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IMMUNE RESPONSE OF RABBITS TO HEMORRHAGIC SEPTICEMIA VACCINE FORMULATIONS ADJUVANTED WITH MONTANIDE ISA-206, PARAFFIN OIL AND ALUM

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ABSTRACT

Based on safety and sound immunological principles for animal vaccines, there arises a need for identifying rational standards for selection of different adjuvant formulations. Montanide ISA206 (W/O/W) was used in this study to prepare the vaccine and was compared with already available paraffin oil and alum adjuvanted hemorrhagic septicemia vaccines. All the three vaccines were prepared according to the standard procedures keeping the antigen amount same in all the three vaccine formulations. The goal of the study was to ascertain immune response in rabbits against several *Pasteurella multocida* vaccine preparations including Montanide adjuvanted, paraffin oil based and alum-precipitated vaccines. Forty male adult rabbits were divided into four groups namely A, B, C and D and kept in separate cages. Rabbits in groups A, B and C were vaccinated via subcutaneous route with 0.3ml dose of Montanide vaccine, oil based vaccine and alum-precipitated vaccine respectively. A booster dose of 0.3 ml of same preparation was given 15 days post-vaccination. Rabbits in group D were kept unvaccinated as negative control. All rabbits were bled pre-vaccination and then every 7 days post-vaccination for nine weeks and sera were subjected to indirect haemagglutination assay (IHA) and geometric mean titres of anti-*Pasteurella multocida* antibodies were determined. The mean and standard errors were also determined. Results showed that Montanide ISA206 (W/O/W) adjuvanted vaccine gave higher antibody titres as compared with oil based and alum-precipitated vaccines.

Keywords: Montanide ISA-206; *Pasteurella multocida*; Hemorrhagic septicemia vaccine; Geometric mean titre; Immune response

INTRODUCTION

Hemorrhagic septicemia (HS), an economically important disease of dairy animals (cattle, buffalo) is caused by *Pasteurella multocida* serotypes which is normally found in the upper respiratory tract of animals (De Alwis, 1992). It is the major infectious and principal killer disease in ruminants (Maslog, 1998). Mortality rate due to this disease is about 70% and it causes annual losses of hundred million dollars to world's economy (May *et al*., 2001). The serotype B: 2 of Asian origin and the serotype E: 2 of African origin (Carter and Heddleston system), conforming to 6: B and 6: E (Namioka-Carter system) is responsible for this disease (Hopkins, 1998). It is a disease of bacterial origin and thought to be treated by a broad spectrum of antibiotics, but animals may be saved only if they have been medicated in the very initial phase of the disease (Benkiranei and De Alwis, 2002). Furthermore, after a short interval following the treatment infection recurs signifying that antibiotics are not an effective remedy to this condition; therapeutic failure usually results due to per-acute nature of disease, febrile condition of animals and development of resistance against antibiotics. Therefore, an effective control of disease could only be achieved by vaccination (Ali *et al*., 2000).

HS bacterin has been used in the past for vaccination but the period of immunity conferred upon was very short. Therefore, bacterin was switched over to adjuvanted vaccines. At present alum precipitated vaccine is being used as mass scale vaccine in Pakistan. Immunity conferred by this vaccine lasts for 3-4 months only, which reflects an unprotective state of vaccinated animals between two consecutive vaccinations (Vancheswara *et al*., 1955; Israil & Qauder, 1960) and its protective

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efficacy is only 60% (FAO, 1991). Therefore alum precipitated vaccine has been switched over to oil adjuvanted vaccine (OAV) at many places. The oil adjuvant vaccine protects the animals from the problem for one year as compared with alum precipitated vaccine (Neramitmansook, 1993).

Adjuvant technology has seemed significant advancement; as a result the researchers have used various adjuvants to improve immunogenicity of different vaccines. In the present study, alum precipitated, oil based and Montanide ISA206 adjuvanted vaccines were prepared according to the standard protocols. Antigen amount was same in all the three preparations and the experimental trial were conducted in rabbits to compare the humoral immune response of these prepared vaccines.

