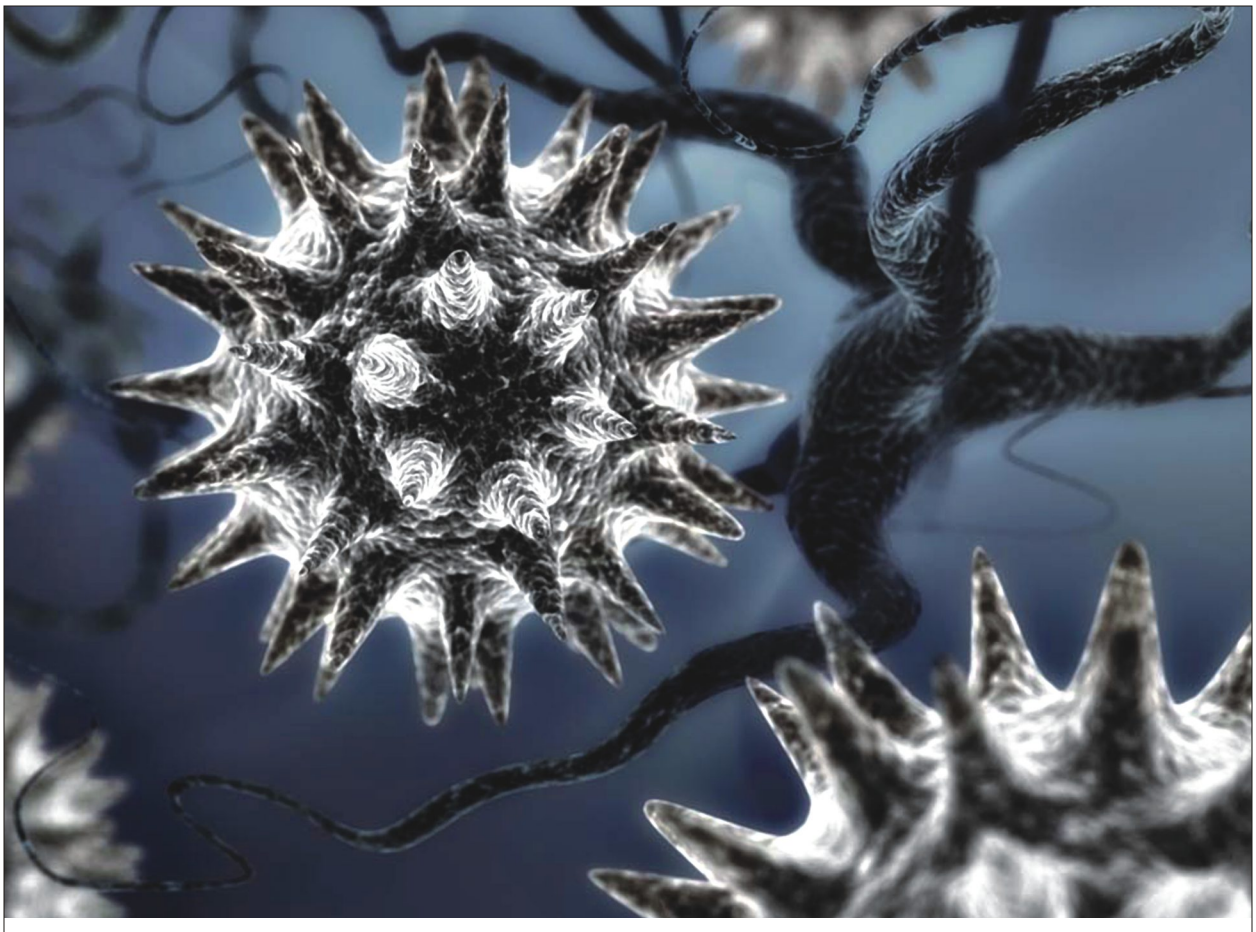

# International Journal of Virology

ISSN 1816-4900

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS International Journal of Virology

ISSN 1816-4900
DOI: 10.3923/ijv.2017.43.52

# Research Article
# Humoral Immune Response and Protective Efficacy of Binary Ethylenimine (BEI) Inactivated Pentavalent Bluetongue Vaccine after Challenge with Homologous Virus in Sheep

[1]Molalegne Bitew, [2]Sukdeb Nandi, [2]Chintu Ravishankar and [2]Asit Sharma

[1]National Veterinary Institute (NVI), P.O. Box 19, Debere Zeit, Ethiopia
[2]Virus Laboratory, Center for Animal Disease Research and Diagnosis (CADRD), Indian Veterinary Research Institute (IVRI), 243 122 Izatnagar, Uttar Pradesh, India

## Abstract

**Background:** Recent invasion of multiple bluetongue virus serotypes (BTV) in different regions of the world necessitates urgent development of efficient vaccine that aims numerous serotypes. **Materials and Methods:** In this experimental study, humoral immune response and protective efficacy of binary ethylenimine (BEI) inactivated montanide adjuvanted pentavalent (BTV 1, 2, 10, 16 and 23) vaccine was evaluated in sheep against challenge with homologous serotypes in their respective group. **Results:** All sheep were seronegative at day 0 before vaccination. After first vaccination, mean PI value was gradually declined in all vaccinated sheep. Vaccinated sheep were seroconverted to bluetongue virus starting from 10 days of primary vaccination (DPV) with Mean±SD PI value of 36.05±10.78 and $\log_{10}$ Mean±SD neutralizing antibody slightly increased from 0.85-1.42 starting from 3-21 days of post primary vaccination while, unvaccinated group of sheep had Mean±SD PI value of 111.79±12.36 and no $\log_{10}$ serum neutralizing antibody at this point of time. At 28 Days Post Vaccination (DPV), all vaccinated animals registered an abrupt increment in antibody level. Strong seropositivity was remained up to the date of 49 DPV steadily in all vaccinated sheep. After challenge at 49 DPV, vaccinated sheep registered high level of group specific antibody and neutralizing antibody. This level persisted up to 180 days and declined slowly to 270 days post vaccination whereas, unvaccinated challenged showed seropositivity between 7-14 DPC which started to decline after 21 DPC. Increased level of rectal temperature (Mean = 40.8°C) and clinical signs were evident between 5-13 days post challenge in control animals. There was significant difference ($p<0.05$) between vaccinated and unvaccinated animals in the Mean±SD PI value, $\log_{10}$ Mean±SD of neutralizing antibody, mean rectal temperature and development of clinical signs after homologues virus challenge. However, there was no significant difference ($p>0.05$) in all above parameters due to variability of challenge virus serotypes. **Conclusion:** All the findings, clearly suggested that binary ethylenimine (BEI) inactivated montanide adjuvanted pentavalent bluetongue vaccine was effective in protecting sheep from BTV 1, 2, 10, 16 and 23 infections.

