

Original Research Article

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Production and Standardization of Combined Vaccine against Haemorrhagic Septicaemia and Black Quarter

Nivedita Kushram¹, Rakesh Sharda², Kripa Shankar Misraulia¹,
Sanjay Madhukar Gholap³, Daljeet Chhabra², Hemant Mehta² and Rakhi Gangil^{2*}

¹Department of Veterinary Medicine, NDVSU, ²Department of Veterinary Microbiology,
College of Veterinary Science and Animal Husbandry, Mhow -453446, India
³Institute of Animal Health and Veterinary Biologicals, Mhow -453446, India

*Corresponding author

ABSTRACT

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The enormosity of infectious diseases continues to be a major constraint to the economical viability of livestock industry. Combined vaccines against more than one infectious disease reduce the cost, time, and labour of vaccination. In present study, formalized vaccines against Haemorrhagic Septicaemia and Black Quarter diseases were prepared using P₅₂ strain of *Pasteurella multocida* and *Clostridium chauvoei* strain 49. Different proportions of these two vaccines were mixed to formulate various groups of combined vaccines, which were subsequently standardized for sterility, safety and potency. Amongst all combinations a group containing 1 ml of H.S. casein sucrose yeast extract agar washed vaccine and 4 ml of B.Q. thioglycolate broth and culture vaccine with shaking was found most efficacious in our study. This combined vaccine conferred 100% protection against H.S. disease and 75% against B.Q. disease in rabbits and guinea pigs, respectively in potency test performed for individual vaccines. The study also revealed that casein sucrose yeast agar and fluid thioglycolate broth are better medium for cultivation of *P. multocida* and *Cl. chauvoei*. Enhanced growth of *Cl. chauvoei* was observed when incubated on a shaker as compared to static culture.

Introduction

Livestock sector has a significant contribution in Indian economy and it also plays an important role in prosperity of rural India. India is the largest milk producer in the world with annual production of 165.4 million tons in 2017 (Singh, 2018). It can continue to hold this position if some of the diseases affecting the productivity of the animals is taken care

off. There are number of bacterial, viral and fungal pathogens which cause severe diseases in cattle and buffaloes affecting animal health and lead to decreased milk production.

In India haemorrhagic septicaemia (H.S.) and black quarter (B.Q) constitute the major bacterial diseases of ruminants and have an adverse effect on the economic viability of the livestock industry. Haemorrhagic Septicaemia

is an acute and fatal septicaemic disease of cattle and buffaloes, primarily caused by serotype B:2 and E:2 of *Pasteurella multocida* in Asia including India.

The disease is endemic and is of great economic importance (OIE, 2002); the annual losses exceeding 10 million rupees per annum in India (Singh *et al.*, 1996). Black Quarter is a predominant disease of cattle, goat and sheep caused by *Clostridium chauvoei*. It is characterized by development of focal gangrenous and emphysematous myositis and gives rise to crepitating and serohaemorrhagic swelling in gluteal muscles (Radostits *et al.*, 2009).

Vaccination is an important tool for controlling infectious diseases. Regular prophylactic vaccination of animals against infectious diseases in developing countries has become an important input to maintain milk production and to reduce economic losses.

In an exercise to reduce the cost of vaccination, many workers attempted to prepare and use combined vaccines (Salt *et al.*, 1996). Present work has been aimed to develop and standardize a combined vaccine against H.S. and B.Q. diseases.

Materials and Methods

Experimental animals

Apparently healthy laboratory animals including Swiss albinomice (approx.18-20g body weight), albino rabbits (approx. 2kg body weight) and guinea pigs (approx. 300-450g body weight) of either sex were procured from the Institute of Animal Health and Veterinary Biological (IAH and VB), Rasalpara, Mhow (M.P.).This study was approved by the Institutional Animal Ethics Committee.

Seed strains

The seed of *Pasteurella multocida* strain P₅₂ was procured from I.V.R.I. Izatnagar, Bareilly (U.P.) India and *Clostridium chauvoei* strain 49 was obtained from the Institute of A.H. and Veterinary Biologicals, Mhow.

Preparation of seed stock

The freeze-dried culture of *P. multocida* P₅₂ strain was reconstituted in normal saline solution and inoculated on blood agar plate. Purity was tested by Gram's staining followed by subculture on blood agar slant and incubated at 37°C for 18 hrs. The seed strain of *Clostridium chauvoei* strain 49 was cultured in Fluid thioglycolate broth (FTB) (Himedia, India) and incubated for 24-48 hrs.at 37°C and purity was confirmed by Gram's staining. Seed stock of both strains was preserved at 4°C.

