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# Comparative efficacy of montanide and aluminium hydroxide gel adjuvanted trivalent inactivated bluetongue virus vaccines

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Bluetongue (BT), an OIE notifiable, infectious, non-contagious, arthropod-borne viral disease of ruminants owing to bluetongue virus (BTV) causes annual loss of US$3 billion across the world (Tabachnick *et al.* 1996). In India bluetongue accounted for 77.1% of the total economic losses due to all diseases in sheep. The annual loss was maximum in the year 2005 (2606.96 lakh) according to Division of Livestock Economics, Statistics and Information Technology, IVRI. There are at least 24 serotypes of BTV in the world of which 21 (except 19, 22 and 24) are reported to be circulating in India based either on serology or virus isolation studies (Ramakrishnan *et al.* 2005). In general, vaccination using either live attenuated or killed or recombinant vaccines is the main strategy to combat BT (Murray *et al.* 1996). Attenuated vaccines are widely used in many countries including South Africa, USA (Stott *et al.* 1985) and China. However, live vaccines have several disadvantages including abortions in pregnant ewes, genetic reassortment with field virus and possible persistence in animals (Campbell 1985). Further, attenuated vaccines may revert back and cause disease outbreaks (Foster *et al.* 1968). Therefore, the present investigation was taken up to study the efficiency of montanide and algel adjuvants in developing humoral and cellular immunity for a BT inactivated vaccine.

*Virus and cells:* Bluetongue virus serotypes namely BTV-2, BTV-9 and BTV-15, isolated from sheep (Bommineni *et al.* 2008) during the outbreaks occurred at different times in different areas of Andhra Pradesh, are being maintained at the Department of Veterinary Microbiology, College of Veterinary Science, Hyderabad, Andhra Pradesh. Vero (African green monkey kidney) cell lines obtained from Veterinary Biologicals Research Institute (VBRI), Shanthinagar, Hyderabad, Andhra Pradesh were used in the present study.

*Experimental animals:* The sheep (Deccani breed) used in this experiment were procured from Livestock Research Station, Mahaboobnagar, Andhra Pradesh and screened for the presence of precipitating antibodies to BTV by agar gel immuno diffusion test (AGID) and competitive enzyme linked immunosorbant assay (c-ELISA). Bluetongue seronegative sheep (17) aged 6 months to 1 year were selected for use in the present study and shifted to a fly proof experimental animal house. All the animals were dewormed with fenbendazole (7.5 mg/kg body weight) and held for 1 week before the start of the experiment for acclimatization. Animal experimentation was performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), India.

*Inactivation of the virus:* The virus was inactivated as per Bahnemann (1990) with few modifications (Barteling and Cassim 2003). The inactivated viral harvest was given 3 consecutive passages in vero cells to check for residual infectivity, and also the double stranded RNA of the inactivated BTV was extracted and checked for the typical banding pattern.

*Formulation of vaccines:* The vaccines were prepared by mixing equal volumes of the above inactivated viral harvest with montanide (kindly provided by Dr Y K M Reddy, TANUVAS) and Al-gel adjuvants and kept for rapid stirring at 4°C for 30 min for proper mixing of the inactivated virus with the adjuvants. The vaccines were stored in aliquots of 30 ml each at 4°C until further use.

*Experimental design:* Seronegative sheep (17) of Deccani breed were selected and randomly divided into 3 groups. One group (Montanide group) of 6 sheep received 2 ml of Montanide adjuvanted vaccine via subcutaneous route and another group (Al-gel group) of 6 sheep received 2 ml of Al-

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gel adjuvanted vaccine via the same route. Third group of 5 sheep (uninoculated) were kept as controls.

*Collection of blood samples:* Blood samples were collected on 0, 7, 10,14, 21, 28, 35 and 42 days post inoculation (dpi) in heparin vacuutainers and were used for lymphocyte proliferation assay (LPA) by MTT method on the same day, and the serum collected from non-heparinized blood was inactivated at 56°C for 30 min and stored at −20°C for serological testing.

*Humoral immune response:* The presence of group specific, precipitating, non-neutralizing antibodies were detected by using standard methods of AGID (Jochim *et al.* 1969) and c-ELISA (Hubshle *et al.* 1981) while, serotype specific neutralizing antibodies were detected by standard serum neutralization test (SNT) (Parker *et al.* 1975).

*Cell mediated immune response : lymphocyte stimulation index -MTT assay:* The procedure for lymphocyte proliferation assay (LPA) using MTT tetrazolium salt was performed as per Bounous *et al.* (1992). The mean stimulation indices of the groups were compared by using ANOVA. Statistical significance was expressed as significant differences against 'F' critical values at 5% levels.

The residual infectivity of the inactivated virus was not detected as revealed by the absence of cytopathic effect (CPE) in vero cells. Further, the band pattern of BT viral nucleic acid was absent in the PAGE indicating the complete inactivation of the viruses and safety of the vaccine preparations.

*Humoral immune response:* The 6 sheep in Al-gel group and 5 sheep in Montanide group were positive for AGID and c-ELISA by 21 dpi and remained so throught the study i.e 42 dpi. The detailed results were given in Table 1.

The average group serum neutralization titers for all the serotypes were higher in the montanide group when compared to the Algel group and the detailed SN titers were presented in Table 2.