Key words: Bluetongue, vaccine, sheep, clinical signs, humoral immune response, inactivated, pentavalent, montanide

Received: September 01, 2016 Accepted: October 04, 2016 Published: December 15, 2016

Citation: Molalegne Bitew, Sukdeb Nandi, Chintu Ravishankar and Asit Sharma, 2017. Humoral immune response and protective efficacy of binary ethylenimine (BEI) inactivated pentavalent bluetongue vaccine after challenge with homologous virus in sheep. Int. J. Virol., 13: 43-52.

Corresponding Author: Molalegne Bitew, National Veterinary Institute (NVI), P.O. Box 19, Debere Zeit, Ethiopia



Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Bluetongue (BT) disease is caused by bluetongue virus (BTV) which is the prototype species of the genus *Orbivirus* in the family Reoviridae[1,2]. The BTV genome is composed of 10 linear segments of double-stranded RNA (dsRNA), each of which codes for 1 of 10 distinct viral proteins. Currently, there are 27 recognized serotypes (BTV 1-27) with recent additions of the 25th serotype ("Toggenburg orbivirus") from Switzerland in goat and 26th from Kuwait in sheep and goat with little cross-reactivity and BTV-27 from goats in Corsia[3-7] in 2014 and 2015.

According to World Organisation for Animal Health[8], BT is a multiple species disease and notifiable to veterinary authorities in many countries and is therefore subject to strict regulations regarding the trade of animals and their products[9]. It can have considerable economic impact. The worldwide economic loss due to bluetongue is $3 billion in a year[10]. The direct losses are death, abortions, weight loss and reduced milk and meat productions and indirect losses are export restrictions of live animals, semen and foetal calf serum[11].

The major control methods for BT disease intensification include restriction of animal movement, vector control, slaughter of infected animals and vaccination. The first three ways are very difficult to attain in developing countries like India. However, the option at hand at this point of time is to vaccinate animals and this is the most practical measure for combating BT infection[12]. Vaccination can be accomplished either by immunization with live attenuated viruses, inactivated virus particles or sub-particle units[9]. Until 2003, the only vaccines available to control the BT disease were live attenuated vaccines[13]. Due to various risks associated with the use of live vaccines including reversion to virulence, teratogenicity, immuno-suppression and genetic reassortment of gene segments inactivated vaccines are considered safer[13,14]. Since 2005, Inactivated monovalent vaccines have been already used successfully in field trials[9,12-19] against BTV 1, 2, 4, 8, 11 and 16. However, studies on the inactivated pentavalent vaccine haven't been studied earlier.

Assessment of vaccine efficacy is based on clinical and virological data as well as on immunogenicity[13]. Cell mediated immune response and expression profile of cytokine transcripts in peripheral blood mononuclear cells of sheep following vaccination with the newly introduced binary ethylenimine (BEI) inactivated montanide adjuvanted pentavalent bluetongue vaccine has shown very promising result. However, there is little information about the humoral immune response and protective efficacy of the multivalent BT vaccines and newly produced binary ethylenimine (BEI) inactivated montanide™ ISA 206 VG adjuvanted pentavalent bluetongue vaccine in particular. The aim of the present study was to evaluate the humoral immune responses and protective efficacy following vaccination with binary ethylenimine (BEI) inactivated montanide adjuvanted pentavalent bluetongue vaccine in sheep and challenge with homologous BTV 1, 2, 10, 16 and 23 serotypes to their respective groups. The protective efficacy of vaccine in sheep has been evaluated in terms of development of pyrexia and average clinical score in comparison with control animals.

## MATERIALS AND METHODS

**Study animals and sample collection:** A total of 40 sheep (25 vaccinated and 15 unvaccinated) of 1.5–2 years of old, were procured from BT free area and tested sero-negative by c-ELISA (Pourquier c-ELISA kit (IDEXX, UK)) and were randomly divided in to 5 groups (Table 1). Animals were dewormed and maintained in insect proof sheds and the experiments were performed in accordance with the guidelines of the Institute's Animal Ethics Committee.

**Virus for vaccine preparation:** The bluetongue virus serotypes (BTV 1, 2, 10, 16 and 23) used in the production of pentavalent inactivated vaccine and for the challenge were received at the Virus Laboratory, Center for Animal Disease Research and Diagnosis (CADRAD), Indian Veterinary Research Institute (IVRI), Izatnagar. These serotypes were incorporated in the vaccine because they are predominantly distributed in most part of India. The identity of serotype was checked by RT-PCR amplification of the segment 2 by serotype specific primers[5].

**Vaccine preparation:** Vaccine was prepared as described by Ramakrishnan *et al.*[20] and Umeshappa *et al.*[18]. Briefly, BTV 1, 2, 10, 16 and 23 infected BHK-21 cells showing 90% cytopathic effect were harvested and centrifuged (Sorvall DuPont RC-5B, California, USA). The supernatant was collected and pellet

Table 1: Sheep involved in the humoral immune response

| Vaccine group | Vaccinated | Unvaccinated | Challenge virus |
|---|---|---|---|
| 1 | 5 | 3 | BTV 1 |
| 2 | 5 | 3 | BTV 2 |
| 3 | 5 | 3 | BTV 10 |
| 4 | 5 | 3 | BTV 16 |
| 5 | 5 | 3 | BTV 23 |
| Total | 25 | 15 | 40 |

suspended in 2 mM tris-HCl (pH 8.8) buffer each separately. The suspension was sonicated (Sonics, USA) at 30 µ amplitude for 1 min to release the intra cellular virus, again centrifuged and supernatant was mixed with previous collected supernatant. The collected supernatant then concentrated by 8% w/v PEG-6000, centrifuged and pellet was re suspended in tris-HCl buffer. Before inactivation, the virus suspension had a titre of $>10^6$ TCID 50 $mL^{-1}$ for all serotypes.