Preparation of production flask of *Pasterulla multocida* for HS vaccine

Pasteurella multocida P₅₂ strain was inoculated in nutrient broth followed by Seed culture of incubation at 37°C for 18 hrs. Roux flasks of nutrient agar (Himedia, India) and casein sucrose yeast extract (CSY) agar were inoculated with this over night broth culture and incubated at 37°C for 18 hrs. The bacterial growth was harvested in 0.5% normal saline by using sterile rolling glass beads. Subsequently the harvest was filtered through muslin cloth to remove coarse agar particles and glass beads and purity were examined with Gram's staining.

Preparation of production flask of *Clostridium chauvoei* for BQ vaccine

The production flasks, each of 4 L volume, containing Fluid thioglycolate broth (Himedia, India) and a commercial BQ

vaccine medium (Himedia, India) were inoculated with seed culture of *Clostridium chauvoei* strain 49 followed by the addition of 30 ml 50% glucose solution in each flask. The production flasks were divided into two batches. One batch was incubated in static condition (without shaking) at 37°C for 7 days, while the other batch of production flask was incubated at 37°C in a shaker incubator at speed of 120 rpm for 7 days.

Inactivation by formalization

The formal saline solution (0.5%, v/v) was added at final concentration in both bacterial suspensions. For complete inactivation of *Pasteurella multocida* the harvest was kept in the incubator at 37°C for 2-3 days while to prepare BQ anaculture (bacteria + toxoid) vaccine up to 7 days incubation was given at same temperature.

Determination of antigenic mass

Antigenic mass of *Pasteurella multocida* grown in nutrient agar and CSY agar was determined as per prescribed method (Cruickshank *et al.*, 1975). Dry weight of *P. multocida* was found higher in CSY agar than nutrient agar and, therefore, formalized CSY agar washed H.S. bacterin was used to prepare combined vaccine against H.S. and B.Q.

Preparation of combined HS and BQ vaccine

Four groups of combined H.S and B.Q. vaccines were prepared by mixing different proportions of individual formalized bacterial suspensions of *P. multocida* and *Cl. chauvoei* prepared as above to give a final volume of 5ml of vaccine in each group (Table1). Alum solution was added in each group of combined vaccine at a final concentration of 1%.

Standardization of combined vaccine

All groups of combined vaccines were standardized as per described procedure for H.S. and B.Q. vaccine (Kher, 1991; Indian Pharmacopeia 2007; IVRI 1977; OIE 2012).

Sterility test

Sterility of each group of combined vaccine was confirmed by inoculating in nutrient broth, Robertson cooked media and Sabouraud's broth (Himedia, India). The inoculated media were incubated at 37°C for up to 5 days and growth/turbidity, if any, was recorded.

Safety test

The safety test was conducted by injecting 1 and 2ml of combined vaccine from each group in six guinea pigs and two rabbits, respectively. The animals were observed for any local or systemic reaction and/or mortality up to 5 days.

Potency test

H.S. vaccine

The potency test of combined vaccine against H.S. disease was conducted in rabbits. Twenty four rabbits were divided into four equal groups (A_H to D_H). All animals within each group received 2ml of combined vaccine of respective group (A to A_H, B to B_H.) by I/M route on day 1 and a booster dose of equal volume on day 14 of experiment. Two rabbits kept as control were injected with equal volume of sterile nutrient broth I/M on day 1 and 14. On 21st day all rabbits were challenged by injecting 0.2ml of 18h old broth culture of virulent *P. multocida* (Table2) and observed for any untoward clinical symptoms/mortality upto 5 d.p.i.

B.Q. vaccine

The potency test of combined vaccine against B.Q. disease was evaluated in guinea pigs. Thirty two guinea pigs were divided into four equal groups (A_B to D_B). All animals within each group were injected 3ml of combined vaccine of respective group (A to A_B, B to B_B.) by S/C route on day 1 and a booster dose of equal volume on day 14 of experiment. Four guinea pigs kept as control were injected with equal volume of sterile nutrient broth S/C on day 1 and 14. On 21st day all guinea pigs were challenged by injecting 25 viable spores of *Cl. chauvoei* along with 0.2 ml of 0.5% CaCl₂ (Table 3) and observed for any untoward clinical symptoms/mortality upto 5 d.p.i.

Results and Discussion

Haemorrhagic septicaemia and Black quarter are the most common bacterial diseases affecting cattle and buffalo in India. Prophylactic programme is carried out by vaccination against various types of infectious diseases. Combined Haemorrhagic Septicaemia and Black Quarter vaccine, which was found to confer dependable levels of immunity in cattle, was used in zones where both diseases existed (Bharsefat *et al.*, 1977). This research was conducted to prepare and standardize a combined vaccine

against these two diseases.

