COMPARATIVE EVALUATION OF ANTIBODY TITER AGAINST *PASTEURELLA MULTOCIDA* IN RABBITS BY USING FOUR DIFFERENT ADJUVANTS IN HS+BQ COMBO VACCINES

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ABSTRACT: Hemorrhagic Septicemia (HS) caused by *Pasteurella multocida* is an economically significant disease of bovines that instigates high monetary losses due to unexpected mortality of animals in emerging countries like Pakistan. Similarly Black Quarter (BQ) caused by *Clostridium chauvoei* is also an acute infectious ailment of cattle and buffaloes in which severe inflammation of skeletal and cardiac muscles, severe systemic toxicity and a high mortality occurs. The only way to prevent losses due to both diseases is an effective and long lasting vaccination programme. Four HS+BQ combo vaccines adjuvanted with four different adjuvants were prepared. Two HS+BQ vaccines were prepared by addition of two oil adjuvants i.e Montanide ISA-50V-2 and Eolane-170 while one HS+BQ vaccine was prepared by addition of gel while fourth HS+BQ vaccine was prepared by addition of alum. The bacterial dry weight of *P. multocida* was adjusted to 2 mg/0.75 mL while the bacterial dry weight for *Cl. chauvoei* was set at 2.5 mg/0.75 mL. The dose of all vaccines was adjusted to 3 mL /animal. Four groups of rabbits having five rabbits in each group were selected at Veterinary Research Institute, Lahore. From four groups of rabbits one was inoculated with HS+BQ oil based vaccine containing Montanide ISA-50 V2 as adjuvant while other group was inoculated with HS+BQ oil based vaccine containing Eolane-170 oil as adjuvant. Third group was vaccinated with HS+BQ vaccine adjuvanted with gel while fourth group of rabbit was vaccinated with HS+BQ vaccine adjuvanted with alum. All rabbits were vaccinated at dose rate of 1mL / rabbit. Rabbits were again vaccinated with the same vaccines as booster dose after 27 days. Indirect HemAgglutination test was conducted after 141 days intervals on serum samples of all groups. The results showed that both HS+BQ combo oil based vaccines have higher antibody titers as compared to gel and alum based vaccines against HS disease.

Keywords: Black quarter, Combo vaccine, Hemorrhagic septicaemia, Rabbits.

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INTRODUCTION

Hemorrhagic Septicaemia (HS) is a highly disastrous and economically disturbing disease of cattle and water buffaloes in continents of Asia, Africa and Middle East with highest incidence in South East Asia (Shahzad *et al.,* 2013; O.I.E., 2017; Almoheer *et al.* 2022). This disease is caused by bacteria *Pasteurella multocida* (*P. multocida*) serotype B:2. Different studies have signalled that *P. multocida* serotype B:2 causes high transience (upto 50 %) in numerous parts of Pakistan (Khan *et al*., 2011). This disease is comparatively predominant with maximum mortality in 6-12 months old buffalo calves (Farooq *et al*., 2011). The disease mostly occurs in rainy season. The environment (high temperature), management (overloading, scanty aeration and transference) and malnourishment are amongst the probable reasons which are concerned to strengthen the incidence of outbreaks of HS in Pakistan (Tariq *et al*., 1997). The sudden onset and short duration of the disease are major reasons of treatment failures in diseased animals. The mortality of HS among cattle and buffaloes has amplified in 2017–2019 as compared to the period between 2014 and 2016 (Almoheer *et al.* 2022). Similarly Black Quarter (BQ) is a soil-born Clostridial infection of bovines caused by *Clostridium chauvoei* (*Cl. chauvoei*) which produces severe inflammation of skeletal and cardiac muscles resulting spongy look at necropsy, severe systemic toxicity which ultimately causes a high mortality (Nasir *et al*., 2020). Young cattle and buffaloes with 6 to 24 months of age and good body condition are highly vulnerable (Saminathan *et al*., 2016). The survival of Clostridial spores in different soil types is still under observation and there might be a strong bond between the soil types and number of rainy days for BQ & HS to participate in a locality (Sivakumar *et al*., 2012; Khan,

2010). Poor carcass disposal of infected bodies adds to the frequent soil contamination with infected spores to build-up the soil infection. The key to prevent losses due to both diseases is a good and long lasting immunization programme.

