Evaluation of three oil-adjuvant vaccines against *Pasteurella multocida* in buffalo calves

R. MUNEER, S. AKHTAR and M. AFZAL *

**Summary:** Three oil-adjuvant vaccines of *Pasteurella multocida* 6:B were evaluated with respect to the level and duration of the humoral immune response produced in buffalo calves. Preparation 1 was a water-in-oil emulsion containing Marcol 52, Montanide 888 and antigen at a ratio of 6:1:3. Preparation 2 was a double emulsion containing Marcol 52, Arlacel A and Tween 80 in addition to antigen. Preparation 3 contained α-tocopheryl acetate (vitamin E), Montanide 888 and antigen. All three preparations induced a similar sustained immune response in buffalo calves beyond 270 days post-vaccination.

KEYWORDS: Adjuvants – Buffalo – Haemorrhagic septicaemia – Oil adjuvant – *Pasteurella multocida* – Vaccines.

**INTRODUCTION**

Haemorrhagic septicaemia is a highly contagious disease of cattle and buffalo, caused by *Pasteurella multocida* 6:B. Vaccination is used for the control of this disease in all countries where it occurs. Formalin-killed, alum-precipitated bacterin (APB) is the most widely-used vaccine, but this confers a relatively low level of immunity for a short duration. Water-in-oil adjuvant vaccines of *P. multocida* using mineral oil and lanoline have been developed and are used in some countries. Although these vaccines confer a higher level of immunity for approximately one year, injectability is poor due to high viscosity.

In a previous publication, Muneer and Afzal (10) reported the development and properties of two emulsions of lower viscosity and higher stability prepared from highly-purified mineral oils. The above study involved only a small number of buffalo calves, and the results still need to be verified in a larger trial.

Vitamin E has been reported as being a potent immuno-enhancer when administered as a dietary supplement or as an immuno-adjuvant in vaccines against various infectious diseases in a number of animal species (11, 12, 13, 14). Vitamin E as an antioxidant and cell-membrane stabiliser enhances humoral antibody production and phagocytosis, probably by regulating prostaglandin and cyclic nucleotide biosynthesis.

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The objectives of the present study were as follows:

a) to formulate an oil-adjuvant vaccine of low viscosity against *P. multocida*
b) to compare the effect of vitamin E with that of other standard adjuvants.

**MATERIALS AND METHODS**

**Animals**

Forty-five buffalo calves of comparable age and weight were used in this study; the animals were kept at the Livestock Research Station of the Animal Sciences Institute in Islamabad, Pakistan. The animals were fed seasonal green fodder supplemented with concentrates. The animals were randomly divided into five groups. Animals in group 1 were vaccinated with APB, while groups 2, 3 and 4 received oil-adjuvant vaccines 1, 2 and 3 (OAV$_1$, OAV$_2$, and OAV$_3$), respectively (Table I). Group 5 was comprised of five animals serving as non-vaccinated controls. All vaccines were administered subcutaneously in 3-4 ml doses. A booster dose of each vaccine was administered two months after the sensitising dose. Prior to vaccination, a serum sample was collected from each calf in all five groups. Post-vaccination serum samples were collected at monthly intervals for the first three months, and subsequently four samplings were conducted at approximately two-month intervals. Sera were separated from blood samples and stored at $-30^\circ$C until analysed.

**TABLE I**

*Biochemical composition of different oil-adjuvant vaccines (OAVs) against *Pasturella multocida*

<table>
<thead>
<tr>
<th>Constituent</th>
<th>OAV$_1$</th>
<th>OAV$_2$</th>
<th>OAV$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marcol 52 (oil)</td>
<td>60.0</td>
<td>63.0</td>
<td>–</td>
</tr>
<tr>
<td>$\alpha$-tocopherol acetate (oil)</td>
<td>–</td>
<td>–</td>
<td>62.0</td>
</tr>
<tr>
<td>Montanide 888 (emulsifier)</td>
<td>10.0</td>
<td>–</td>
<td>8.0</td>
</tr>
<tr>
<td>Arlacel A (emulsifier)</td>
<td>–</td>
<td>7.0</td>
<td>–</td>
</tr>
<tr>
<td>Tween 80 (emulsifier)</td>
<td>–</td>
<td>1.5</td>
<td>–</td>
</tr>
<tr>
<td>Pasteurella suspension (antigen)</td>
<td>30.0</td>
<td>28.5</td>
<td>30.0</td>
</tr>
</tbody>
</table>