**Virus inactivation:** The virus was inactivated individually with 0.02 M BEI at 37°C for 48 h. BEI was freshly prepared from bromoethylamine hydrobromide (BEA) (Sigma, Milwaukee, WI, USA) at a concentration of 0.2 M by keeping at water bath in the presence of 0.2 M NaOH for 20 min at 37°C. The inactivation was stopped by chilled 1 M sodium thiosulphate to obtain final concentration of 0.1 M at the end of incubation period. Once inactivation was completed, inactivated virus (BTV 1, 2, 10, 16 and 23) preparations were pooled and mixed in equal proportion.

**Blending with adjuvant:** Following sterility and innocuity tests, 208 mL of this pooled inactivated BTV was mixed with 242 mL of Montanide™ ISA 206 VG, (SEPPIC, France) adjuvant (water oil water emulsion on weight by weight basis) in 500 mL measuring cylinder and homogenized with homogenizer as recommended by Ramakrishnan *et al.*[20] and Umeshappa *et al.*[18].

**Vaccination of animals:** Each animal of vaccinated group received 2 mL of vaccine by subcutaneous (S/C) route. An equal quantity of vaccine was inoculated on two sites (neck and posterior thigh). A booster dose of vaccine was given on 21st day with similar dose and route. Similarly, unvaccinated animals were injected with two doses of 2 mL of physiological saline by same route.

**Virus challenge:** Each of the 5 different BTV serotypes were passaged 3 times in BHK-21 cells before used as challenge virus. Forty nine days after primary vaccination (49 DPV) (booster vaccination), both vaccinated and unvaccinated groups were challenged by intradermal inoculation of 4 mL of clarified BTV 1, 2, 10, 16 and 23 virus suspension having a titer of $>10^6$ TCID 50 $mL^{-1}$ to their respective group of animals at multiple sites in the neck and under the thigh region.

**Sample collection:** Blood samples without anticoagulants were collected at day 0, 3, 7, 10, 14, 21, 28 and 38 (before challenge), 52, 56, 63, 70, 120, 180 and 270 days of post vaccination by jugular vein puncture under sterile conditions from all the animals. Plain tube blood samples were centrifuged at 3,000×g for 5 min, serum was removed using a sterile transfer pipette. Separated serum was heat inactivated at 56°C for 30 min and used for serum neutralization test (SNT) and competition enzyme linked immune sorbent assay (cELISA).

**Serological analysis**

**Competition enzyme linked immunosorbent assay (cELISA):** The BTV specific antibodies in serum was detected using competition ELISA assays (Pourquier cELISA kit (IDEXX, UK) according to the manufacturer's instructions. Results of the cELISA were expressed as percent inhibition (PI) of the optical density of the negative reference sera based on optical density readings at 450 nm in ELISA reader. The PI value can be expressed as:

$$\text{Percentage inhibition (PI)} = \frac{\text{OD of sample}}{\text{OD of negative reference}} \times 100$$

The PI≤70 correspond to seropositive sera whereas, PI≥80 are considered seronegative for BT antibody. The sera samples with PI greater than 70% and less than 80% are doubtful.

**Micro-serum neutralization test (m-SNT):** The m-SNT was performed according to the method of Oura *et al.*[21] and Batten *et al.*[6]. About 50 µL duplicate serial 2 fold serum dilutions with Dulbecco's minimum essential medium (DMEM, GIBCO®) starting from 1:2 to 1:64 or 1:16 to 1:512 were added to each well of flat-bottomed microtitre plates and mixed with 50 µL of standard reference BTV serotypes 1, 2, 10, 16 and 23 containing 100 TCID 50 (each serotype at separate plate) followed by incubation at 37°C for 2 h in the humidified $CO_2$ incubator (Heal Force, China) containing 5% $CO_2$ tension. About 100 µL of BHK-21 cell suspensions having $2\times10^5$ cells $mL^{-1}$ suspended in DMEM supplemented with 10% fetal calf serum was added per well and incubated similarly for 72 h. The plates were examined daily under the inverted microscope for the presence of cytopathic effect (CPE). A sample was considered positive when it showed a CPE of more than 50% neutralization at the lowest dilution (1:2). The neutralization titer was determined as the dilution of serum giving a 50% neutralization end point. The SNT antibody titers were expressed as $\log_{10}$ reciprocal of the highest serum dilution that neutralizes 100 TCID 50 of different BTV serotypes.

**Rectal temperature and clinical sign:** The rectal temperature and clinical sign score of all vaccinated and unvaccinated animals were recorded on 49 DPV (0 DPC) (before inoculums injection), 50 DPV (1 DPC), 52 DPV (3 DPC), 54 DPV (7 DPC), 57 DPV (8 DPC), 59 DPV (10 DPC), 62 DPV (13 DPC), 64 DPV (15 DPC), 67 DPV (18 DPC) and 70 DPV (21 DPC). The general health condition (depression, anorexia) and BTV-specific clinical manifestations, such as the mouth lesions, feet lesions and respiratory lesions were recorded and quantified using a modified version of the average clinical scoring system developed by Darpel *et al.*[22] and Moulin *et al.*[23]. The average clinical scores were made as follows: Fever: 1 point for each day of temperature >40°C, anorexia: 1 point for each day of anorexia and scores from 0-4 were provided depending on the severity to lesions like mouth lesions (such as conjunctivitis, rhinitis, facial oedema, ulcers of the mucosa and tongue oedema), foot lesion (lameness), respiratory tract lesions (bronchitis and/or pneumonia). Six points were provided to score veterinary intervention; 8 points were allotted when an animal was euthanized and 30 points were scored for natural death from BT. The duration of clinical signs was also taken into account by adding clinical scores, between 3-21 DPC.

**Statistical analysis:** All the statistical analyses were performed using SPSS version 17 software program (SPSS Inc. Chicago, IL, USA). The statistical differences between the mean percent inhibition (PI) value, $\log_2$ mean serum neutralizing (SN) antibody titer, mean rectal temperature and mean clinical scores of the vaccinated and unvaccinated groups of sheep before and after challenge were analyzed using unpaired student's t-test for independent means. The $p \leq 0.05$ was considered significant.

## RESULTS

Out of total of 40 sheep included in this study 25 were vaccinated and 15 were controls. Under vaccinated group 1-5 were challenged with BTV 1, 2, 10, 16 and 23, respectively. Similarly, group 1-5 sheep under unvaccinated were challenged with BTV 1, 2, 10, 16 and 23, respectively. To measure the humoral immune response after vaccination and challenge cELISA and SNT were used. The cell mediated immune response and viraemia study showed that the vaccine significantly ($p<0.05$) reduced BTV RNA load in PBMCs of vaccinated animals than unvaccinated animals following the challenge.