In South and South-East Asian countries, HS and BQ vaccines are majorly dealt with different immunization programmes (Srinivasan *et al*., 2001). Vaccination with HS and BQ combo vaccine is advocated in bovines in endemic areas as many workers have recorded outbreaks in bovines in countries in Subcontinent (Rajasekhar, 2005; Khan, 2010; Mondal and Yamage, 2014; Ahmad *et al*., 2016; Saminathan *et al*., 2016; Krishnamoorthy *et al*. 2018). Previously the efficacy and potency of combined HS and BQ antigens has been studied by Sinha and Prasad (1973); Srinivasan *et al*. (2001); Saseendranath *et al*. (2009). Montanide ISA-50 V2 (SEPPIC, France) is a readymade oil adjuvant and has been documented as user friendly and thinner emulsion for vaccine preparation (Aucouturier *et al*. 2001; Afrooz *et al*. 2016). Similarly Eolane-170 (TOTAL, PARCO, Pakistan) is a modern mineral oils being used as adjuvant for veterinary vaccines preparations throughout world having advantages of being cost effective, user friendly and cheap (Shahzad *et al.* 2020). Alum and gel precipitated vaccines are also being used for immunization against both diseases in different countries including Pakistan. The HS and BQ combine vaccine has been advocated by Kushram *et al.* 2020.

The present study has been conducted to prepare four HS+BQ combo vaccines by using (1) Montanide ISA-50 V2 oil adjuvant (2) Eolane-170 oil adjuvant (3) alum hydroxide (4) gel adjuvant and to evaluate their efficacies in rabbits by estimating serum antibody titers against *P. multocida* using Indirect HemAgglutination (IHA) test.

MATERIALS AND METHODS

Working seeds of *Pasteurella multocida* and *Clostridium chauvoei:* Lyophilized seed of *P. multocida* type B:2 was liquefied with nutrient broth and incubated at 37 °C for a period of 4-5 hours. The incubated seed was later on inoculated sub-cutaneously in Swiss Albino mice at abdominal region. After 16-18 hours of inoculation, mice died and heart blood was collected aseptically after postmortem, cultured on Brain Heart Infusion (BHI) broth and incubated at 37 °C for 24 hours. Diluted heart blood was also cultured on Nutrient broth, MacConkey's agar, Sabouraud's dextrose agar slants and thioglycolate media to check the sterility (O.I.E., 2018). After purity checking microscopically as well as on the basis of cultural characteristics, the pure seed in BHI was stored at 4 °C (O.I.E., 2018). *Cl. chauvoei* seed was activated as per FAO, 1991. Freeze dried seed of *Cl. chauvoei* (local strain) was activated by culturing in Reinforced Clostridium Medium (RCM) in 250 mL flask and incubated at 37 °C for 24 hours under anaerobic conditions. Purity of activated *Cl. chauvoei* was checked microscopically after Gram's staining.

HS & BQ antigens preparation: For HS antigen preparation, Brain Heart Infusion (BHI) broth (Merck, India) was autoclaved with final pH 7.4. The prepared media was found sterile after incubating at 37 °C for 24 hrs. Media supplementations were prepared and sterilized as described in Table 1 and were checked for sterility similarly as described previously. Sterile BHI broth was cultured with the pure working seed of *P. multocida* type B:2 @ 5% and supplementations were added aseptically in cultured BHI broth(O.I.E., 2018).