In the present study, the antigenic mass of *P. multocida* on CSY agar was found to be higher (9.8 mg/ml) than on Nutrient agar (7.8 mg/ml). Better growth of *P. multocida* B:6 was also observed in CSY and BHI broth compared to Nutrient broth on dry weight basis (Mahmood 2001, Shah 2007). An antigenic mass of 3.35-3.68 mg/ml was obtained in CSY broth while only 0.45-0.62 mg/ml in nutrient broth (Kavitha *et al.*, 2011), indicating that CSY broth is a better medium for producing H.S. vaccine, which is in concurrence to our results.

The B.Q. vaccine was prepared by cultivating *Cl. chauvoei* in FTB and a commercial B.Q.vaccine media. Better growth was observed in FTB than the latter. Better growth of *Cl. perfringens* for vaccine production was obtained in reinforced Clostridium media than nutrient broth, in terms of higher biomass and maximum haemolytic units (Bhatti, 2005).

Incubation of *Cl. chauvoei* in FTB with shaking at a speed of 120 rpm for 7 days at 37°C yielded better growth of bacterium than when incubated as static culture. Similar to our findings it was observed that the biomass production of clostridia increased twice on incubation with shaking (Shoshtary *et al.*, 2007).

Table.1 Different groups of H.S. and B.Q. combined vaccine

Group	Combinations	Volume
Group A	H.S. CSY agar washed vaccine	1 ml
	B.Q. Fluid thioglycolate broth vaccine without shaker	4 ml
Group B	H.S. CSY agar washed vaccine	1 ml
	B.Q. Fluid thioglycolate broth vaccine with shaker	4 ml
Group C	H.S. CSY agar washed vaccine	1.5 ml
	B.Q. Fluid thioglycolate broth vaccine without shaker	3.5 ml
Group D	H.S. CSY agar washed vaccine	1.5 v ml
	B.Q. Fluid thioglycolate broth vaccine with shaker	3.5 ml

Table.2 Potency test of combined vaccines against H.S. in rabbits

Group of combined vaccine	Group A	Group B	Group C	Group D	Control
Total no. of animals	6 (A _H)	6 (B _H)	6 (C _H)	6 (D _H)	2
Primary Dose (I/M) (day 1)	2ml	2ml	2ml	2ml	2ml sterile nutrient broth I/M
Booster Dose (I/M) (day 14)	2ml	2ml	2ml	2ml	2ml sterile nutrient broth
Challenge with 18 hrs old culture of <i>P. multocida</i> strain P ₅₂ (I/M)(day 21)	0.2ml	0.2ml	0.2ml	0.2ml	0.2ml

I/M: Intra muscular

Table.3 Potency test of combined vaccines against B. Q. in Guinea pigs

Group of combined vaccine	Group A	Group B	Group C	Group D	Control
Total no. of animals	8 (A _B)	8 (B _B)	8 (C _B)	8 (D _B)	4
Primary Dose (day 1) (S/C)	3 ml	3 ml	3ml	3 ml	3ml sterile nutrient broth
Booster Dose(day 14) (S/C)	3 ml	3ml	3ml	3 ml	3ml sterile nutrient broth
Challenge with 25 viable spores of <i>Cl. chauvoei</i> + 0.2 ml of 5% CaCl ₂ (I/M) (day 21)	0.2ml	0.2ml	0.2ml	0.2ml	0.2ml

S/C – Subcutaneous, I/M – Intramuscular

Fig.1 Results of potency test of different groups of combined vaccine Against Haemorrhagic septicaemia in rabbits

