Preliminary studies on improved oil-adjuvant vaccine for haemorrhagic septicaemia in buffalo calves

R. MUNEEER and M. AFZAL *

Summary: Two oil-adjuvant vaccines of Pasteurella multocida Robert's type 1 were prepared and evaluated for induction of an immune response in buffalo calves. Adjuvant 1 was a water-in-oil emulsion and contained Marcol 52, Montanide 103 and antigen in ratios of 6:1:3. Adjuvant 2 was a double emulsion and contained Marcol 52, Arlacel A and Tween 80 besides antigen. Viscosity of the adjuvants was low (easily injectable) and the oil phase did not separate upon centrifugation at 1,000 g for 30 minutes or standing at room temperature for 30 days. Both preparations induced sustained high antibody titres in buffalo calves beyond 230 days after vaccination.

KEYWORDS: Buffalo - Haemorrhagic septicaemia - Inactivated vaccines - Pasteurella multocida.

INTRODUCTION

Haemorrhagic septicaemia (HS) is the commonest contagious bacterial infection of cattle and buffaloes in Pakistan and other South-East Asian countries (2, 5). The disease is caused by Pasteurella multocida Robert's type 1. It is highly fatal, with a mortality rate above 70%. The disease occurs in both cattle and buffaloes, but at least in South-East Asia, many more buffaloes die from HS than cattle (6).

Formalin-killed, alum-precipitated bacterin of Pasteurella multocida is presently the most widely used vaccine for preventing HS. However, immunity lasts for 4 to 5 months and protective efficacy is only 60% (4). Water-in-oil adjuvant vaccines of P. multocida using mineral oil and lanoline have been developed and used in some countries. However, the poor stability of the emulsion and high viscosity have hampered the widespread use of these vaccines (4, 5).

This report describes two new oil adjuvants which are stable and easily injectable, for use in HS vaccines.

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MATERIALS AND METHODS

Animals

Ten unvaccinated buffalo calves more than four months old, born at the Livestock Research Station of the Animal Sciences Institute, were used. Nine of them were randomly divided into three groups and vaccinated with *P. multocida* alum-precipitated bacterin or oil adjuvant vaccines. One served as unvaccinated control. Calves in all groups were similar in age and weight. The animals were fed green fodder and were kept in a herd of 26 calves at the Station.

Vaccines

**Alum-precipitated bacterin**

Commercially available *P. multocida* vaccine prepared by Veterinary Research Institute, Lahore (Batch No. 45) was used in the experiment. It is a formalinised alum-precipitated bacterin of *P. multocida* Robert's type 1 and contains $1 \times 10^{10}$ cells per 5 ml.

**Adjuvant vaccine 1**

*P. multocida* Robert’s type 1 was grown in tryptose broth (Difco Laboratories, Detroit, USA) at 37°C for 24 hours in a shaking water bath. The bacterial growth was centrifuged at 3,000 rpm for 30 minutes. The pellet was washed and treated with 0.5% formaldehyde overnight at 4°C. The 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 alum-precipitated bacterin.

Adjuvant was prepared by mixing Marcol 52, Montanide 103 (kindly donated by SEPPIC, Paris, France) and *Pasteurella* suspension in the ratio of 6:1:3. Marcol 52 and Montanide 103 were mixed at low speed (13,000 rpm) in a Hamilton Beach Drink Mixer (Hamilton Beach Div., Scovill, NC, USA). *Pasteurella* suspension was gradually added over a period of five minutes. High-speed mixing (18,000 rpm) was then done for fifteen minutes to prepare the adjuvant.

**Adjuvant vaccine 2**

This adjuvant was prepared by mixing 6.3 ml Marcol 52 and 0.7 ml Arlacel A for five minutes in the same mixer at low speed. Separately, Tween 80 (5%) was mixed with 3.0 ml *Pasteurella* suspension. Both constituents were gradually mixed for five minutes, followed by high-speed mixing for fifteen minutes.

All vaccines were injected in a 2 ml dose subcutaneously. A booster dose of 2 ml of each vaccine was given two months after the sensitising dose.

Antibody titration

Serum samples were collected at regular intervals before and after vaccination. They were stored at $-30^\circ$C until antibody titration.

Antibody titration was carried out in quadruplicate by an indirect enzyme-linked immunosorbent assay (3). Briefly, U-shaped 96-well microtitre plates (Flow Laboratories, Irvine, UK) were sensitised with 100ml of 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 containing 0.05% Tween 20 (PBS-Tween 20). Test serum (100µl diluted 1:50 in PBS-Tween 20) was added to the wells, and the plates were incubated for 2 h at 37°C. After washing five times, 100µl of rabbit antibovine peroxidase-conjugated IgG (Cappel Labs., Malvern, PA, USA) was added and the plates incubated at 37°C for 2 h. The plates were washed five times. Substrate (O-phenylenediamine dihydrochloride + 0.01% hydrogen peroxide) was added and the plates kept in the dark for 20 min for colour development. The reaction was stopped by adding 2N sulphuric acid. The plates were read in an ELISA reader (Titertek Multiskan MCC/340) at 492 nm. Proper controls including a sample without antigen, a positive serum and a negative serum were run on each plate.

RESULTS

The adjuvant preparations possessed satisfactory physical properties (Table I). The aqueous phase did not separate from the oil phase upon centrifugation at 1,000 g for 30 min or standing at room temperature for 30 days.