Cultured vessels were aerated by filtered air through syringe filters 0.2 µm pore size, (Maxipore, England) by using a compressor and continuous rotation on shakers @ 120 rpm was applied. Incubation was provided to vessels at a temperature of 37 °C for 16-24 hours. After completion of incubation, purity of bacterial growth was checked microscopically and growth was stopped by the addition of formalin (Merck, Germany) @ of 0.5% of culture. The inactivated culture was kept at 37 °C for 24 hours after shaking briskly. The Bacterin was inoculated on Nutrient agar, MacConkey's agar, Sabouraud's dextrose agar, Thioglycolate medium and Nutrient broth to check the complete inactivation and sterility. Dry mass of the bactrin was estimated as described by Bratbak and Dundas, 1984 and adjusted to 2mg / 0.75 mL (O.I.E., 2018). BQ antigen (*Cl. chauvoei*) was prepared by using BQ medium enriched with 0.5 % sterilized glucose solution and kept incubated at 37 °C for 24 hours (FAO 1991). Growth of bacterium was ended by the addition of formalin (Merck, Germany) @ of 0.7 % final concentration and antigen dry mass was set at 2.5 mg/0.75 mL.

Preparation of HS+BQ vaccines with different adjuvants: Four different adjuvants were used for development of four (A,B,C & D) HS+BQ combo vaccines by homogenisation at 10000 rpm - 15000 rpm using a heavy duty homogenizer (PAMCO, Pakistan). First vaccine (A) was prepared by addition of equal volume of Montanide ISA-50V2 oil (Seppic, France) and HS+BQ antigens in the ratio of 1:1. Similarly second vaccine (B) was prepared by adding equal volume of Eolane-170 oil (Total, PARCO) alongwith emulsifiers (Lanolin & Span-80) and HS+BQ antigens in the ratio of 1:1. Third vaccine (C) was prepared by using alum with HS+BQ antigen. Fourth vaccine (D) was prepared by using gel with HS+BQ antigen. Preservative Thiomersal (Bio world, USA) was used @ of 0.003% and addition of formalin (Merck, Germany) was performed to a final concentration of 0.5%. Following overnight storage at room temperature, the emulsions were re- homogenised

and stored at 4 °C for 10 days (O.I.E., 2018). The dose of all vaccines was adjusted to 3 mL / animal by inoculating deep intra muscular route in neck region.

Stability test: Stability testing was executed at different storage temperatures for oil based vaccine emulsions as well as for alum and gel vaccines. For this purpose, vaccine samples were placed at 4±0.1 °C (refrigerator) and 25 °C±0.1 (room temperature) .Samples was monitored after 24 hours, 14, 90, 180, 270 and 365 days.

Organoleptic properties: Newly developed oil adjuvant, alum as well as gel based vaccines were inspected organoleptically for color, viscosity and phase separation (Bain *et al*., 1982; Koh *et al*., 2006; Kumar *et al*., 2015). Organoleptic propertied of newly developed vaccines were studied after 24 hours, 14 , 90, 180 , 270 and 365 days at 4±0.1 °C (refrigerator) and 25 °C±0.1 (room temperature).

Centrifugation test: Centrifugation test (Koh *et al*., 2006; Kumar *et al*., 2015) was carried out for each vaccine after 24 hours, 14 , 90, 180 , 270 and 365 days.

Drop test: Drop test as done by (Bain *et al*., 1982; Bomford 1997; Mowat *et al*., 1997; Aucouturier *et al*., 2001) was carried out for each oil based preparation after 24 hours, 14, 90, 180, 270 and 365 days. One drop of each vaccine was poured in ice cold water and was observed for clear margins, dispersion and stay at the surface.

Sterility test: Each vaccine was inoculated on different culture media which include Nutrient agar slant, MacConkey's agar slant, Sabouraud's agar slant, Thioglycolate medium and Nutrient broth and kept at 37 °C for 7 days (FAO, 1991; Kumar *et al*., 2015; O.I.E., 2018). All test media were daily observed for any contamination or growth.