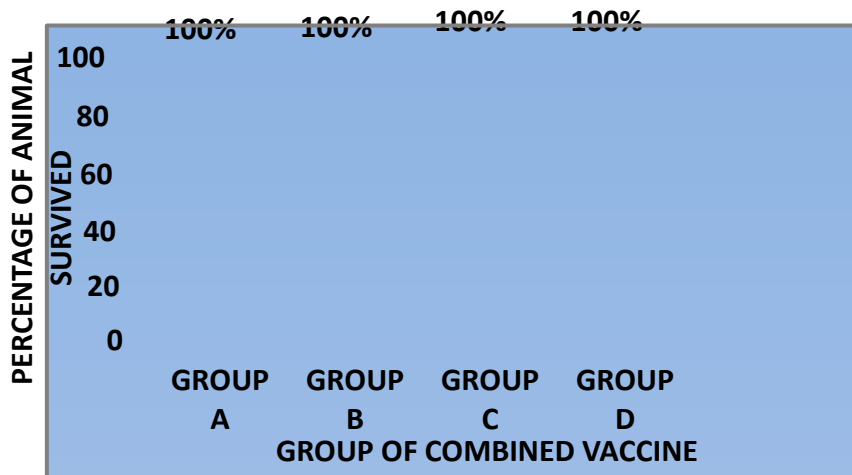
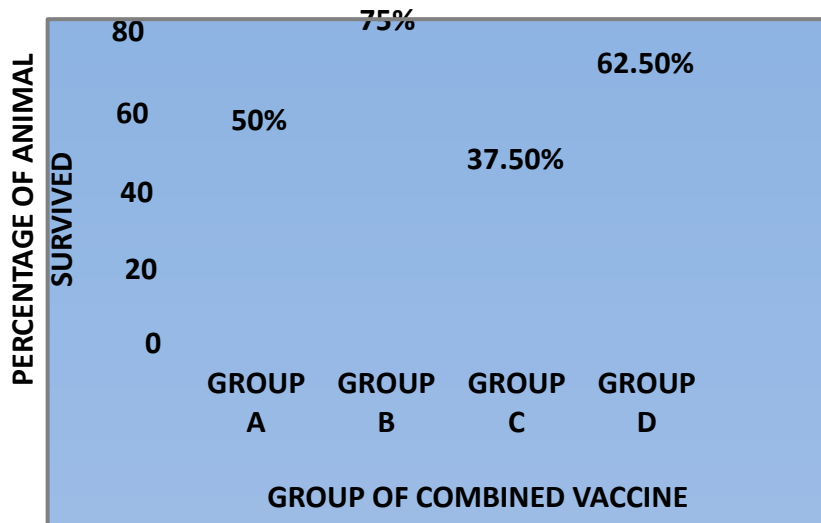


Fig.2 Result of potency test of different groups of combined vaccine against Black quarter in guinea pigs



In the present investigation, different formulations of combined H.S. and B.Q. formalised vaccines along with alum (1% final concentration) as an adjuvant were prepared to give a final volume of 5 ml and further standardized as per standard procedures. The combined vaccines of all four groups were tested for sterility, safety and potency. The results of potency test are presented in figure 1 and figure 2. Some researchers also prepared and standardized combined H.S. and B.Q. vaccines (Sinha and Prasad 1973; Srivastava *et al.*, 1975; Srinivasan *et al.*, 2001; Reddy *et al.*, 2001). All the groups of combined vaccine were found safe and sterile. Anaphylactic reaction was also not observed against any of the combined vaccine, which is in agreement to others (Jabbari *et al.*, 2008).

Amongst all groups, group B combined vaccine containing 1 ml H.S. Casein sucrose yeast extract agar washed formalized vaccine and 4 ml B.Q. Fluid thioglycolate broth formalized anaculture vaccine with shaking was found most efficacious in our study. This combined vaccine conferred 100% protection

against H.S. disease and 75% against B.Q. disease in rabbits and guinea pigs (figure 1 and figure 2), respectively in potency test performed for individual vaccines. In another study, H.S. agar washed alum precipitated-B.Q. broth vaccine provided absolute protection in vaccinated calves against a fatal challenge infection of the homologous strain of *P. multocida* and *Cl. Chauvoei* (Sinha and Prasad, 1973). Some studies observed that the performance of oil adjuvanted vaccines containing *P. multocida* and *Cl. chauvoei* antigen in comparison with individual component vaccines; combined vaccine provided better immunity than individual vaccine (Srivastava *et al.*, 1975; Reddy *et al.*, 2001). Combined vaccines were found potent and induced a sustainable seroconversion against individual infectious agent (Jang *et al.*, 2009; Altaf *et al.*, 2012).

In the present study, group B vaccines gave 100% (6/6 survived) protective against virulent *P. multocida* and 75% (6/8 survived) against *Cl. chauvoei* in rabbits and guinea pigs, respectively. This may be attributed to lower antigenic ratio of *Cl. chauvoei* and its

toxoids in the combined vaccine. This can be overcome either by increasing volume of B.Q. anaculture vaccine with effort to concentrate protection unit (minimum 10^4 /dose) of H.S. vaccine in lesser volume or by increasing antigenic mass of *Cl.chauvoei* anaculture in above vaccine.

This study conclude that a combined vaccine for HS and BQ consisting of 1 ml of CSY agar washed *P. multocidabacterin* and 4 ml of FTB cultivated *Cl. chauvoei* anaculture with shaking was found most potent in protecting against virulent bacterial infection in experimental animals. Further studies also suggested that potency test of combined vaccine should be made in natural host that is cattle and buffalo. Long-term studies are also required to determine the duration of immunity against two diseases.

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