

PREPARATION AND EVALUATION OF HAEMORRHAGIC SEPTICAEMIA
OIL ADJUVANTED VACCINE WITH A NEW READY TO USE
OIL ADJUVANT FOR CATTLE AND BUFFALOES

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ABSTRACT

Haemorrhagic Septicaemia (HS) caused by *Pasteurella multocida* serotype B:2 is an economically important disease of cattle and buffaloes, which causes heavy economic losses due to sudden death of animals in developing countries like Pakistan. In this country, animals were being vaccinated by alum (adjuvant) precipitated vaccine twice a year. Immunity induced through this prophylactic measure lasts for 3 to 4 months only, which reflect an un-protective state of the animals between two vaccinations. A new HS oil based vaccine has been developed by adding an adjuvant Montanide ISA-50V2 with the ratio of 1:1. The bacterial dry weight has been adjusted to 2 mg/ml which results in to reduction of dose per animal from 5 ml (alum precipitated) to 2 ml. The addition of enrichments and aeration (sparging and vortexing) has resulted in dense bacterial growth of *Pasteurella multocida*. The new vaccine has passed sterility, safety and potency tests as per OIE, 2017. This new product has low viscosity and single shot is expected to confer solid immunity against HS for one year. Active Mouse Protection

Test, Passive Mouse Protection test and Indirect Haem-Agglutination Tests have been used to evaluate its potency. Properties like easy to inject with no side effects such as swelling at the injection site, have motivated the livestock owners to use this new product to protect their animals against fatal HS disease which will ultimately result in the increased productivity of livestock in Punjab, Pakistan.

Keywords: *Bubalus bubalis*, buffalo, haemorrhagic septicaemia, oil adjuvanted vaccine, active mouse, protection test, passive mouse protection test, IHA

INTRODUCTION

Haemorrhagic septicemia (HS) caused by *Pasteurella multocida* serotype B:2 is a highly fatal and economically devastating bacterial disease of cattle and water buffalo in Asia, Africa and Middle East with highest incidence in South East Asia (Shahzad *et al.*, 2013; OIE, 2018). Different studies indicated that *Pasteurella multocida* serotype B: 2 causes high mortality (up to 50%) in

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several parts of Pakistan (Khan *et al.*, 2011). The disease is more prevalent with highest mortality in buffalo calves of age 6 to 12 months (Farooq *et al.*, 2011). The disease is seasonal in its occurrence. The environment (high ambient temperature), management (overcrowding, inadequate ventilation and transportation) and malnutrition are among the plausible factors which are incriminated to potentiate the incidence of HS disease in Pakistan (Tariq *et al.*, 1997).

Acute nature and short duration of the disease are main causes of therapeutic failure in affected animals. The disease can only be controlled through mass vaccination programme. Different types of vaccines for HS disease are being prepared such as Bacterin, alum and aluminium hydroxide gel based vaccine and oil adjuvanted vaccine. Bacterins are readily absorbed from the site of inoculation and immunity is of limited duration. While alum and aluminium hydroxide gel based vaccine give the immunity only for 3 to 4 months. Oil adjuvanted vaccine has advantage over alum precipitated vaccine that it confers immunity for one year. In past, oil adjuvanted vaccine prepared from liquid paraffin and lanoline was being used. The vaccine prepared thus gave the immunity for one year (Bain *et al.*, 1982; Jaffri *et al.*, 2006; Kumar *et al.*, 2011) but having disadvantages of possessing high viscosity (difficult to inject), high dose rate (5 cc) and swelling at injection site. Montanide ISA-50 V2 is a ready to use oil adjuvant and has been proved for preparation of a user friendly, thinner emulsion/vaccine (Aucouturier *et al.*, 2001; Afrooz *et al.*, 2016).

Keeping in view the advantages Montanide ISA-50V2, the present study has been conducted to prepare oil based HS vaccine by using this oil and to evaluate its efficacies in lab and large animals.