Safety test: Each vaccines was inoculated in two Swiss Albino mice @ 0.2 mL, Intra-muscular and in two guinea pigs @ 0.5 mL, Intra-muscular. All vaccinated mice and guinea pigs along with control were observed for 5-7 days. Vaccines were also inoculated in two Nili-Ravi Buffalo calves and two Sahiwal Cattle calves @ 6 mL, deep intramuscular that were kept at Veterinary Research Institute, Lahore. Vaccinated calves along with control were kept under observation for absence of untoward reactions for 14 days (FAO, 1991; O.I.E., 2018).

Potency testing by Active Mouse Protection Test (AMPT): AMPT was executed on HS-BQ combo vaccines emulsified with Montanide ISA 50 V2, Eolane-170, alum and gel for *Pasteurella multocida* as per protocol mentioned by O.I.E, 2018. A group of 50 Swiss Albino mice was inoculated intramuscularly with 0.2mL of each vaccine and again booster dose was given 14 days later. At day 21, the mice were distributed in ten groups, each containing five mice and each group was challenged with 10 fold serial dilutions (10^1 to 10^{10}) of a 6-8 hours live culture of *P. multocida* serotype B:2. A group of fifty unvaccinated mice were also inoculated in the same way as vaccinated groups. All vaccinated and non-vaccinated groups of mice were kept under observation for 5 days to check the mortality. Presence of *P. multocida* was checked microscopically from heart blood of the dead mice after post mortem. The method of Reed and Muench, 1938 was followed to calculate the LD_{50} of vaccinated and non-vaccinated groups. By calculating the difference in the LD_{50} of vaccinated and non-vaccinated groups Log of protection was calculated.

Potency testing by Passive Mouse Protection Test (PMPT): PMPT was performed with HS+BQ combo vaccines emulsified with Montanide ISA-50 V2 and Eolane-170 against *Pasteurella multocida* as per protocol mentioned by Bain *et al*., 1982; Jabbari and Moaeni, 2004; Jaffri *et al.,* 2006. For the execution of this test antiserum was raised in rabbits. Ten rabbits were vaccinated intramuscularly @ 0.5mL separately with HS+BQ combo vaccine adjuvant with Montanide ISA-50 V2 and Eolane-170 at 0 day and 2nd dose was given on 21st day. Blood samples were collected on day 30 and 45 day after booster and serum was separated and pooled for each vaccine. 0.5mL of the sera was injected in each of 10 mice. Two groups (1 and 2) of treated mice were prepared. Mice which were being kept as control were also divided in two groups; 3 and 4. For challenge purpose, a fresh six to eight hour growth of *P. multocida* was used. Two Groups i.e. 1 and 3 were challenged with $100LD_{50}$ and remaining groups 2 and 4 were challenged with $1000LD_{50}$ of live *P. multocidaB:2*. The mice were kept under observation for 7 days and mortality was recorded daily. Post mortem of died mice was done to examine the presence of bipolar micro-organism in the blood smears. The results are depicted in the percentage surviving out of 5 after challenge treatment.

Potency testing by Challenge Protection Test: Challenge protection test was conducted in gunaei pigs for *Cl. chauvoei* as described by FAO, 1991.

Antibody Titre Estimation in Rabbits at Veterinary Research Institute, Lahore: For testing the efficacy of different HS+BQ combo vaccines in lab animals, four groups of rabbits having five rabbits in each group was selected at Veterinary Research Institute, Lahore. These animals were not immunized with HS or BQ vaccines previously. Before the start of trials, rabbits were dewormed with an anthelmintic using Albendazole drug. One group of rabbits was immunized with HS+BQ combo emulsified vaccine adjuvant with Montanide ISA-50 V2 (1 mL intramuscular / rabbit) while second group was vaccinated with HS+BQ combo oil based vaccine adjuvanted with Eolane-170 oil adjuvant (1 mL

intramuscular / rabbit), third group was vaccinated with HS+BQ vaccine adjuvanted with alum and fourth group was vaccinated with HSBQ vaccine adjuvanted with gel. The immune status of each group of vaccinated rabbits was estimated by Indirect Haemagglutination (IHA) test against *P. multocida*. Five rabbits were also kept as control. The rabbits were re-vaccinated (1mL intramuscular/rabbit) with the same vaccines as booster dose after 27 days. Serum samples were obtained from experimental and control rabbits on 0 day and then after 141 days post immunization. IHA test was performed on these samples following Tariq *et al*., 1997; Bain *et al*., 1982.