* vitamin E
** each preparation contained the same number of bacterial cells ($2 \times 10^9$/ml) as alum-precipitated bacterin

**Vaccines**

*Alum-precipitated bacterin*

Commercially-available formalinised APB of *P. multocida* containing $2 \times 10^9$ cells/ml was used in the experiment.

*Oil-adjuvanted vaccine*

The methods of preparation of OAV$_1$ and OAV$_2$ were essentially as reported elsewhere (10) and are briefly outlined below.
*P. multocida* 6:B was grown in tryptose broth at 37°C for 24 h in a shaking water bath. The bacterial growth was centrifuged at 3,000 rpm for 30 min. The pellet was washed and treated with 0.5% formaldehyde overnight at 4°C. This suspension was centrifuged and suspended in normal saline. The bacterial concentration was adjusted so that the vaccine contained the same number of organisms as the APB.

**OAV₁**

Adjuvant 1 was prepared by mixing Marcol 52, Montanide 888 and *P. multocida* 6:B suspension at a ratio of 6:1:3. Marcol 52 and Montanide 888 were mixed at low speed (13,000 rpm). *Pasteurella* suspension was gradually added over a period of 5 min. High-speed mixing (18,000 rpm) was then performed for 15 min to prepare the adjuvant.

**OAV₂**

Adjuvant 2 was prepared by mixing together 6.3 ml Marcol 52 and 0.7 ml Arlacel A for 5 min at low speed. Separately, Tween 80 (5%) was mixed with 3.0 ml *Pasteurella* suspension. Both sets of constituents were then gradually mixed together for 5 min, followed by high-speed mixing for 15 min.

**OAV₃**

The third vaccine was prepared by mixing 6.3 ml α-d-tocopheryl acetate (vitamin E) with 0.8 ml Montanide 888 at low speed (13,000 rpm). *Pasteurella* suspension (3 ml) was gradually mixed in for 5 min, followed by high-speed mixing for 15 min.

The bacterial concentration in all three oil-adjuvant vaccines was the same as in the APB. The stability of this emulsion was similar to that of similar vaccines using other adjuvants.

**Enzyme-linked immunosorbent assay**

Antibody titration of the serum samples was performed by indirect enzyme-linked immunosorbent assay (ELISA) (10). Briefly, flat-bottomed 96-well microtitre plates were sensitised with 100 µl sonicated *P. multocida* in carbonate coating buffer (pH 9.6) for 18 h at room temperature. The plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS–Tween 20). Test serum (100 µl diluted 1:50 in PBS–Tween 20) was added to the wells and the plates were incubated at 37°C for 2 h. After washing five times, 100 µl of rabbit anti-bovine peroxidase-conjugated IgG were added, and the plates were incubated at 37°C for 2 h. The plates were then washed five times. Substrate (O-phenylenediamine dihydrochloride with 0.01% hydrogen peroxide) was added, and the plates were kept in a dark place for 20 min to enable colour development to occur. The reaction was stopped by adding 2N sulphuric acid. The optical density of the plates was then read in an ELISA reader at 492 nm. The appropriate controls – including a sample without antigen, a positive serum and a negative serum – were run on each plate.

**Experimental challenge**

Two animals from each of the vaccinated groups and one animal from the control group were challenged with virulent *P. multocida* (6 × 10⁸ colony-forming units) administered subcutaneously at 6 months post-vaccination. At 12 months post-vaccination, experimental challenge was repeated using different animals (two from each experimental group and one from the control group).
RESULTS

The geometric mean ELISA titres recorded for buffalo calves in the three vaccination groups are depicted in Figure 1. Throughout the study period of 270 days, the mean titres of groups of calves vaccinated with OAV1, OAV2 and OAV3 remained substantially higher than those of both the group vaccinated with APB and the non-vaccinated control group. Although the mean ELISA titres elicited by OAV1 and OAV2 were persistently higher than titres against OAV3 throughout the study, the titres for all three vaccines were very similar.