MATERIALS AND METHODS

Isolation and Identification of *Pasteurella multocida*

Pure culture of *Pasteurella multocida* B: 2 was used for vaccine preparation. The organism was obtained from Nuclear Institute of Agriculture and Biology (NIAB), Faisalabad where it was preserved on agar slants for the production of NIAB HS vaccine. The organism was identified and confirmed on the basis of morphology, staining reaction, culture characteristics, sugar fermentation tests, biochemical tests, serological test and pathogenicity test according to techniques described by Buxton and Fraser (1977), Sarma and Boro (1980).

Preparation of Vaccines

Pasteurella multocida was cultured in brain heart infusion broth for 8 hours at 37°C and 0.2 ml of this culture was injected intra peritoneally in laboratory mouse. Death of the mouse occurred within 24 hours. Blood was collected asceptically from the heart of mouse and then it was inoculated on nutrient and blood agar plates. The plates were incubated at 37°C for 24 hours. Single colony of *Pasteurella multocida* was picked from the uncontaminated agar plate and inoculated into 200 ml of CSY broth. It was incubated at 37°C for 24 hours in a rotatory incubator shaker at 200 rpm. This broth culture was centrifuged at 4000 rpm for 20 minutes to obtain pellet of the bacterial antigen. The bacterial pellet was mixed in 100 ml of normal saline and 0.3% of formaldehyde was added to inactivate the bacteria. After 24 hours incubation at 37°C, the formaldehyde was removed by centrifugation. Inactivated bacterial pellet was harvested in normal saline and optimization of the bacteria per ml was done by spectrophotometer at 640 nm wavelength. 20% transmittance was adjusted by adding normal saline. It was equal to 2×10^9 organisms per ml.

Vaccines were formulated such that number of inactivated bacterial cells per unit volume of each vaccine were kept same i.e. 6.4×10^8 bacterial cells/ml. This was achieved by preparing an initial stock of inactivated antigen with 20% transmittance containing 2×10^9 bacterial cells/ml (Muneer, 2005).

Vaccine formulations:

Montanide ISA-206 was agitated gently in the beaker and the aqueous part was added in the ratio 1:1(Reddy *et al*., 1995). Thirty two ml of inactivated bacterial antigen containing 2×10^9 bacterial cells/ml was diluted to a total of 50 using normal saline. This 50 ml was mixed with the adjuvant (50 ml) at a moderate speed at 31°C for 10 minutes.

While preparing oil based Haemorrhagic Septicaemia Vaccine, in solution I, antigen (32%) was mixed with Tween 80 (3%) i-e, 3 ml of Tween 80 was added slowly to a 32 ml antigen containing 2×10^9 bacterial cells/ml with continuous agitation. This solution I was homogenized at 1200 rpm for half an hour. In solution II, span 80 (7.5%) was mixed with paraffin oil (57.5%) i-e, 7.5 ml of span 80 was added slowly to 57.5 ml of paraffin oil undergoing continuous stirring. Homogenization was done at 1200 rpm for 0.5 hr. Afterwards Solution I was poured slowly into solution II and homogenized at 1200 rpm for 3 hr.

In alum-precipitated vaccine 10% sodium alum was added to the antigen solution (32 ml inactivated bacterial antigen was diluted to 90 ml by incorporating normal saline into it) to achieve the final concentration of 1% alum. Optimal pH was worked out to be 6.5±0.2. The precipitate was then centrifuged and washed twice with physiological saline. The precipitate was resuspended in physiological saline to the original volume of antigen.