RESULTS

The HS+BQ combo oil adjuvant vaccine developed with two different oil preparations i.e. Montanide ISA-50 V2 and Eolane-170 were stable and firm upto 365 days at 4 °C & 25 °C. The colour remain unchanged, no liquefaction and no separation in phases of the vaccines was upto 365 days at 4 °C & 25 °C. On performing centrifugation test, the vaccines were also found stable upto 365 days. The drop test showed no dispersion in cold water upto 365 days. The preparations were found sterile and safe as per O.I.E, 2018 and FAO, 1991 as no death / adverse reaction was recorded in mice, guinea pigs and large animals. In current study, 100 % protection was observed by HS-BQ combo oil adjuvant vaccines after challenge at 30 and 45 days post immunization by 100 LD_{50} and 1000 LD_{50} in PMPT and a 5 log protection was achieved in AMPT. Challenge protection test was conducted in Guinea pigs for *Cl. chauvoei* for potency testing and both groups of vaccinated Guinea pigs were found protective after challenge with 20 LD_{50} of pure culture of *Cl. chauvoei.*

IHA tests was conducted on serum samples from four groups of rabbits immunized with HS+BQ combo vaccines preparation adjuvant with Montanide ISA- 50 V2, Eolane- 170, alum and gel. The results of IHA tests showed that HS+BQ combo oil based vaccine emulsified with Montanide ISA-50 V2 and Eolane-170 both gave better (GMT = 31.98 and 29.21) antibody titer verses gel and alum (GMT = 13.89 and 8.00) upto 141 days post vaccination in rabbits against HS disease.