### Humoral immune response

**Competition enzyme linked immune sorbent assay:** The VP7 cELISA assays were carried out before and after vaccination and challenge with homologous BTV serotype and results were calculated. At day 0 before vaccination, all sheep were seronegative with mean percent inhibition (PI) value (Mean±SD) of 117.02±25 and unvaccinated sheep were remained seronegative in all serological assays until challenge. After first vaccination, mean PI was slowly declined in all vaccinated sheep. Vaccinated sheep were seroconverted to bluetongue virus starting from 10 days of primary vaccination (DPV) with Mean±SD PI value of 36.05±10.78 while, unvaccinated group of sheep had Mean±SD PI value of 111.79±12.36 at this point of time. The group specific VP7 antibody levels remained steady state up to day 21 of primary vaccination. Booster (second) vaccination was given at 21 days of post primary vaccination. At day 28, days post vaccination (DPV) (7 days after booster), all vaccinated animals registered an abrupt increment in antibody level tested by cELISA with Mean±SD PI value of 8.44±4.46 compared to unvaccinated sheep with Mean±SD PI value of 106.73±10.47. Strong seropositivity (mean PI value <10) was remained up to the date of 49 DPV steadily in all vaccinated sheep. At 49 DPV (0 days post challenge (DPC)) all sheep (vaccinated and unvaccinated) were challenged with the homologous BTV serotypes (BTV 1, 2, 10, 16 and 23). After challenge, vaccinated sheep were registered very high level of antibody (PI<8.0) starting from 52 DPV (3 DPC) and this level persisted up to 180 DPV and then declined slowly to 270 DPV whereas unvaccinated challenged showed seropositivity between 56 DPV (7 DPC) to 63 DPV (14 DPC) with Mean±SD PI value of 30.86±7 and this was shortly declined. There was no significant difference ($p>0.05$) in antibody titer produced by sheep vaccinated and challenged with different serotypes. All groups of animals responded in a similar fashion irrespective of difference in challenge serotype. On the other hand, there was very significant difference in the Mean±SD PI value of the antibody between vaccinated challenged and unvaccinated challenged sheep (Fig. 1a-f).

**Micro serum neutralization assay (mSNT):** At 0 day of first vaccination, all sheep from vaccinated and unvaccinated control had no detectable neutralizing antibodies (NA) measured by SNT. In vaccinated sheep the $\log_2$ Mean±SE neutralizing antibody titer slightly increased from 0.85-1.42

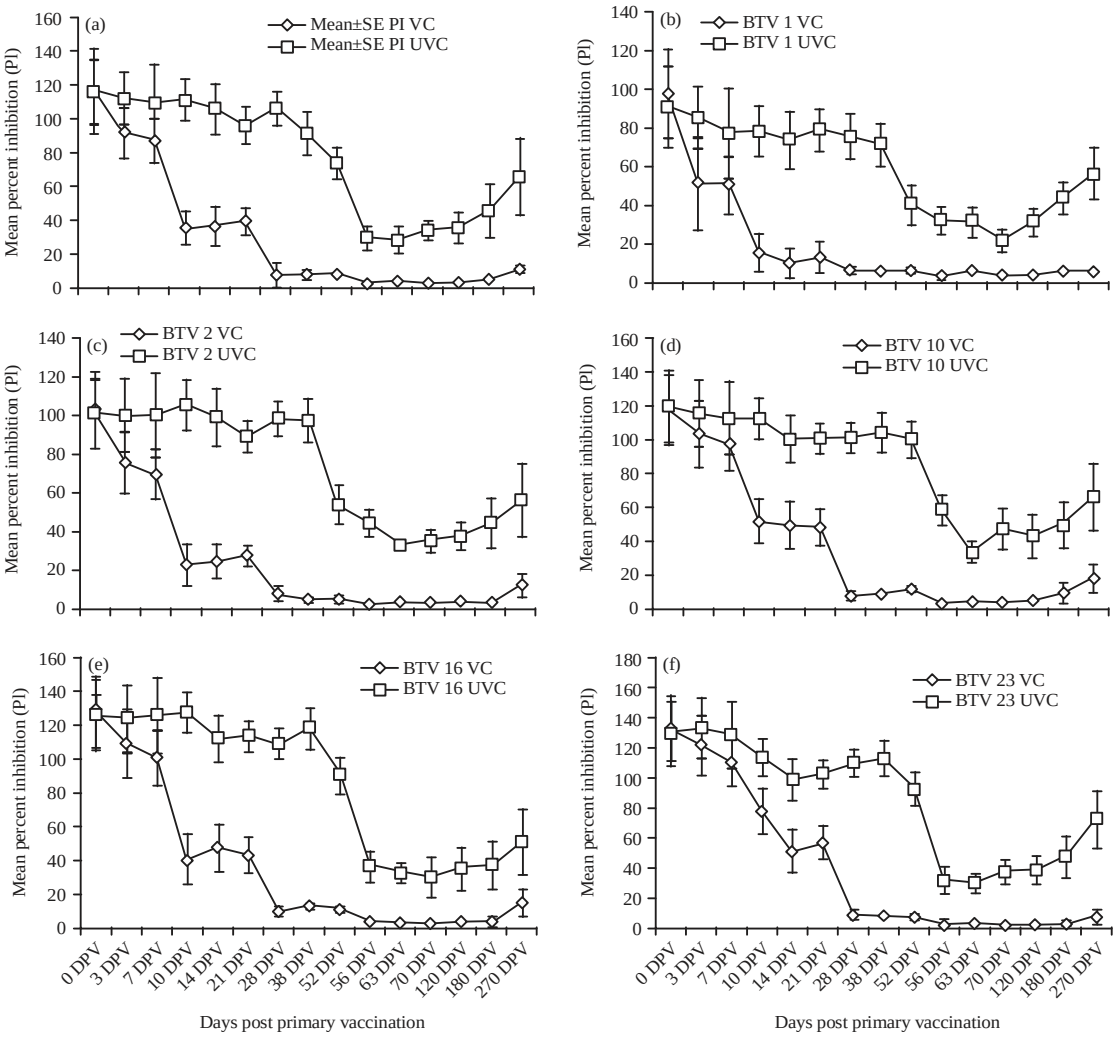


Fig. 1(a-f): Mean±SD percent inhibition (PI) value of each group of vaccinated and unvaccinated sheep with different BTV serotype challenge. DPV: Days post vaccination, VC: Vaccinated challenged (n = 5 in each group), UVC: Unvaccinated challenged (n = 3 in each group)