MATERIALS AND METHODS

Activation of *Pasteurella multocida* seed

Freeze dried seed of *Pasteurella multocida* serotype B:2 was reconstituted with nutrient broth and incubated at 37°C for 4 to 5 h. The reconstituted seed was injected sub-cutaneously in Swiss Albino mice. Immediately after the death of mice, postmortem was conducted and heart blood was collected aseptically, cultured on Brain Heart Infusion (BHI) broth and incubated at 37°C for 24 h. Heart blood was also cultured on Nutrient, Mac-Conkey's and Sabouraud's agar slants and thioglycolate media to check the purity of the seed. After clearing the purity microscopically as well as on the basis of growth characteristics, the cultured seed in BHI was stored at 4°C (OIE, 2018).

Media preparation and vaccine production

For HS vaccine production BHI broth (Merck, India) was prepared with final pH 7.4. The media was checked for sterility by incubating it at 37°C for 24 h. Enrichments were prepared with following concentrations and sterilization temperatures as shown in Table 1.

All enrichments were checked for sterility by incubating at 37°C for 24 h. BHI broth prepared was cultured with the pure seed of *P. multocida* serotype B:2 at the rate of 50 ml per liter of media. The enrichments were transferred aseptically in BHI (OIE, 2018).

Aeration process

Cultured vessels were provided sterile air by syringe filters (0.2 µm pore size, Maxipore, England) through a compressor using sparging aeration and vortexing technique and air was dispersed through a sparger. The vessels were incubated at 37°C for 15 to 18 h on a shaker having

to-and-fro motion with 60 to 80 rpm. The purity of flasks was checked microscopically and growth was terminated by the addition of formalin (Merck, Germany) at the rate of 0.5% final concentration. The formalized cultured media was kept at 37°C for 24 h after shaking vigorously.

The Bacterin was checked for sterility on Nutrient agar, Mac Conkey's agar, Sabouraud's agar, Thioglycolate medium and Nutrient broth. Dry weight of the formalized culture was calculated as described by Bratbak and Dundas (1984) and adjusted to 2 mg per ml (OIE, 2018).

Preparation of HS oil adjuvanted vaccine

Oil adjuvanted vaccine was prepared by emulsification (10000 rpm to 15000 rpm) using a homogenizer (PAMCO, Pakistan) of equal volumes of light mineral oil Montanide ISA-50V2 (Seppic, France) and Bacterin. Preservative Thiomersal (Bio world, USA) was added at the rate of 0.003% and formalin (Merck, Germany) was added to a final concentration of 0.5%. Following overnight storage the mixture was re-emulsified and stored at 4°C for 10 days (OIE, 2018).

Stability testing

Stability test was performed at different storage conditions for oil based vaccine emulsion. The test was performed on samples kept at 4±0.1°C (Refrigerator) and 25°C±0.1 (Room temperature) after 24 h, 14 days and 90 days.

Organoleptic characteristics

Freshly prepared oil adjuvanted vaccine was investigated organoleptically for colour, liquefaction and phase separation (Bain *et al.*, 1982; Koh *et al.*, 2006; Kumar *et al.*, 2015). Organoleptic characteristics of freshly prepared vaccine were noted after 24 h, 14 days and 3 months at different

storage conditions i.e. refrigerator and room temperature.

Centrifugation test

Centrifugation test (Koh *et al.*, 2006; Kumar *et al.*, 2015) was performed for the emulsion after 24 h, 14 days and 90 days.

Drop test

Drop test as described by Bain *et al.* (1982); Bomford (1997); Mowat *et al.* (1997); Aucouturier *et al.* (2001) was performed for the emulsion after 24 h, 14 days and 90 day. A drop of vaccine was poured in a glass beaker of cold water and was checked for stay at the surface.