Evaluation of humoral immune response in rabbits

The trial of Montanide adjuvanted vaccine (MAV), oil based vaccine (OBV) and alum precipitated vaccine (APV) was conducted in rabbits. 40 male rabbits of 5-6 months age and about 1.5-2.0 kg weight were divided into four groups (A, B, C and D) with each group containing 10 rabbits. Groups A, B and C were vaccinated with 0.3 ml of Montanide, oil based and alum precipitated vaccine respectively. Group D was kept as unvaccinated control. A booster dose of 0.3 ml was given after 15 days post vaccination.

The serum samples were collected on 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63 days post vaccination and anti-HS antibodies were titrated by indirect haemagglutinating assay (IHA) (Fraser *et al*., 1983).

Statistical analysis

Data were recorded and processed for calculation of GMT (the ultimate degree of positivity found in an antigen-antibody reaction, commonly expressed as the reciprocal of a serum dilution) to evaluate the immune response in rabbits (Villegas & Purchase, 1989).

RESULTS

HS-causing type B isolates of *P. multocida* when amplified with the primer pair (KTSP61-KTT72) produces a product of approximately 620 bp. (Fig 1)

KTT72	AGGCTCGTTTGGATTATGAAG
KTS P61	ATCCGCTAACACACTCTC

Conditions: initial denaturation at 95°C for 4 min, followed by **30 cycles of** denaturation at 95°C for 1 min, annealing at 55°C for 1min, extension at 72°C for 1 min, and a final extension at 72°C for 9 min

The Comparative Geometric Mean titres of Anti-*Pasteurella multocida* Antibodies in Rabbits at Different Days Post Vaccination are presented in the figure 2.

The Comparative mean antibody titers determined by IHA test in various groups of Rabbits is also found out and given in the tabulated (table 1) as well as in graphical layout (figure 3).

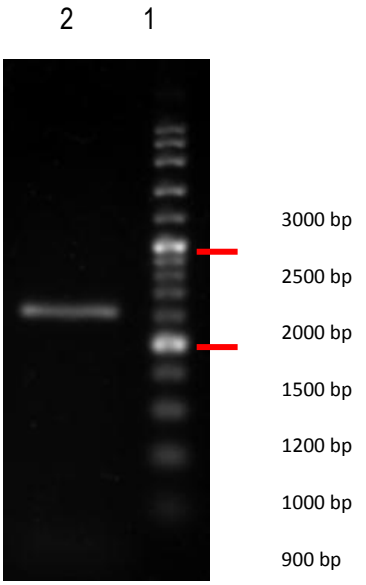

Figure 1: Lane1): 100 bp DNA ladder; Lanes2): Agarose gel electrophoresis (1.5%) of PCR product 618 bp obtained with primer pair KTSP61/KTT72 1F/1R.

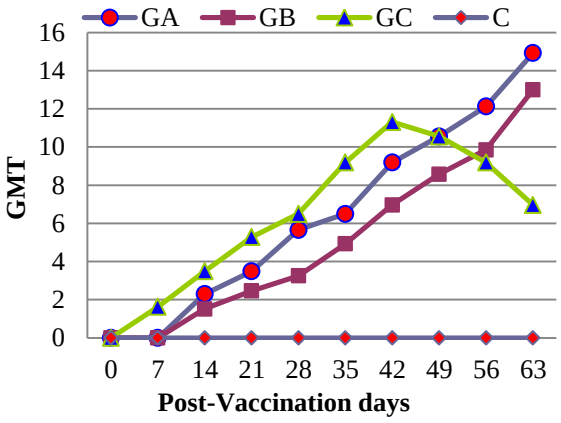

Figure 2: Comparative Geometric Mean titres of Anti-*Pasteurella multocida* Antibodies in Rabbits at Different Days Post Vaccination. GA = Montanide adjuvanted, GB= oil based, GC= Alum precipitated & GD= Control