from 3-21 days of post primary vaccination (Fig. 2a-e). At 21 DPV, vaccinated sheep received booster dose of vaccine with the same amount and route of inoculation. After 28 DPV (7 days after booster), vaccinated sheep had higher levels $\log_2$ of neutralizing antibody titer (2.38±0.42). This value was gradually declined up to 49 DPV. No neutralizing antibody titer was detected in the unvaccinated control animals until the challenge. Both vaccinated and unvaccinated animals were challenged at 49 DPV and vaccinated animals showed abruptly elevated $\log_2$ Mean±SE neutralizing antibody titer at 52 DPV or 3 days post challenge (2.11±0.4) and registered very high value (2.456±0.32) at 56 DPV (7 DPC). After 56 DPV, the $\log_2$ neutralizing anybody titer was slightly declined up to the 270 DPV where as unvaccinated challenged animals showed low level of $\log_{10}$ Mean±SE neutralizing antibody titer at 56 DPV (7 DPC) (0.26±0.07). On the later days, unvaccinated challenged sheep started to decline in the SN titer at 63 DPV and finally diminished to 0 at 270 DPV. The difference in $\log_{10}$ Mean±SE SN titer between vaccinated and unvaccinated sheep was statistically significant ($p<0.05$) for the period ranging from day 0-270 DPV. On the other hand, there was no statistical significant difference ($p>0.05$) on $\log_2$ Mean±SD SN titer among the different BTV serotype challenge (Fig. 2a-f).

**Rectal temperature:** The mean rectal temperatures of the 5 groups, starting on 49 day are presented in Fig. 3a-e. Increase of rectal temperature was observed in unvaccinated sheep compared to vaccinated sheep from days 5-13 post challenge (54-62 days). All vaccinated sheep showed only

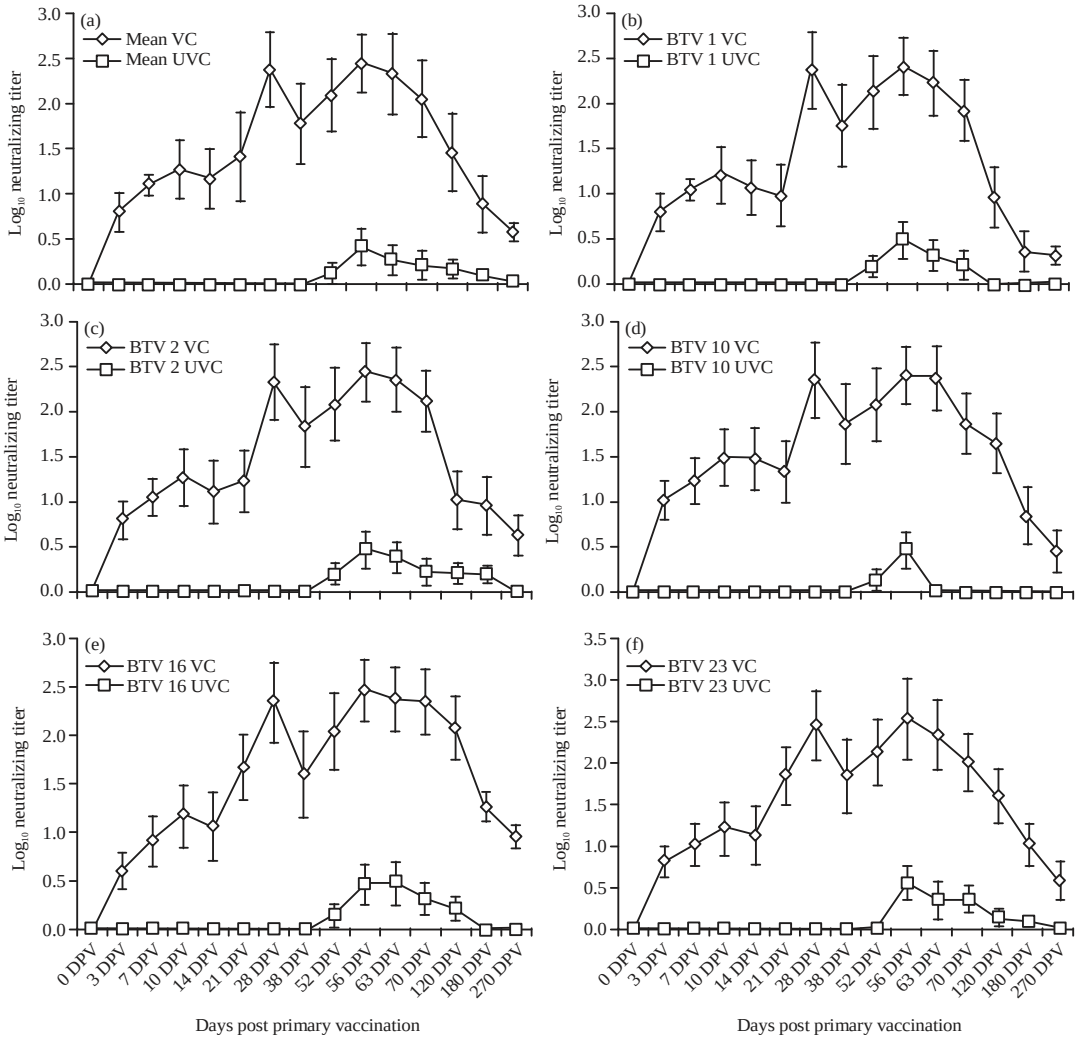


Fig. 2(a-f): $Log_2$ Mean±SD serum neutralizing antibody titer detected in vaccinated and unvaccinated sheep at different time interval after vaccination

short peak of temperature between 8 and 10 day post challenge (57-59 days) where as unvaccinated animals showed a peak ranging from 5-13 days post challenge and average value reached upto 40.8. There was no significant difference ($p>0.05$) in mean rectal temperature over time due to the variability in the BTV serotype challenge. However, there was significant difference ($p<0.05$) in rectal temperature recorded between vaccinated and unvaccinated sheep in which unvaccinated animals experienced very high level of fever than vaccinated animals (Fig. 3a-e).