Sterility test

A sample of prepared vaccine was taken and cultured on Nutrient agar, MacConkey agar, Sabouraud's agar, Thioglycolate medium and Nutrient broth and incubated at 37°C for 7 days (Kumar *et al.*, 2015; OIE, 2018). All the media were daily checked for absence of growth.

Safety test

A sample of prepared vaccine was taken and injected (0.2 cc, Intra-muscular) in Swiss Albino mice. All vaccinated mice together with control were observed for five days. Sample was also injected (4 cc, deep intramuscular) in two Nili-Ravi buffalo calves kept at Veterinary Research Institute, Lahore. Vaccinated calves together with control were observed for absence of adverse reactions for 14 days (OIE, 2018).

Potency testing by active mouse protection test (AMPT)

AMPT was conducted as described by OIE, (2018). Each of 50 Swiss Albino mice was

vaccinated intramuscularly with 0.2 ml of vaccine and again 14 days later for boosting. On day 21, the mice were divided into ten groups of five and then each group was challenged with 10 fold dilutions of a 6 to 8 h broth culture of *P. multocida* serotype B:2 in the range of 10^1 to 10^{10} . Fifty unvaccinated mice were also challenged in the same manner as vaccinated groups. All the mice vaccinated and non-vaccinated were observed for 5 days for mortality. Heart blood was checked for the presence of *P. multocida* of the dead mice. LD_{50} was calculated for vaccinated and non-vaccinated groups by using the method of Reed and Muench, (1938). Log of protection was calculated with the difference in the LD_{50} of vaccinated and non-vaccinated groups.

Potency testing by Passive Mouse Protection Test (PMPT)

PMPT was done as described by Bain *et al.*, 1982; Jabbari and Moaeni (2004); Jaffri *et al.*, 2006. To perform this test rabbit serum was used. 10 rabbits were vaccinated (0.5 ml intramuscularly) at 0 day and 2nd vaccination was done on 21st day. Serum samples were collected on day 30 and 45 day after booster. PMPT was done with pooled sera which was obtained from rabbits. 0.5 ml of the pooled sera was injected in each of 10 mice. The treated mice were divided in two Groups 1 and 2. Control group was also divided in two Groups 3 and 4. Fresh 6 to 8 h broth culture of *P. multocida* serotype B:2 was prepared for challenge. Groups 1 and 3 were challenged with 100 LD_{50} and Groups 2 and 4 were challenged with 1000 LD_{50} of challenge culture. All the mice were observed daily for 7 days for mortality. Mice that died during the observation period were examined for the presence of bipolar micro-organism in the blood smears and *P. multocida* in blood agar culture media. The

results are expressed as the percentage surviving out of 5 after challenge.

Antibody titer estimation in large animals

The immune status of vaccinated cattle and buffaloes after mass vaccination in three districts of Punjab (Rajan Pur, Bhakkar and Khanewal) was estimated by Indirect Haem-Agglutination (IHA) test. The representative 50 serum samples were obtained from each district after 2 to 3 months after mass vaccination. IHA test was conducted on these samples as described by Tariq *et al.* (1997); Bain *et al.* (1982).

RESULTS AND DISCUSSION

Haemorrhagic Septicaemia (HS) is a seasonal disease of cattle and buffaloes and is controlled by mass vaccination programme before rainy season in Punjab, Pakistan. Immune response to the Bacterin is poor and is of short duration (Tariq *et al.*, 1997). Immune response in vaccinated cattle and buffaloes is required against capsule of *P. multocida* which is composed mainly of lipopolysaccharide (LPS) with minor fraction of proteins (Bain *et al.*, 1982). LPS induces B cell response and cannot be presented along with MHC-II antigen by Antigen Presenting Cells (APC) of the animal body, and hence the responsive B cells (plasma cells) cannot get cooperation of T cells for enhanced antibody production. The B cell response to LPS is primary and the immunity is of low level and short duration (Abbas *et al.*, 1991). This property of LPS in Bacterin necessitated the farmers to vaccinate their animals quarterly (Tariq *et al.*, 1997).