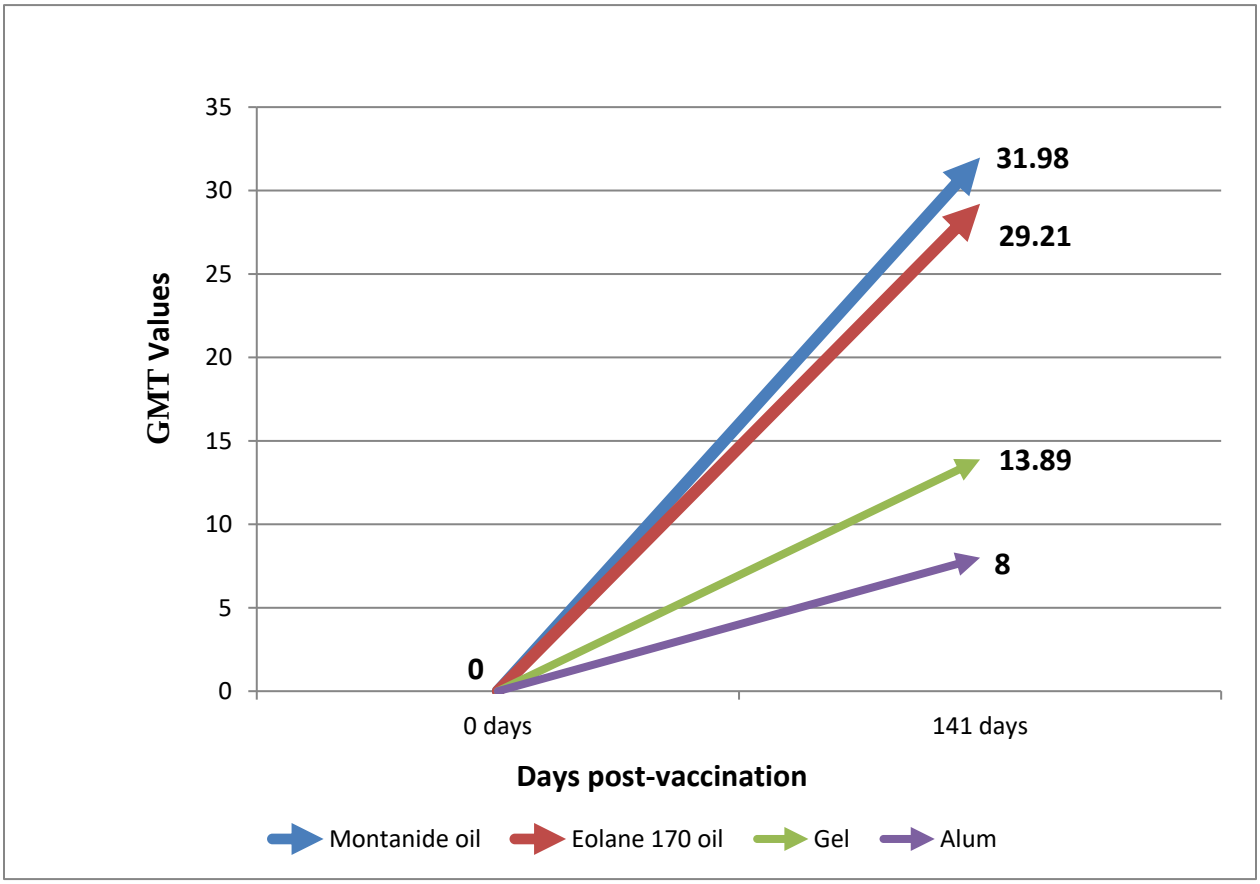

Figure.1 Graph showing GMT values for antibodies titer estimated by Indirect Haemagglutination test in rabbits by using HS+BQ vaccines emulsified with Montanide ISA- 50 V2, Eolane-170, Gel and Alum at Veterinary Research Institute, Lahore.

Table 1. Detailed list of chemicals for dense growth of *P. multocida*.

Ingredients	Grams /litre	Autoclave Temperature	Autoclave Time	pH maintained
Casein hydrolysate	2 Gms	107 °C	10 mints	7.4
Sucrose	6 Gms	107 °C	10 mints	7.4
Yeast Extract	6 Gms	107 °C	10 mints	7.4
Sodium Chloride	5 Gms	121 °C	20 mints	7.4
Anhydrous di-potassium hydrogen orthophosphate	8.6 Gms	121 °C	20 mints	7.4
Anhydrous potassium dihydrogen orthophosphate	1.36 Gms	121 °C	20 mints	7.4

DISCUSSION

Haemorrhagic Septicaemia (HS) is a seasonal bacterial contagious infection while Black Quarter (BQ) is a clostridial spore soil origin malfunctioning of cattle and buffaloes (especially affecting younger and healthy calves) and is prevented by mass immunization programmes in Punjab, Pakistan as the best measure to control the disease is vaccination (Almoheer *et al*. 2022). In vaccination against Haemorrhagic Septicaemia, the immune response against the Bacterin is poor and is of small duration (Tariq *et al*., 1997). Immune response in vaccinated cattle and buffaloes is developed against capsule of *P. multocida* which is collected mainly of lipopolysaccharide (LPS) with insignificant fraction of proteins (Bain *et al*., 1982). B cell response is induced by LPS and this B cell response cannot be presented along with MHC-II antigen by Antigen Presenting Cells (APC) of the animal body, and hence these responsive B cells (plasma cells) cannot get aid 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 stimulated livestock farmers and breeders to conduct vaccination quarterly for protection of their animals against diseases (Tariq *et al*., 1997).

Vaccines containing oil as adjuvants work by forming depot and ultimately slow release over long period of time, protects the antigen from speedy degradation by enzymes and invites the APCs at the site of injection and augments the antigen uptake by these APC (Aucouturier *et al*., 2001; Aguilar and Rodiguez, 2007). There is small portion of bacterial proteins in the capsule of *P. multocida*, protection against which can be strengthened by having it in required quantity of dose and adding adjuvants in the Bacterin.