**Average clinical score:** Fever and clinical signs were recorded in unvaccinated sheep rather vaccinated sheep after challenge. After 6-7 DPC, the mean rectal temperature of unvaccinated sheep had peaked to 40.8°C. Clinical manifestations were first appeared 5 DPC and there were hyperaemia of the buccal, labial and nasal mucosa, facial oedema and conjunctivitis was starting to develop. They also developed early signs of painful coronitis resulting in a reddening of the area around the coronary band and because of the severity of the clinical signs, two of them challenged with BTV 2 and 10 were treated with antibiotic in order to prevent secondary complications. The clinical scores of the animals during the study are shown in Table 2. Most unvaccinated sheep developed moderate clinical signs from 7-14 DPC where as vaccinated sheep showed negligible clinical signs. There was a significant difference ($p<0.05$) between vaccinated and unvaccinated animals in the development of clinical signs after homologues virus challenge. However, there was no significant difference ($p>0.05$) in the clinical manifestation as a result of difference in the BTV serotype used in challenge.

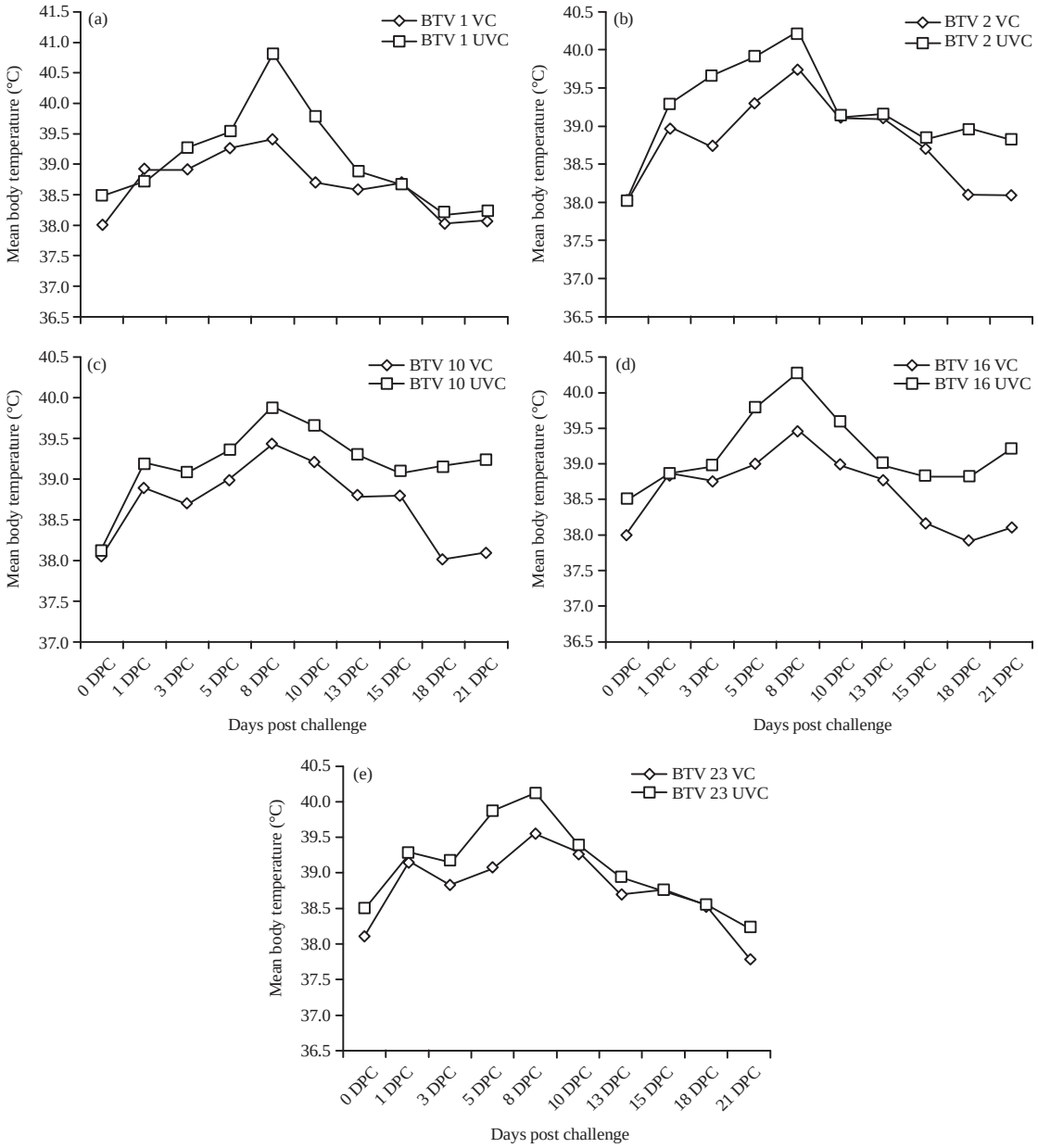


Fig. 3(a-e): Mean rectal temperature of vaccinated and unvaccinated animals after challenge with homologous BTV serotypes

Table 2: Mean clinical scores* of the vaccinated and unvaccinated sheep challenged with homologues BTV serotypes

| Challenge virus | Animal | Fever (0-6) | Anorexia (0-5) | Face (0-4) | Feat (0-4) | Respiratory tract (0-4) | Veterinary intervention (6) | Euthanasia (8) natural death (30) | Total score |
|---|---|---|---|---|---|---|---|---|---|
| BTV 1 | VC | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 3 |
| | UVC | 3 | 1 | 1 | 2 | 2 | 6 | 0 | 15 |
| BTV 2 | VC | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 3 |
| | UVC | 2 | 2 | 2 | 2 | 2 | 0 | 30 | 40 |
| BTV 10 | VC | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | UVC | 3 | 2 | 3 | 3 | 1 | 6 | 0 | 18 |
| BTV 16 | VC | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 2 |
| | UVC | 3 | 2 | 3 | 3 | 3 | 1 | 0 | 15 |
| BTV 23 | VC | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| | UVC | 3 | 2 | 2 | 2 | 2 | 0 | 0 | 11 |

*Mean clinical scores were calculated for the clinical signs developed between 3-21 DPC to each respective group members

## DISCUSSION

The major control methods against BT disease include restriction of animal movement, vector control, slaughter of infected animals and vaccination. The first three ways are very difficult to attain in developing countries like India. However, the option at hand at this point of time is to vaccinate animals and this is the most practical measure for combating BT infection[12].