The Oil Adjuvanted Vaccine (OAV) work by forming depot of antigen and letting its

slow release over long course of time, protect the antigen from rapid degradation by enzymes and recruit the APCs at the injection site and enhances the antigen uptake by these APC (Aucouturier *et al.*, 2001; Aguilar and Rodriguez, 2007). There is a minor fraction of bacterial proteins in the capsule, immunity against which can be potentiated by having it in required level in the dose and adding adjuvants in the Bacterin. In the present study, use of enrichments media and provision of fresh filtered air during incubation resulted 2 mg bacterial dry weight per ml of the culture. These results are in agreement with Afzal and Muneer (1990); Tariq *et al.* (1997).

During this study Brain Heart Infusion (BHI) broth was used for cultivation of *P. multocida* which is in accordance with Khan *et al.* (2013) who found that BHI is highly enriched media that supports *P. multocida* growth as compared to plain broth and nutrient broth.

According to Aucouturier *et al.* (2001) water in oil emulsions allows the reduction of the vaccine dose or the antigen concentration. During this study 2 mg antigen was achieved in one ml and equal part of Montanide ISA-50V2 oil adjuvant was mixed to formulate a single dose of 2 ml vaccine per animal. Hence a reduction in dose was achieved (2 ml/animal) as compared to alum precipitated vaccine (5 ml/animal) which was previously used in the field to vaccinate the animals against HS in Punjab, Pakistan.

OAV was found stable upto 90 days at 4°C and 25°C (Aucouturier *et al.*, 2001; Sotoodehnia *et al.*, 2005). There was no change in colour, no liquefaction and no phase separation of the vaccine upto 90 days at 4°C and 25°C (Bain *et al.*, 1982; Koh *et al.*, 2006; Kumar *et al.*, 2015). The vaccine was also found stable after performing centrifugation test up to 90 days (Koh *et al.*, 2006; Kumar *et al.*,

2015). The drop test was cleared for water in oil emulsion up to 90 days (Bain *et al.*, 1982; Bomford 1997; Mowat *et al.*, 1997; Aucouturier *et al.*, 2001). The vaccine was found sterile and safe as per OIE, (2018). In present study, 100% protection was observed by Montanide ISA-50V2 oil adjuvanted vaccine after challenge at 30 and 45 days post vaccination by 100 LD₅₀ and 1000 LD₅₀ in PMPT and a five log protection was obtained in AMPT. These results are in consonance with earlier findings that observed OAV as potent vaccine (Dutta *et al.*, 1990; Chandrasekaran *et al.*, 1994; Sotoodehnia *et al.*, 2005).

The results of IHA also indicated that OAV gave protective antibody titre (IHA Titre 1:32) upto 60 to 90 days post vaccination in three districts of Punjab in cattle and buffaloes (Jaffri *et al.*, 2006; Afrooz *et al.*, 2016). Indirect Haem-Agglutination (IHA) test and Passive Mouse Protection Test (PMPT) were used to calculate the potency of oil based vaccine as described by Nagarajan *et al.* (1972); Gupta and Sareen (1975); Chandrasekaran and Yeap (1978); Sotoodehnia *et al.* (2005); Jaffri *et al.* (2006). Both of these tests were also found satisfactory in evaluation of HSOBV prepared from Montanide ISA-50V2 oil adjuvant. PMPT and AMPT have been described as satisfactory for measuring immunity in either vaccinated or naturally immune animals and survival of any mice in this test group identifies an immune serum, provided that all of an equal numbers of control mice die (Bain, 1963; Thomas, 1970; Sotoodehnia *et al.*, 2005).

Properties like easy to inject with no side effects such as swelling at the injection site, have motivated the livestock owners to use this new product to protect their animals against fatal HS disease which will ultimately result in the increased productivity of livestock in Punjab, Pakistan.

