Comparative Assessment of Serum Biochemical Markers from Cattle and Buffaloes Vaccinated with Three Different Oil Adjuvant Vaccines of Hemorrhagic Septicemia from Punjab, Pakistan

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Abstract

Haemorrahgic Septiceamia (H.S) is a deadly, sub-acute livestock disease of cattle and buffalo in Southeast Asia including Pakistan. A whopping 98% case fatality rate renders vaccination a mandatory prophylactic control measure to counter HS. Very little information is available on serum biochemical profile of the HS vaccinated animals. This study was conducted to investigate the serum biochemistry of the vaccinated cattle and buffalo calves and adults alike. Three different experimental oil adjuvant vaccines were produced for *Pasturella multocida* serotype B:2 (locally prevalent). N=80 healthy cattle and buffalo (calves and adults) were divided into four groups. Each group consisted of 20 animals (10 calves and 10 adults). Group A was injected with vaccine prepared with Montanide ISA-50; Group B with vaccine prepared from Montanide ISA-206 while Group C with a vaccine produced from Liquid paraffin and Lanolin. Group D animals served as non-immunized control. Blood samples were collected from each animal at zero-day, after booster at 90 days, and then every quarter for one year. Twelve biochemical parameters were assessed by ANOVA and Kruskal Wallis Test. Post hoc multiple comparisons were done for glucose levels. It was concluded that vaccinating both cattle and buffalo calves and adults with H.S. vaccine formulated with three different adjuvants did not have a statistically significant effect on serum biochemistry of vaccinated animals compared to controls.

Keywords: Biochemical markers, Hemorrhagic septicemia, Vaccine, Montanide, ISA-50, ISA-206, LP+LA, *Pasteurella multocida*.

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1

Introduction

Hemorrhagic Septicemia is an extremely fatal and acute disease of cattle and buffalo caused by *Pasteurella multocida*. Serotype B:2 is the most prevalent in Asia while in Africa, serotype E:2 is mostly responsible for the disease. This specific pathogen is classified into five serogroups based on capsular typing i.e. A, B, D, E, and F. Additionally, there is a very strong relationship between the capsular serotyping and disease predilection [1, 2]. *P. multocida* also affects a wide range of other animal species like pigs, sheep, goats, camels, poultry, ducks as well as wild animals and resides as a normal inhabitant of the respiratory tract [3]. In Pakistan, this disease is considered to be among the most lethal endemic and/or a sporadic infections and is known to cause 31.4 % of all deaths in susceptible cattle and buffalo [4]. Once clinical signs appear, the case fatality rate becomes 100%. It causes heavy economic losses to animal production by causing primarily respiratory or systemic diseases [2] [5], [6]. During a respiratory disease outbreak in Pakistan in 2011, this bacteria was also isolated from clinical and morbid samples in the dromedary population of camels [7].

From a prophylactic viewpoint, the only method of prevention and control of this disease is timely vaccination. Alum precipitated H.S. vaccine has been in wide use for many years. Recently, the alum-based vaccine has been replaced by an oil adjuvant vaccine for the following reasons: (i) induction of a long-term specific immune response owing to sustained release (ii) a single injection to animals per year compared to two with alum precipitated vaccine (iii) improving animals' welfare and finally to reduce the logistical costs for dissemination and administration of vaccine across the country. Now, a single oil adjuvant-based vaccine shot is being practiced with an annual booster [4]. For long-term immunity, generally, water in oil emulsions is recommended for bovine, small ruminants, poultry, and fishes [8]. For laboratory diagnostic evaluations, the measurement of serum proteins like albumin and globulin is found to have a vital role in animal

immunization. After vaccination, the serum protein ratio of albumin to globulin is decreased which acts as an indicator of antibody production. In case of pasteurellosis in sheep, a decline in the total serum protein, albumin, and increased globulin was found [9]. -Post-vaccination, the immune responses are mediated via the interaction of antioxidant systems along with reactive oxygen free radicals [10]. It has been observed from the past studies conducted on murine models that the injection of either lipopolysaccharide (LPS), outer membrane protein (OMP) or *Pasteurella multocida* whole cells results in alteration in the biochemical parameters within 24-48 hours [1]. Petite information is available in the literature on the analysis of serum biochemistry profile post vaccination against *Pasteurella multocida* serotype B:2 in cattle and buffaloes. To see the impact of HS vaccination on serum biochemical markers, the current study has been conducted on cattle and buffalo calves and adults vaccinated with HS vaccines manufactured by using three different oil adjuvants (Montanide ISA-50; Montanide ISA-206 and Liquid Paraffin/Lanolin). A comparison with non-immunized control has also been provided.