\[ \text{ FIG. 1 } \]

*Geometric mean antibody titres of buffalo vaccinated with vaccine against haemorrhagic septicaemia, measured by enzyme-linked immunosorbent assay (ELISA)*
On experimental challenge at 6 and 12 months post-vaccination, the animals vaccinated with OAV₁, OAV₂ and OAV₃ withstood experimental challenge, and no untoward reaction (e.g. elevation of body temperature or oedema at the inoculation site) was observed. However, animals injected with APB showed clinical symptoms of disease on post-vaccination challenge at 6 and 12 months, although these animals subsequently achieved complete recovery from clinical disease. The animals in the control group died, showing overt disease, on challenge at both 6 months and 12 months post-vaccination.

**DISCUSSION**

Use of vitamin E as a dietary supplement is known to enhance humoral response against bacterial infection by promoting co-operation between the T and B lymphocytes and macrophages (4, 5, 12). Administration of vitamin E as an adjuvant has been reported as inducing greater enhancement of antibody production in immunising sheep against Clostridium perfringens type D than administration as a dietary supplement (14). Vitamin E adjuvant has also been reported as improving the protection conferred by vaccine against experimental infection with Brucella ovis (1). Recently, the possibility of using vitamin E as an adjuvant in vaccine against P. multocida has been indicated (3).

In the present study, the authors sought to verify the previous results obtained using two oil-adjuvant vaccines (10), and to evaluate the inoculation of buffalo calves using P. multocida vaccine emulsified with vitamin E. The ELISA titres elicited by OAV₁, OAV₂ and OAV₃ remained substantially higher than those obtained using APB following sensitising and booster vaccination. This difference in titres was sustained until at least 270 days post-vaccination. These observations confirmed the results of previous studies (2, 10).

The results of the present study did not enable the authors to prove the working hypothesis that OAV₃ might induce a better immune response than other oil-adjuvant vaccines against P. multocida. The mean ELISA titres in the group inoculated with OAV₃ were slightly lower than those in groups injected with OAV₁ and OAV₂, but were very similar. However, the above results are in agreement with those of previous studies, wherein partial replacement of mineral oil with vitamin E failed to improve immune response against Escherichia coli in chickens (6, 7, 9) and against Pasteurella anatipestifer in turkeys (8). The present results may reflect a weak association between the immune response to these bacterial antigens and the activity of T lymphocytes influenced by vitamin E (12).

The results presented above on the use of vitamin E as a complete replacement for mineral oil differed from those of Afzal et al. (1) and Tengerdy et al. (14), who reported improved immune response in sheep when mineral oil was completely replaced by vitamin E in emulsified vaccines against B. ovis (1) and C and D toxins of C. perfringens (4). This may have been due to different sensitivity to these antigens in sheep, or species variation in response to vitamin E adjuvant.

To the knowledge of the authors, this is the first report on the use of vitamin E as an adjuvant in vaccine against P. multocida in buffalo calves. The above results therefore require verification in a large-scale study.
ÉVALUATION DE TROIS VACCINS ADDITIONNÉS D’UN ADJUVANT HUILEUX CONTRE PASTEURELLA MULTOCIDA CHEZ DES BUFFLONS. - R. Muneer, S. Akhtar et M. Afzal.


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**RESUMEN** : Se presenta aquí la evaluación de tres vacunas con adyuvante oleoso contra Pasteurella multocida 6:B, realizada según el nivel y la duración de la respuesta inmune humoral inducida en crías de búfalos. La preparación N°1 era una emulsión «agua en aceite» que asociaba Marcol 52, Montanide 888 y antígeno en proporción 6:1:3. La preparación N°2 era una doble emulsión que incluía Marcol 52, Arlacel A y Tween 80, además del antígeno. Por último, la preparación N°3 estaba compuesta de acetato de α-tocoferol (vitamina E), Montanide 888 y antígeno. Las tres vacunas produjeron una respuesta inmune superior a 270 días en las crías de búfalos.


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**REFERENCES**


