/laminitis. In trial 2, all vaccinations were done during mid-winter when the weather was very pleasant (environmental temperature was between 18.3°C-21.1°C).

Vaccination of pregnant ewes with this polyvalent inactivated BTV vaccine adjuvanted with mineral oil appeared to be safe as evidenced by the absence of any vaccine-associated adverse systemic reactions and relatively low injection site reactions. Compared to the bluetongue attenuated live virus vaccines which have several disadvantages including its lack of safety in the pregnant animals (Monaco et al., 2004; Veronesi et al., 2009; McVey and MacLachlan, 2015), the inactivated vaccines containing only one serotype of BTV have been reported to have multiple advantages, particularly with regard to their safety and higher serotype specific seroconversion in the vaccinated animals (DiEmidio et al., 2004; McVey and MacLachlan, 2015). However, no published information was yet available on the safety and efficacy of inactivated vaccines containing three or more serotypes of the virus in pregnant sheep or in any other ruminants. This study shows that this polyvalent vaccine was equally safe for use in pregnant ewes.

The results of serological evaluation of the vaccinated pregnant ewes of trial 2 indicated that, besides being reasonably safe, the vaccine was also capable of inducing VN antibody response when administered as a two-dose regimen at a monthly interval. The VN antibody titers increased in most of the ewes following vaccination and revaccination after one month (Table 5), with some increasing by more than fourfold compared to the pre-vaccination titers. In order to avoid frequent handling of the pregnant ewes during adverse environmental conditions (unusually high environmental temperatures), no serological evaluation was done for the pregnant ewes of trial 1.

Previous BTV vaccine evaluation studies in other ruminants indicated an appropriate correlation between VN antibody titer and protection against challenge (Oura et al., 2009; McVey and MacLachlan, 2015; Sarma et al., 2026). An animal with a VN antibody titer of eight or above could be protected against the disease. In this trial, the post-vaccination antibody titers were all above this threshold. The authors of the current study also observed that there was a variation in individual immune response from animal to animal to the virus serotypes used in the vaccine. A few animals did not respond robustly to vaccination, as evidenced by a lack of significant increase in post-vaccination antibody titers compared to their pre-vaccination status. This could be due to individual animal response to the immunogen, concurrent infection with other pathogenic microorganisms, or other immunosuppressive factors associated with the health status of the animal. No consistent interference was observed with respect to the ability of the animals to seroconvert to each serotype of the virus present in this polyvalent vaccine. Moreover, the authors of the current study also observed that induction of injection site reactions did not apparently influence antibody production in the vaccinated animals. The VN antibody titer responses in the study animals that developed injection site tissue reactions were similar to the animals that did not develop injection site tissue reactions.

The presence of VN antibodies in the pre-vaccination serum samples of all pregnant ewes used for trial 2 was likely due to either prior exposure to the field strains of the virus inducing clinical or sub-clinical infection. This could also be

due to vaccination with either live or inactivated BTV vaccine prior to initiation of this trial. The previous vaccination history, however, did not indicate use of any live virus vaccine in the ewes used in this trial. An experimental inactivated BTV vaccine manufactured by Hygieia Biological Laboratories, Woodland, California 95776, USA was, however, used to vaccinate some of the ewes aged six years or older about five years prior to initiation of this trial. The pre-vaccination VN antibodies in the pregnant ewes of trial 2 could, therefore, be either the residual antibodies from the previous bluetongue virus vaccination with the inactivated vaccine or induced by field virus exposure causing subclinical infection. Long-term persistence of post-vaccination antibodies following administration of inactivated BTV vaccines has been reported in other studies (Oura *et al.*, 2009; McVey and MacLachlan, 2015). This could be an added advantage in using inactivated vaccines since vaccination with the adjuvanted inactivated BTV vaccine in the presence of residual antibodies will likely boost immunity by increasing the antibody titer and thus help protect the animals against field virus infection.