The HS+BQ combo oil adjuvanted vaccines prepared for cattle and buffaloes were found constant upto 360 days while being kept at 4 °C & 25 °C and these results are in agreement with Aucouturier *et al*., 2001; Sotoodehnia *et al*., 2005 and Shahzad *et al.,* 2020 who also obtained a stable oil based vaccine at 4 °C & 25 °C. No change in colour of vaccine was observed, there was also no liquefaction and there was no phase separation of the vaccines upto 365 days at 4 °C & 25 °C as comparable outcomes have been perceived by Bain *et al*., 1982; Koh *et al*., 2006; Kumar *et al*., 2015 and Shahzad *et al.,* 2020. The HS+BQ vaccines were also found unchanged after execution centrifugation test upto 365 days and these outcomes are in agreement with Koh *et al*., 2006; Kumar *et al*., 2015 and Shahzad *et al.,* 2020 who have perceived comparable type of outcomes. The drop test for the newly prepared HS+BQ vaccines was cleared upto 365 days and this outcome is in agreement with the conclusions of Bain *et al*., 1982; Bomford 1997; Mowat *et al*., 1997; Aucouturier *et al*., 2001 and Shahzad *et al.,* 2020.

During this study, usage of supplementations media and provision of fresh filtered air through syringe filter having pore size of 0.2 µ during cultivation produced 2 mg dry microbial mass per 0.75 mL of the bacterial culture of *P. multocida* and these results are inconformity to Afzal and Muneer (1990); Tariq *et al*., 1997 and Shahzad *et al.,* 2020.

Brain Heart Infusion (BHI) liquid medium was employed for growth of *P. multocida* which is in agreement with Khan *et al*., (2013) who concluded that BHI is much improved media that enhances *P. multocida* development in comparison to simple broth & Nutrient broth. Bestowing to Aucouturier *et al*., 2001, water in oil emulsions permits the decline of the vaccine dosage or the antigen absorption. In the course of this study, 2 mg dry weight of *P. multocida* antigen was achieved in 0.75 mL while 2.5 mg dry weight of *Cl. chauvoei* antigen was also achieved in 0.75 mL. These two antigens (1.5 ml) were then mixed with equal part (1.5 mL) of Montanide ISA-50V2 oil and Eolane-170 adjuvants to frame a single dosage of 3ml vaccine injection for each animal. Alum precipitated HS+BQ vaccine and gel precipitated HS+BQ vaccines were also have 2 mg of *P. multocida* antigen and 2.5 mg dry weight of *Cl. chauvoei* in each dose.

In current experiment, 100 % safety was perceived by Montanide ISA-50V2 and Eolane-170 oil adjuvanted emulsions after challenge at 30 & 45 days post immunization by 100 LD_{50} and 1000 LD_{50} in Passive Mouse Protection Test and a 5 log fortification was attained in Active Mouse Protection Test. These outcomes are in accordance with previous conclusions that perceived Oil Adjuvanted Vaccines as effective

inoculations (Dutta *et al.,* 1990; Chandrasekaran *et al*., 1994, Sotoodehnia *et al*., 2005; Shahzad *et al.,* 2019 and Shahzad *et al.,* 2020).

The IHA outcomes which designated that HS+BQ Montanide ISA-50 V2 oil adjuvanted vaccine and HS+BQ Eolane-170 oil adjuvanted vaccines gave better antibody titer (GMT = 31.98 and 29.21) verses HS+BQ gel precipitated vaccine and Alum precipitated vaccine (GMT = 13.89 & 8.00) upto 141 days post vaccination in rabbits against *P. multocida* are in accordance with earlier outcomes of Jaffri *et al*., 2006; Afroz *et al*., 2016 who have obtained satisfactory protective antibody titer with oil based vaccines against HS disease. In order to evaluate the effectiveness of oil based emulsions, Indirect Haem-Agglutination (IHA) test and Passive Mouse Protection Test (PMPT) are being employed as designated by Nagarajan *et al*., 1972; Gupta and Sareen, 1975; Chandrasekaran and Yeap, 1978; Sotoodehnia *et al*., 2005; Jaffri *et al*., 2006. Satisfactory results have been obtained by applying both of these tests in valuation of HS-BQ oil adjuvanted emulsions manufactured from Montanide ISA-50V2 oil and Eolane-170.