Table 1. Comparison of sero-conversion of sheep vaccinated with montanide and Al-gel adjuvanted bluetongue virus vaccines

| Days P.I | *Number of sheep seroconverted | | | |
|---|---|---|---|---|
| | *Montanide group | | Al-gel group | |
| | c-ELISA | AGID | c,-ELISA | AGID |
| 0 | - | - | - | - |
| 7 | 1 | - | 3 | - |
| 10 | 4 | 4 | 3 | 3 |
| 14 | 5 | 5 | 4 | 4 |
| 21 | 5 | 5 | 6 | 6 |
| 28 | 5 | 5 | 6 | 6 |
| 35 | 5 | 5 | 6 | 6 |
| 42 | 5 | 5 | 6 | 6 |

PI, Post inoculation; *1 animal did not show any serological response to the vaccine in any of the serological tests throughout the experiment.

Table 2. Comparison of group average SN titers ($Log_2$ serum dilutions) in montanide and Al-gel groups for different serotypes

| Days PI | Montanide group | | | Al-gel group | | |
|---|---|---|---|---|---|---|
| | BTV-2 | BTV-9 | BTV-15 | BTV-2 | BTV-9 | BTV-15 |
| 7 | - | - | - | - | - | - |
| 10 | 5.26 | - | - | 5.40 | - | - |
| 14 | 5.26 | 3.88 | 4.0 | 5.11 | 3.1 | 4.13 |
| 21 | 5.6 | 4.83 | 4.8 | 5.05 | 4.38 | 4.83 |
| 28 | 5.86 | 4.86 | 5.89 | 4.6 | 5.88 | 4.88 |
| 35 | 6.09 | 5.69 | 5.26 | 5.41 | 5.26 | 4.22 |
| 42 | 5.92 | 5.61 | 5.17 | 5.26 | 5.69 | 5.01 |
| Avg.SN titers | 5.67 | 4.97 | 5.02 | 5.14 | 4.86 | 4.61 |

*Cell mediated immune responses*

The mean stimulation indices for all the 3 groups were calculated and the results were compared statistically by using one way ANOVA (Table 3).

The aim of the present study was to develop a trivalent inactivated bluetongue vaccine using 2 different adjuvants, viz. montanide and Al-gel, and compare their efficacies. In eliciting humoral immune response, montanide adjuvanted vaccine was slightly more potent (Emidio *et al.* 2004) when compared to Al-gel adjuvanted vaccine. BEI inactivated and Al-gel adjuvanted vaccine showed low serum neutralizing antibodies as observed by Anselmo *et al.* (1999). Similar observations were also seen in this study. Group specific non neutralizing antibodies were detected as early as 7 dpi by c-ELISA and 10dpi by AGID and this could be due to the more sensitivity of c-ELISA compared to AGID. All the sheep in montanide group seroconverted early (14 dpi) as detected by AGID and c-ELISA; whereas, Al-gel group seroconverted by 21dpi. This can be attributed to the efficacy of the adjuvant montanide. The mean SN titers for BTV-2, 9 and 15 (Table 2) for montanide group are higher when compared to Al-gel group (individual titers not given). Though the neutralizing antibodies for different serotypes were detected at the same time in both the groups, the average neutralizing antibody titers were higher in montanide group and this variation could be due to adjuvant. However one sheep in montanide group remained seronegative during the entire period of the

Table 3. Comparison of CMI response in montanide and Al-gel vaccinated groups

| Groups | Mean stimulation index on days PI | | | | | |
|---|---|---|---|---|---|---|
| | 7 | 14 | 21 | 28 | 35 | 42 |
| Montanide | 0.758 [a] | 0.873 [a] | 1.992 [a] | 0.612 [a] | 0.887 [a] | 0.172 [a] |
| Al-gel | 1.054 [a] | 1.044 [a] | 1.821 [a] | 0.723 [a] | 0.450 [a] | 0.185 [a] |
| Control | 0.031 [b] | –0.015 [b] | 0.123 [b] | 0.136 [b] | 0.062 [b] | 0.008 [b] |

[a,b] Mean values with different superscripts vary significantly ($P \leq 0.05$).

experiment and considered as non responder as it did not react to any of the serological tests. This might be due to idiosyncrasy of the animal as previously explained by Neitz (1948) and Luedke *et al.* (1964). The results of lymphocyte stimulation index showed no significant difference between montanide and Al-gel groups with regard to CMI response. Hence it can be concluded that the Montanide is a better adjuvant in eliciting humoral immunity than that of Al-gel. Further, there was no significant difference with respect to CMI response; as most of the inactivated vaccines stimulate cellular immunity poorly. Prospectively, efficacy of the vaccines can be evaluated by boosting the animals with vaccines and challenging them with field strains.

Inactivated vaccines are safer than the live attenuated vaccines especially in the bluetongue disease because of adverse reactions such as abortions in pregnants and reversion to virulence in immunocomprimised animals may be seen with attenuated vaccines. Further montanide is better adjuvant in eliciting humoral immunity when compared with Aluminium hydroxide gel.

## SUMMARY

In the present investigation, an attempt was made to evaluate and compare montanide and aluminium hydroxide gel (Al-gel) adjuvants for a trivalent inactivated bluetongue vaccine. The vaccine was prepared with three indigenous bluetongue virus serotypes i.e BTV-2, 9 and 15. All the serotypes were inactivated using Binary Ethylene Imine (BEI) plus formaldehyde and mixed with 2 different adjuvants, viz. montanide and Al-gel. The effect of adjuvants was studied by inoculating the vaccine and measuring the antibody titers and cell mediated immune (CMI) responses in sheep. It was found that montanide adjuvanted vaccine was slightly more potent in eliciting neutralizing antibodies when compared with Algel adjuvanted vaccine and there was no significant difference regarding CMI response for both the adjuvants.

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