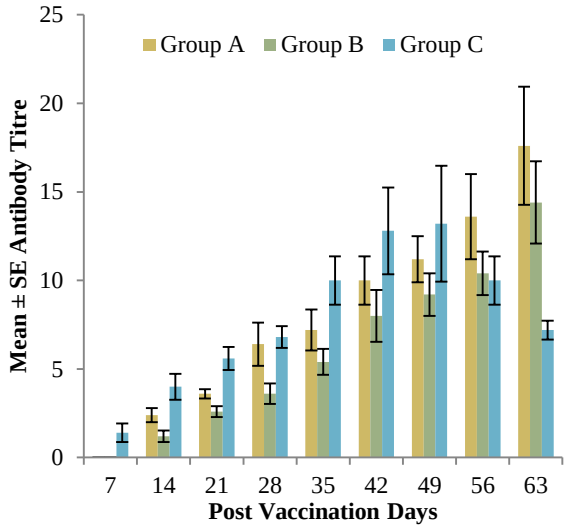

Figure 3: Comparative mean antibody titers in various groups of Rabbits. Progressive increase in antibody titers was more pronounced in case of Group A (Montanide adjuvanted vaccine group) from days 07 to 63 post vaccination. Significantly elevated antibody titers were found in Group C (Alum Precipitated vaccine), as compared to the Group A (Montanide adjuvanted vaccine) and Group B (Oil based vaccine), which dropped rapidly after 7th week.

Table 1: Antibody response following vaccination

Group	Days post-vaccination								
	7	14	21	28	35	42	49	56	63
A	0.00±0.00^{a}	2.40±0.40^{a}	3.60±0.27^{a}	6.40±1.22^{a}	7.20±1.16^{a}	10.00±1.37^{a}	11.20±1.31^{a}	13.60±2.40^{a}	17.60±3.33^{a}
B	0.00±0.00^{a}	1.20±0.33^{a}	2.60±0.31^{a}	3.60±0.58^{a}	5.40±0.73^{a}	8.00±1.46^{a}	9.20±1.20^{a}	10.40±1.22^{a}	14.40±2.32^{a}
C	1.40±0.52^{b}	4.00±0.73^{b}	5.60±0.65^{b}	6.80±0.61^{b}	10.00±1.37^{b}	12.80±2.44^{b}	13.20±3.27^{b}	10.00±1.37^{b}	7.20±0.53^{b}
D	0.00±0.00^{a}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}

Group A vaccinated with Montanide adjuvanted vaccine; Group B vaccinated with oil based vaccine; Group C vaccinated with alum precipitated vaccine; Group D unvaccinated control group; [data are expressed as mean ± SE; Means with no common superscript differ significantly ($P < 0.05$)]

DISCUSSION

The results obtained are summarized in table. To potentiate the immune response, adjuvants were added to inactivated bacterial cultures. Commonly used adjuvants include aluminium salts, lanolin, sodium alginate, saponin, mineral and vegetable oils (Bain *et al*., 1982; Muneer, 1993). Adjuvants in this experimental trial were Montanide ISA 206, paraffin oil and alum. Montanide ISA 206 is a Ready-to-use oily vaccine adjuvant which is based on high-grade injectable mineral oil. It produces double-emulsion vaccine very fluid, stable, well tolerated and induces short- and long-term immune response. This promising adjuvant was compared with the adjuvants that were already used in the preparation of hemorrhagic septicemia vaccines.

In this study, subcutaneous route was used for vaccination of rabbits instead of deep intramuscular or intra peritoneal route of inoculation. This route was proved to be safe without any adverse local reaction at the site of injection as was observed in the case of mice (Bhatti, 2005). A booster dose of 0.3 ml was given after 15 days post vaccination as was done by Gupta and Sareen, 1976; Tasneem, 1993. As in oil adjuvant vaccine antigen is slowly released from depot, the time interval of booster and challenge was increased. Serum sampling was done at 0, 7, 14, 21, 28, 35, 42, 49, 56, and 63 days post inoculation to keep fine track of antibody profile after vaccination.