PMPT & AMPT have been categorised as acceptable tests for evaluating and quantifying protection in either immunized or naturally immune animals and existence of any mice in this test group categorises an immune serum, provided that all of an equal numbers of control mice die (Bain, 1963; Thomas, 1970; Sotoodehnia *et al*. 2005). Similarly Kushram *et al.* (2020) obtained 100% protection against H.S. disease and 75% against B.Q. disease in rabbits and guinea pigs, in potency test performed for individual vaccines.

During the study, HS+BQ combo oil adjuvanted vaccine was applied intramuscularly to rabbits and a satisfactory immune titer (GMT= 31.98) was achieved after 141 days post vaccination. The research outcomes of this study are covenant with Saseendranath *et al*. (2009) and Kushram *et al.* (2020) who obtained satisfactory immune level after 21 days post vaccination with HS+BQ combo oil adjuvanted vaccination by intramuscular route.

HS+BQ combo oil adjuvanted vaccine induced a satisfactory immune response against *P. multocida* in this study. Similar types of results were obtained by Srinivasan *et al*. (2001) who observed no effective variation in the serological response induced by individual component vaccines and combo vaccine containing HS and BQ antigens.

Eolane-170 is a new cohort mineral oil and is being employed for manufacturing of oil adjuvanted emulsions throughout the world. Eolane-170 or Eolane-150 oil adjuvants fulfil the Food and Drug Administration (FDA) necessities of mineral oil. Furthermore, Eolane-170 and Eolane-150 meets the requirements of pureness of United States Pharmacopeia and is in agreement with the general requirements of European Union regulations (TOTAL FLUIDES, FRANCE). The oil emulsion developed by Eolane is homogeneously distributed having micron size droplets, guaranteed extreme emulsion constancy and solidity, reduced thickness, stress-free to inoculate in animals, negligible or no unwanted side effects and is very extremely cost effective (Shahzad et al. 2020). Sri Lanka and Indonesia have efficaciously used lesser levels of lanoline as the blending agent in an effort to reduce viscidness of oil adjuvanted HS emulsion (Lubroth et al. 2007).

Benefits of oil based emulsions like easy to inoculate with no special effects such as inflammation at the inoculation spot, reduced dose rate (3Ml /animal), protection against two major fatal economic diseases with a single injection, reduced animal stress, vaccination once in a year and reduced vaccination cost will encourage the livestock owners to use this new invention to safeguard their animals against deadly HS and BQ diseases which will ultimately result in the increased productivity of livestock in Punjab, Pakistan.

Conclusions: Oil adjuvant HSBQ combo vaccines prepared from Montanide ISA-50V2 and Eolane-170 produced better antibody in rabbits as compared to Gel and Alum precipitated vaccines, hence both oil adjuvanted vaccines are potent vaccines against HS and BQ diseases which can produce minimum one year protection in large animals against both endemic diseases, easy to inoculate, compact dose rate, handler friendly, decreased inflammation / reaction at inoculation site, reduced stress due to single injection instead of two injection, reduced transportation cost and infrequent visits of vaccinator to farmer.

Recommendations: HS+BQ combo oil adjuvanted vaccine is a killed vaccine and is recommended for protection against HS and BQ diseases in cattle and buffaloes. The recommended dose for protection of animals is 3mL and inoculation route is deep Intra muscular in the neck region at the age of 4-6 months. Booster dose in claves is necessary after 12 weeks of 1st vaccination and then repeat annually. For good results animal should be dewormed against ecto and endo parasites about 10-14 days prior to vaccination.

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