Different kinds of BT vaccines have been developed so far for use in sheep and cattle. The vaccines comprise from conventional Live Attenuated (LA) or inactivated vaccines to recombinant or vectored vaccines as well as subunit vaccines[15]. The live attenuated vaccines have been employed with substantial success in South Africa and Italy but they are often associated with reduced milk production in lactating sheep, spread by natural infection, risk of reversion to virulence, reassortment of the vaccine strain with field viruses and clinical disease due to under-attenuation[15,24,25]. However, there is an urgent need to develop safer and effective vaccines to circumvent infection of multiple BTV serotypes and virus spread. Recombinant DNA technology based vaccines such as VLP, CLP, subunit vaccines utilizing a canary pox vector have been advocated by many researchers and could be promising although there have been debates with the production on a commercial scale in addition to the stability of the products and cost. These vaccines could be promising vaccine candidates in the years to come although additional development is needed[15,25-27].

Few countries in Europe (France, Spain, Italy, Portugal and others), India, the USA and China utilized inactivated bluetongue vaccine to avert BT disease outbreak and to prevent and avoid virus circulation caused by various BTV serotypes[12]. Various inactivated vaccines are available, of which some are directed against specific serotypes whereas others are multivalent depending on the serotype prevalence and country's policy. In India inactivated vaccines are still in experimental stage[12]. Monovalent inactivated vaccine directed against BTV 1, 2, 4, 11 and 16 with double dose and bivalent vaccine for BTV 2 and 4 have been successfully protected sheep from clinical disease and viral replication. It has been shown that single dose of BTV 2 inactivated vaccine successfully protected sheep from both clinical signs and viraemia for 12 months although there have been difference of opinions as to whether a single dose of inactivated vaccine is adequate to offer long-term protection[13,15,21].

The present study was undertaken with the aim to develop a BEI inactivated pentavalent (BTV 1, 2, 10, 16, 23) montanide adjuvanted bluetongue vaccine for sheep under All India Network Program on BT disease and to measure the humoral immune response and protective efficacy after challenge with homologous virus. It was found that vaccinated sheep were seroconverted to bluetongue virus starting from 10 days of primary vaccination compared to unvaccinated group of sheep. At 28 days post vaccination (DPV) (7 days after booster), all vaccinated animals registered an abrupt increment in antibody level tested by cELISA compared to unvaccinated sheep. After challenge, vaccinated sheep registered very high level of antibody (PI<8.0) starting from 52 DPV (3 DPC) and this level persisted up to 180 DPV and then declined slowly to 270 DPV whereas, unvaccinated challenge animals showed low level of seropositivity at 63 DPV (14 DPC) which subsequently declined. From this study it has been clearly shown that group specific antibody was strongly developed after initial shot of vaccination and abruptly increased after booster and challenge. From the point of protection of vaccinated sheep, serotype specific antibodies have been registered and less or no clinical signs were observed in vaccinated sheep compared to unvaccinated sheep. This finding was in line with the findings of many researchers[9,13,24,28].

Neutralising antibodies are known to play a key role in protecting animals from disease and viraemia and protective immunity is generally associated with the presence of type-specific neutralizing antibodies[11,13,14]. Several studies have shown that colostrum-fed or vaccinated animals with neutralizing antibodies are protected against infection, however colostrum-fed or vaccinated animals without neutralising antibodies may or may not be protected, even if they remain ELISA positive[11,14]. There is thus a strong correlation between the presence of neutralizing antibodies and serotype specific protection against BT virus[28]. From the present study, the highest titers of neutralizing antibody was detected in vaccinated sheep blood upto 270 days post-vaccination after the homologous virus challenge. It can be postulated that there is a strong likelihood that sheep vaccinated with two shots of BEI inactivated pentavalent bluetongue vaccine will remain protected from homologous (BTV 1, 2, 10, 16 and 23) infections for more than 270 days of post-vaccination as evidenced by the presence of the stronger neutralizing antibody response compared to unvaccinated sheep having titer of <1:2 dilutions. This finding is in agreement with researchers[14,9] and who reported a protection for 6 months of sheep vaccinated with single dose of inactivated vaccine and 12 months if the dose is doubled. Oura *et al.*[28] reported neutralizing antibody up to 3 years of post vaccination however; it was not checked for its protection by challenge study.

There was significant difference ($p<0.05$) between vaccinated and unvaccinated sheep in their Mean±SE PI in cELISA, $\log_2$ Mean±SE value of neutralizing antibody in SNT, mean rectal temperature and development of clinical signs after homologues virus challenge. However, there was no significant difference ($p>0.05$) between different groups of vaccinated and unvaccinated sheep in all above parameters due to variability of challenge serotypes. This finding is in accordance with the findings of different researchers[9,13,29].

## CONCLUSION

While analyzing the humoral immune response, it was found that vaccinated animals showed high amount of group specific and serotype specific (neutralizing) antibody response compared to unvaccinated sheep. In addition to this fever and clinical signs were not evident following challenges with homologues virus. There was statistically significant difference between vaccinated and unvaccinated animals in their group or type specific antibody, fever and clinical sign development. However, there was no significant difference in their group or type specific antibody, fever and clinical sign development as a result of difference in the BTV serotype used as challenge. This result is supported by cellular immune response and viraemia study. The results clearly indicated that BEI inactivated montanide adjuvanted pentavalent bluetongue vaccine was very effective in protecting sheep from infection with any of the 5 serotypes of BT virus and the use of this pentavalent vaccine in susceptible sheep in endemic areas would definitely reduce the incidence of the BT disease and enormous economic losses.

## ACKNOWLEDGMENTS

This study was supported by the All India Network Programme on Bluetongue Disease, Indian Council of Agricultural Research (ICAR), New-Delhi, India. The researchers are thankful to the Director, Indian Veterinary Research Institute for providing the necessary facilities in carrying out the study.