CONCLUSION

Oil adjuvant vaccine prepared from Montanide ISA-50V2 is considered as the most potent vaccine against HS which provide minimum one year protection, easy to inject, reduced dose rate, user friendly and very less swelling/reaction at injection site.

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REFERENCES

- Abbas, A.K., A.H. Lichtman and J.S. Pober. 1991. *Cellular and Molecular Immunology*, W.B. Saunders Company, London, UK. 417p.
- Afroz, H., S. Sattar, A. Rasool, B. Zamir, I. Haq and R. Rafique. 2016. Comparison of immunogenic effect of three oil adjuvant vaccines against haemorrhagic septicaemia in cattle and buffalo. *International Archives of Biomedical and Clinical Research*, **2**: 43-48.
- Afzal, M. and R. Muneer. 1990. Development of combined vaccine of haemorrhagic septicaemia and foot and mouth disease. *Pak. Vet. J.*, **10**: 67-69.
- Aguilar, J.C. and E.G. Rodriguez. 2007. Vaccine

Table 1. Details of chemicals used for enrichments for cultivation of *P. multocida*.

Chemicals	Quantity per liter in distilled water (Gms)	Sterilization temperature (°C)	Sterilization time (minute)	Final pH
Casein hydrolysate (Titan, India)	2	107	10	7.4
Sucrose (Titan, India)	6	107	10	7.4
Yeast extract (Titan, India)	6	107	10	7.4
Sodium chloride (Merck, Germany)	5	121	20	7.4
Anhydrous di-potassium hydrogen orthophosphate (Titan, India)	8.6	121	20	7.4
Anhydrous potassium dihydrogen orthophosphate (Titan, India)	1.36	121	20	7.4

- adjuvants revisited. *Vaccine*, **25**: 3752-3762.
- Aucouturier, J., L. Dupuis and V. Ganne. 2001. Adjuvants designed for veterinary and human vaccines. *Vaccine*, **19**: 2666-2672.
- Bain, R.V.S. 1963. *Haemorrhagic Septicaemia*. FAO Agricultural Studies No. 62. FAO, Rome, Italy.
- Bain, R.V.S., M.C.L. De Alwis, G.R. Carter and B.K. Gupta. 1982. *Haemorrhagic Septicaemia*. FAO Publication, Rome, Italy.
- Bomford, R. 1997. Adjuvants in veterinary vaccines. *In Vaccine Manual: The Production and Quality Control of Veterinary Vaccines for use in Developing Countries*, FAO, Rome, Italy.
- Bratbak, G. and I. Dundas. 1984. Bacterial dry matter content and biomass estimations. *Appl. Environ. Microb.*, **48**: 755-757.
- Chandrasekaran, S. and P.C. Yeap. 1978. Safety and potency testing of haemorrhagic septicaemia oil adjuvant vaccine by mouse protection test. *Kajian Veterinar*, **10**: 28-34.
- Chandrasekaran, S., I. Kennett, P.C. Yeap, N. Muiandy, B. Rani and T.K.S. Mukkur. 1994. Characterization of immune response and duration of protection in buffaloes immunized with haemorrhagic septicaemia vaccines. *Vet. Microbiol.*, **41**: 213-214.
- Dutta, J., B. Rathore, S. Mullick, R. Singh and G. Sharma. 1990. Epidemiological studies on occurrence of haemorrhagic septicaemia in India. *Indian Vet. J.*, **67**: 893-899.
- Farooq, U., Z. Saeed, M.A. Khan, I. Ali and M.F. Qamar. 2011. Sero-surveillance of Haemorrhagic Septicaemia in buffaloes and cattle in southern Punjab, Pakistan. *Pak. Vet. J.*, **31**: 254-256.
- Gupta, M.I. and R.I. Sareen. 1976. Evaluation of haemorrhagic septicaemia oil adjuvant vaccine by the mouse protection test. *Indian Vet. J.*, **53**: 489-492.
- Jabbari, A.R. and G.R. Moazeni Jula. 2004. Potency testing of modified alum adjuvant haemorrhagic septicaemia vaccine in laboratory and farm animals. *Archives of Razi Institute*, **57**: 97-104.
- Jaffri, K.T., Z.J. Gill, A.R. Bhatti and A. Raza. 2006. Immune response of buffalo calves to Haemorrhagic Septicemia oil adjuvant and alum precipitated vaccine. *Int. J. Agric. Biol.*, **8**: 645-647.
- Khan, A., M.K. Saleemi, M.Z. Khan, S.T. Gul, M. Irfan and M.S. Qamar. 2011. Hemorrhagic septicaemia in buffalo (*Bubalus bubalis*) calves under sub-tropical conditions in Pakistan. *Pak. J. Zool.*, **43**: 295-302.
- Khan, A., S. Munir, Q. Jamal, M. Anees, S.A. Shah, S.K. Sherwani, A. Basit, G. Ali, Asadullah and M. Hussain. 2013. Biomass production of *Pasteurella multocida* using biofermenter. *Int. J. Adv. Res.*, **1**: 142-151.
- Koh, Y., S. Higgins, J. Weber and W.M. Kast. 2006. Immunological consequences of using three different clinical/laboratory techniques of emulsifying peptide-based vaccines in incomplete Freund's adjuvant. *Journal of Transition Medicine*, **4**: 42-42.
- Kumar, S., V.K. Chaturvedi, B. Kumar, P. Kumar, S.R. Somarajan, A.K. Mishra and B. Sharma. 2015. Effect of alum co-adjuvantation of oil adjuvant vaccine on emulsion stability and immune responses against Haemorrhagic Septicaemia in mice. *Iranian Journal of Microbiology*, **7**: 79-87.
- Kumar, S., V.K. Chaturvedi, B. Kumar and P. Kumar. 2011. Immune response and viscosity of haemorrhagic septicaemia oil adjuvant vaccine at different water-oil