TABLE I

<table>
<thead>
<tr>
<th>Physical properties of oil-adjuvant preparations</th>
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<tbody>
<tr>
<td>Parameter</td>
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<tr>
<td>Dispersion in cold water *</td>
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<tr>
<td>Viscosity (flow of 0.5 ml through 1 ml pipette)</td>
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<tr>
<td>Separation upon centrifugation at 1,000 g for 30 minutes</td>
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<tr>
<td>Separation on standing at room temperature for 30 days</td>
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* poor (−) to excellent (+ + +)

Antibody titres against *P. multocida* Robert’s type 1 rose considerably following the sensitising dose (Figure 1). In calves vaccinated with alum-precipitated bacterin, the titres declined sharply, but increased again after the booster vaccination. In both groups vaccinated with oil adjuvants, antibody titres did not decrease and remained at high levels throughout the experiment. The unvaccinated calf showed no rise in antibody titres during the period of study, and ELISA absorbance readings ranged from 0.15 to 0.38.

A natural outbreak of haemorrhagic septicaemia occurred among animals at the farm, and two calves died. None of the vaccinated calves died, and none showed signs of the disease. Two calves vaccinated with alum-precipitated bacterin, two calves vaccinated with oil adjuvant 1 and one calf vaccinated with oil adjuvant 2 had an increased rectal temperature during the outbreak, but recovered within 2-4 days.
1002

1003

Days post-vaccination

0 30 60 90 120 150 180 210 240

ELISA absorbance (492 nm)

\( \text{vaccine 1} \)

\( \text{vaccine 2} \)

\( \text{alum-precipitated bacterin} \)

\( \text{time of sensitising and booster vaccination} \)

\( \text{time when natural outbreak of HS occurred at the farm} \)

FIG. 1

Geometric mean antibody titres of buffalo calves vaccinated with HS vaccines

DISCUSSION

Oil adjuvants enhance the immune response to most antigens and prolong the duration of immunity. Field trials using oil adjuvants have proved their superiority over alum hydroxide and saponin for protecting against different diseases (7, 9, 10, 14). The limiting factors in their widespread use are high viscosity, poor stability and adverse reactions at the site of inoculation (4, 11). The oil adjuvants described in this study were stable, had low viscosity and did not produce any adverse effects. The use of Montanide 103 as emulsifier imparts good properties to the adjuvants (8).

Sustained high antibody titres seen in animals vaccinated with oil adjuvants corroborate the findings of previous studies (1, 11, 12, 13). However, this prolonged
immune response might have been due partly to subclinical exposure of the experimental animals to *P. multocida*, as indicated by a rise in temperature in some of the vaccinated animals. Since the vaccinated animals did not contract the disease in the natural outbreak, the experimental vaccines seem to provide the required protection. Further experimental and field trials of these oil-adjuvant vaccines are needed to evaluate their efficacy.

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**ÉTUDES PRÉLIMINAIRES SUR UN VACCIN AMÉLIORÉ EN ADJUVANT HUILEUX POUR L'IMMUNISATION DES BUFFLONS CONTRE LA SEPTICÉMIE HÉMORRAGIQUE.** - R. Muneer et M. Afzal.

**Résumé :** Deux vaccins en adjuvant huileux, à base de *Pasteurella multocida*, type 1 de Robert, ont été mis au point et évalués du point de vue de l'induction d'une réponse immunitaire chez les bufflons. Le premier était du type émulsion eau dans huile et contenait les adjuvants Marcol 52 et Montanide 103 et l'antigène dans des proportions de 6/1/3. Le second était du type émulsion double et contenait, outre l'antigène, du Marcol 52, de l'Arlacel A et du Tween 80. La viscosité des adjuvants était faible (vaccins faciles à injecter) et la phase huileuse ne se séparait pas après centrifugation à 1 000 g pendant 30 minutes ni après maintien pendant 30 jours à température ambiante. Les deux produits ont induit de façon soutenue des titres d'anticorps élevés chez les bufflons pendant une période s'étendant au-delà de 230 jours après vaccination.

**MOTS-CLÉS :** Buffle - *Pasteurella multocida* - Septicémie hémorragique - Vaccins inactivés.

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**ESTUDIOS PRELIMINARES SOBRE UNA VACUNA MEJORADA EN ADYUVANTE OLEOSO PARA LA INMUNIZACIÓN DE LOS BÚFALOS CONTRA LA SEPTICEMIA HEMORRÁGICA.** - R. Muneer y M. Afzal.

**Resumen:** Dos vacunas en adyuvante oleoso a base de *Pasteurella multocida*, tipo 1 de Robert, fueron elaboradas y evaluadas desde el punto de vista de la inducción de una respuesta inmunitaria en los búfalos. La primera era del tipo emulsión agua en aceite y contenía los adyuvantes Marcol 52, Montanide 103 y el antígeno en proporción de 6/1/3; la segunda era del tipo emulsión doble y contenía, además del antígeno, Marcol 52, Arlacel A y Tween 80. La viscosidad de los adyuvantes era débil (vacunas fáciles de inyectar) y la fase oleosa no se separaba tras centrifugación a 1.000 g durante 30 minutos ni tras manutención durante 30 días a la temperatura ambiente. Los dos productos indujeron de manera sostenida títulos de anticuerpos elevados en los búfalos durante un período superior a los 230 días después de la vacunación.
PALABRAS CLAVE: Búfalo - Pasteurella multocida - Septicemia hemorrágica - Vacunas inactivadas.

**REFERENCES**