## REFERENCES

1. Van Regenmortel, M.H.V., 2003. Viruses are real, virus species are man-made, taxonomic constructions. Arch. Virol., 148 : 2481-2488.
2. Mertens, P.P., J. Diprose, S. Maan, K.P. Singh, H. Attoui and A.R. Samuel, 2004. Bluetongue virus replication, molecular and structural biology. Veterinaria Italiana, 40: 426-437.
3. Hofmann, M.A., S. Renzullo, M. Mader, V. Chaignat, G. Worwa and B. Thuer, 2008. Genetic characterization of Toggenburg orbivirus, a new bluetongue virus, from Goats, Switzerland. Emerg. Infect. Dis., 14: 1855-1861.
4. Maan, S., N.S. Maan, K. Nomikou, C. Batten and F. Antony *et al*., 2011. Novel bluetongue virus serotype from Kuwait. Emerg. Infect. Dis., 17: 886-889.
5. Maan, N.S., S. Maan, D.J. Johnson, M.N. Belaganahalli and E.N. Ostlund *et al*., 2012. Identification and differentiation of the twenty six Bluetongue virus serotypes by RT-PCR amplification of the serotype-specific genome segment 2. PLoS ONE, Vol. 7. 10.1371/journal.pone.0032601
6. Batten, C.A., M.R. Henstock, H.M. Steedman, S. Waddington, L. Edwards and C.A.L. Oura, 2013. Bluetongue virus serotype 26: Infection kinetics, pathogenesis and possible contact transmission in goats. Veterinary Microbiol., 162: 62-67.
7. Schulz, C., E. Breard, C. Sailleau, M. Jenckel and C. Viarouge *et al*., 2016. Bluetongue virus serotype 27: Detection and characterization of two novel variants in Corsica, France. J. Gen. Virol., 97: 2073-2083.
8. OIE., 2013. OIE-Listed diseases, infections and infestations in force in 2013. http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2013/
9. Eschbaumer, M., B. Hoffmann, P. Konig, J.P. Teifke and J.M. Gethmann *et al*., 2009. Efficacy of three inactivated vaccines against bluetongue virus serotype 8 in sheep. Vaccine, 27: 4169-4175.
10. Sperlova, A. and D. Zendulkova, 2011. Bluetongue: A review. Vet. Med., 56: 430-452.
11. Verwoerd, D.W. and B.J. Erasmus, 2004. Bluetongue. In: Infectious Diseases of Livestock, Coetzer, J .A. and R.C. Tustin (Eds.). Oxford Press, Cape Town, pp: 1201-1220.
12. Bhanuprakash, V., B.K. Indrani, M. Hosamani, V. Balamurugan and R.K. Singh, 2009. Bluetongue vaccines: The past, present and future. Expert Rev. Vaccines, 8: 191-204.
13. Breard, E., G. Belbis, C. Hamers, V. Moulin and T. Lilin *et al*., 2011. Evaluation of humoral response and protective efficacy of two inactivated vaccines against bluetongue virus after vaccination of goats. Vaccine, 29: 2495-2502.
14. Savini, G., N.J. MacLachlan, J.M. Sanchez-Vizcaino and S. Zientara, 2008. Vaccines against bluetongue in Europe. Comp. Immunol. Microbiol. Infect. Dis., 31: 101-120.
15. Hamers, C., S. Rehbein, P. Hudelet, M. Blanchet and B. Lapostolle *et al*., 2009. Protective duration of immunity of an inactivated bluetongue (BTV) serotype 2 vaccine against a virulent BTV serotype 2 challenge in sheep. Vaccine, 27: 2789-2793.
16. Noad, R. and P. Roy, 2009. Bluetongue vaccines. Vaccine, 27: D86-D89.
17. Savini, G., C. Hamers, A. Conte, P. Migliaccio and B. Bonfini *et al*., 2009. Assessment of efficacy of a bivalent BTV-2 and BTV-4 inactivated vaccine by vaccination and challenge in cattle. Vet. Microbiol., 133 : 1-8.

18. Umeshappa, C.S., K.P. Singh, A.B. Pandey, R.P. Singh and R.H. Nanjundappa, 2010. Cell-mediated immune response and cross-protective efficacy of binary ethylenimine-inactivated bluetongue virus serotype-1 vaccine in sheep. Vaccine, 28: 2522-2531.
19. Umeshappa, C.S., K.P. Singh, K.A. Ahmed, A.B. Pandey and R.H. Nanjundappa, 2011. The measurement of three cytokine transcripts in naive and sensitized ovine peripheral blood mononuclear cells following *in vitro* stimulation with bluetongue virus serotype-23. Res. Vet. Sci., 90: 212-214.
20. Ramakrishnan, M.A., A.B. Pandey, K.P. Singh, R. Singh, S. Nandi and M.L. Mehrotra, 2006. Immune responses and protective efficacy of binary ethylenimine (BEI)-inactivated bluetongue virus vaccines in sheep. Vet. Res. Commun., 30: 873-880.
21. Oura, C.A.L., J.L.N. Woods, A.J. Sanders, A. Bin-Tarif and M. Henstock *et al*., 2009. Seroconversion, neutralising antibodies and protection in bluetongue serotype 8 vaccinated sheep. Vaccine, 27: 7326-7330.
22. Darpel, K.E., C.A. Batten, E. Veronesi, A.E. Shaw, S. Anthony and K. Bachanek-Bankowska *et al*., 2007. Clinical signs and pathology shown by British sheep and cattle infected with bluetongue virus serotype 8 derived from the 2006 outbreak in northern Europe. Vet Rec. 161: 253-261.
23. Moulin, V., C.V. Noordegraaf, B. Makoschey, M. van der Sluijs and E. Veronesi *et al*., 2012. Clinical disease in sheep caused by bluetongue virus serotype 8 and prevention by an inactivated vaccine. Vaccine, 30 : 2228-2235.
24. Boone, J.D., U.B. Balasuriya, K. Karaca, J.C. Audonnet and J. Yao *et al*., 2007. Recombinant canarypox virus vaccine co-expressing genes encoding the VP2 and VP5 outer capsid proteins of bluetongue virus induces high level protection in sheep. Vaccine, 25: 672-678.
25. Roy, P., 2004. Genetically engineered structure-based vaccine for bluetongue disease. Vet. Ital., 40: 594-600.
26. Roy, P., T. French and B.J. Erasmus, 1992. Protective efficacy of virus-like particles for bluetongue disease. Vaccine, 10: 28-32.
27. Roy, P., D.H. Bishop, H. LeBlois and B.J. Erasmus, 1994. Long-lasting protection of sheep against bluetongue challenge after vaccination with virus-like particles: Evidence for homologous and partial heterologous protection. Vaccine, 12: 805-811.
28. Oura, C.A.L., L. Edwards and C.A. Batten, 2012. Evaluation of the humoral immune response in adult dairy cattle three years after vaccination with a bluetongue serotype 8 inactivated vaccine. Vaccine, 30: 112-115.
29. Veronesi, E., K.E. Darpel, C. Hamblin, S. Carpenter and H.H. Takamatsu *et al*., 2010. Viraemia and clinical disease in dorset poll sheep following vaccination with live attenuated bluetongue virus vaccines serotypes 16 and 4. Vaccine, 28: 1397-1403.