- proportion. *Indian J. Anim. Sci.*, **81**: 1000-1004.
- Mowat, N. and M. Rweyemamu. 1997. *Vaccine Manual: The Production and Quality Control of Veterinary Vaccines for use in Developing Countries*. FAO Animal Production and Health Series 35, Rome, Italy. 433p.
- Nagarajan, V., S. Sunderman and K. Ramandi. 1972. Evaluation of the potency of haemorrhagic septicaemia alum precipitated vaccine by mouse protection test. *Indian Vet. J.*, **49**: 1080-1083.
- OIE. 2018. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, Office De International Epizootics, Paris, France. p. 732-745.
- Reed, L.J. and H. Muench. 1938. A simple method of estimating 50 percent end points. *Am. J. Hyg.*, **27**: 493-497.
- Shahzad, W., R. Munir, M. Asif, M.S. Sagar, M. Altaf, W. Aslam, G. Akbar and F. Mehmood. 2013. Prevalence, molecular diagnosis and treatment of field isolates of toxogenic *Pasteuralla multocida* in a hemorrhagic septicemia outbreak in Nili-Ravi buffalo calves at livestock experiment station, Bahadurnagar, Okara, Pakistan. *Buffalo Bull.*, **32**: 1046-1050.
- Sotoodehnia, A., G. Moazeni, S. Ataei and B. Omid. 2005. Study on immunity of an experimental oil adjuvant Haemorrhagic Septicaemia vaccine in cattle. *Archives of Razi Institute*, **59**: 95-101.
- Tariq, M.A., K. Muhammad, P. Das and M.A. Sheikh. 1997. Preparation and evaluation of alum precipitated and oil-based haemorrhagic septicaemia vaccines. *Pak. Vet. J.*, **17**: 175-178.
- Thomas, J. 1970. Studies on HS oil adjuvant vaccine III serological studies. *Kajian Veterinarise Vet. Bult. Abst.*, 1718.