The presence of BTV antibodies in sheep under field conditions has been reported earlier from systematic serological and epidemiological surveys in the USA (Hilke *et al.*, 2019). Most of the samples tested showed the presence of antibodies against multiple serotypes of the virus. However, in most cases, the available low-level residual antibodies were believed to be insufficient to provide adequate protection against virulent field strains of the virus, thus indicating the necessity of vaccination for complete protection.

Results of maternal antibody evaluation in the lambs born out of the vaccinated pregnant ewes (Table 6) indicated the benefits of vaccinating pregnant animals with inactivated BTV vaccine. All serum samples tested from the lambs were seropositive against all three serotypes of the virus. The antibody titers in more than 60% of the serum samples were eight or higher, which may be sufficient to provide protection to the lambs against the virulent field strains of BTV early in their life.

In this study, the efficacy of the vaccine in pregnant ewes was evaluated based on the post-vaccination VN antibody titers. Since this study was conducted in pregnant ewes, it was not possible to conduct a controlled vaccination - challenge experiment to correlate antibody titers with protection. Moreover, the ranch owner was not in favor of allowing Hygieia Biological Laboratories to bring virulent strains of BTV onto the premises with a large population of susceptible sheep. Also, due to similar reasons, no other ewes at the trial location could be kept as unvaccinated controls. However, as discussed before, previous BTV vaccination studies conducted in sheep and other ruminants demonstrated a high correlation between VN antibody titers and protection against challenge (Savini *et al.*, 2008; Oura *et al.*, 2009; Wackerlin *et al.*, 2010). On the basis of the results reported from these earlier studies, it is anticipated that animals with a post-vaccination virus-neutralizing antibody titer of eight or higher may be protected in the case of field exposure to bluetongue virus.

CONCLUSION

The vaccine was found to be reasonably safe for use in the pregnant ewes. Although some animals revealed mild to moderate injection site reactions after administering the vaccine subcutaneously, no abscesses or severe site reactions were observed in any of the vaccinated ewes. All injection site reactions resolved completely within 2-7 weeks without further complications or treatment. Only a few animals developed mild to moderate post-vaccination systemic reactions lasting for about 4 days. Vaccination in the pregnant ewes did not affect the pregnancy, fetal development, and delivery of the lambs. There were no premature abortions or any form of teratogenic deformities in any of the lambs delivered. The vaccine also induced VN antibody titers against all three serotypes of BTV. Lambs born to the vaccinated ewes also showed maternally transferred VN antibody titers against all three serotypes of the virus. The levels of VN antibody titers seen in the ewes and lambs were most likely sufficient to provide protection against field virus challenge. The data from this study demonstrated promise for the safety and efficacy of a polyvalent inactivated BTV vaccine in pregnant ewes and further the benefits of vaccination to provide maternal antibody-mediated protection in the neonatal lambs. A vaccination and challenge study using a large number of animals including both positive and negative controls will help to add further confidence to the current findings.

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Authors' contributions

Girish Sarma came up with the concept of this study, designed the experiment, manufactured the experimental vaccine, evaluated the serum samples for VN antibodies, collected, assembled, analyzed, and interpreted the data, and wrote the manuscript. Tammy Burton assisted in vaccination and revaccination of the ewes, collected the serum samples, observed the ewes daily for reactions throughout pregnancy and lambing, and critically reviewed the manuscript. Laurel Moody conducted the vaccination, sample collection and processing, collection of the data, and critical revision of the article. Dale Wallis supervised the study, helped in vaccination, blood collection, evaluation of the post-vaccination reactions, and critically reviewed the manuscript. All authors approved the final manuscript prior to submission for publication.

Availability of the data and materials

The results presented in this article represent a summarized version of the original data collected during this study. The original data pool is maintained by the Hygieia Biological Laboratories and may be available upon reasonable request from the corresponding author.

Competing interests

The authors declare no conflicts of interest.

Ethical considerations

The authors declare that the present original article has not been submitted simultaneously to another journal for publication and no AI tools were used in the preparation of the manuscript. All data were originally collected and statistically analyzed without using AI tools. The authors accept all responsibility for the original text and the present data before submission and at final stage of publication.

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