The early rise in antibody response in case of Montanide vaccine was more pronounced as compared with the oil based vaccine. It seemed to be due to the reason that Montanide ISA 206 was reported to be responsible for increasing the immune response earlier as compared to the other oil based vaccines as described by Jang *et al*. (2010). In fact, Montanide ISA 206 makes a

W/O/W emulsion, the antigen in the external aqueous phase is immediately available to the immune system like aqueous formulations inducing short term immune response as described by Salt *et al*. 1998, whereas antigen in the internal aqueous phase is released slowly like water in oil emulsions inducing long term immune response. Reddy *et al*., 1995 also reported long term immunity and protection in bovine against hemorrhagic septicemia for 1 year after only one vaccination with Montanide adjuvanted vaccine. Ganne, 1994 had shown that W/O/W emulsion i-e, Montanide ISA 206 was based on mineral oil and was the only one able to induce IL6 cytokine and gave the best protection.

Increase in the immune response in case of oil based vaccine was slower but with gradual increase till the end of experiment. It seemed to be due to the absence of external aqueous phase in case of oil based vaccine. Oil based vaccines were mostly used to induce a sustained immune response in animals. These results corresponded to the work of Muneer *et al*. (1994) who evaluated three oil adjuvanted vaccines of *Pasteurella multocida* with respect to the level and duration of humoral immune response produced in buffalo calves. All the three preparations induced a similar sustained immune response beyond 270 days post vaccination. These results also correlated with the work of Shah *et al.* (2001). Muneer and Afzal, 1989 conducted field trials using oil adjuvants and proved their superiority over alum hydroxide and saponin for protecting against different diseases as previously described by Jolles & Paraf, (1973); Mckercher & Graves, (1977); De Alwis, (1981); Zanella & Marchi (1982).

These results conform the studies of Muneer (1993) who recorded higher antibody titres in buffalo calves vaccinated with OBHSV that persisted for about one year. It is also correlated with work of Vancheswara *et al*. (1955); Gomis *et al*. (1988 – 89) who reported the mineral oil and lanolin added vaccine (OAV) had a better and stronger immunogenic activity than the Alum precipitated bacterin, owing to their retention in the tissue as a depot for longer period thus, providing a prolonged antigenic stimulus for antibody formation.

Verma and Jaiswal (1998) reported, the alum based vaccines offer an immunity of 4-6 months, therefore should be administered twice a year. Alum precipitated vaccine is the most widely used vaccine in Asia and Africa. It consists of a bacterin to which Potash alum has been added to give a final concentration of 1 % (Tasneem *et al*., 2003). The disadvantages of this type of vaccine are that it only provides reliable immunity for three to four months and shock reactions can also occur (Bain *et al*., 1982). APHSV does not induce long lasting and effective immunity (Baig & Sheikh, 1982). It was noted that the existing GMT titres were dropped down soon after vaccination with APHSV. Alum might be toxic to the antigen presenting cells (APC) such as macrophages or B cells. It might be a possible reason of immunity breakdown in animals even with high level of antibody titre. Immune response, in the vaccinated animals is required against outermost components of the bacterial body i.e. capsule which is composed of mainly lipopolysaccharide (LPS) and minor fraction of proteins-exotoxins (Bain *et al*., 1982; Shah & Shah, 1998). Purified form of LPS induces B cell response and cannot be presented along with MHC II antigen by APC of the animal body, and hence the responsive B cells (plasma cell) cannot get cooperation of the T cell so, response to LPS is primary and the immunity is of low level and short duration (Abbas *et al*., 1991). Further, it was reported in OIE Manual that the antibody response of animals without or low antibody titre to bacterin containing alum is relatively short lived and antibody level decreases rapidly at 3-4 weeks after injection.

It is obvious from the experiment that both the Montanide and oil based vaccines gave better immune response in rabbits. However, the alum precipitated vaccine gave earlier serum antibody titre than the Montanide and oil based vaccine. So, it is recommended from the studies that primary vaccination should be done with alum precipitated vaccine followed by the secondary vaccination with Montanide or Oil based vaccine.

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