Material & methods

Animals Selection

Eighty (80) cattle and buffaloes were selected for this study at Livestock Production and Research Institute, Bahadurnagar Okara. Among them, forty were young calves while other forty were adults. All the animals were clinically healthy with good body condition scores. The young calves weighed about 100-150 kg while the adult animals weighed around 300-350 kg. All the animals were alert in their general attitude and no abnormality was observed in the animals regarding their walk and gait. There was no previous history of any diarrhea or other infection for fifteen days before the trial. The feeding and management of all the animals were up to the mark. After deworming with broad spectrum anthelmintics (Oxafax), other physiological parameters of the body including temperature and antibody titers against *Pasteurella multocida* serotype B:2 were evaluated [11]. The feed offered during the experiment consisted of a mixture of silage, hay, concentrate, roughages, and green fodder. Clean drinking water was

available ad-libitum.The animals were divided into four groups: each consisting of twenty animals. Ten animals were young calves and ten were adults in all groups. Both male and female animals were included in this study. Female adult animals were in 6th lactation while the young calves were of 6-8 months old. The identification of each animal was marked by ear tagging. Serum collecting tubes marked by tag numbers were used for the serum collections from each animal [12].

Vaccination

The vaccine production was done at the Hemorrhagic Septicemia vaccine section of Veterinary Research Institute (VRI), Lahore, Pakistan. Three different types of oil adjuvants vaccines were produced for this study. The three adjuvants used were Montanide ISA-206, Montanide ISA-50 and LP+ LA (Liquid paraffin and lanolin). The vaccine prepared with Montanide ISA-206 was a double emulsion that was W/O/W (water in oil in water), while ISA-50 was a single emulsion W/O (water in oil). These Montanide adjuvants were imported from Seppic® France. Liquid paraffin and lanolin were purchased from a local market. All the prepared vaccines were checked for in-house quality control testing and were also sent to Quality Control (QC) Laboratory of Veterinary Research Institute (VRI) Lahore for further investigation of sterility, safety, and potency. After successful QC testing, animals in Group A were injected with a vaccine prepared with Montanide ISA-206. Group B animals received a vaccine with ISA-50 while Group C animals were injected with LP+LA-based vaccine. Group D animals were used as non-vaccinated controls. The route of administration for the vaccine was deep intramuscular in the brisket region of the animals and the dose was 2 c.c per animal regardless of age and weight of the inoculated animals.

Sampling

Sample collection was done before sunrise to avoid heat stress on the animals. All the blood samples were collected into plain vacutainer tubes (05 ml plain vacuum tubes from BD® Company). All the collected samples were then stored in containers having ice bags and were sent to the lab quickly. The samples were centrifuged at 3000 rpm for 05 mins and serum was harvested according to standard methodology [13] and two aliquots were prepared for each sample. One aliquot of all samples was stored at 4ºC for serum protein analysis and lipid profile (analyzed within 02 days of sample collection every time). The other aliquot was stored at -20º C. Blood samples were collected on (i) day 0, (ii) day 90 (iii) day 180 (iv) day 270, and finally on day 360 days -post-vaccination [12].

Blood Biochemistry

Biochemical profiling was studied using the indicators: (i) Total serum proteins (ii) albumin (iii) blood glucose (iv) Cholesterol (v) triglycerides (vi) Calcium (vii) Potassium (viii) Magnesium (ix) Phosphorus (x) Sodium (xi) Uric Acid (xii) Creatinine. Spectrophotometric measurements were done for all calculations by using commercial kits Fluitest® Analyticon® (Germany) and UV spectrophotometer (Kontron® T80,Uvicon 930, and Analytikjena® Specord 50).

Statistical Data Analysis and Results

Normal reference values were taken by following Adb Ellah et, al 2014 and Merck Manual 2015.

Hypothesis

H_0=There is no effect of vaccination on the serum biochemistry profile of the vaccinated along with unvaccinated groups of animals.

H_1=There is an effect of vaccination on the serum biochemistry profile of the vaccinated along with unvaccinated groups of animals.

Table 3.1: Descriptive Statistics

Serum Biochemistry	Vaccinated with ISA 206			Vaccinated with ISA 50			Vaccinated with LP LA			Unvaccinated or controlled		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Albumin	20	2.96	0.355	20	2.94	0.45	20	3.15	0.27	20	3.25	0.44
Glucose	20	27.52	7.63	20	30.12	6.09	20	34.27	8.77	20	32.08	9.61
Potassium	20	5.12	1.63	20	5.28	1.94	20	4.8	1.45	20	5.17	1.42
Phosphorus	20	5.37	1.36	20	4.77	1.1	20	5.16	0.96	20	5.23	1.18
Sodium	20	170.01	23.47	20	176.59	22.84	20	179.2	22.73	20	182.75	23.91
Protein	20	6.11	0.81	20	6.17	0.73	20	6.31	0.91	20	6.08	0.79
Cholesterol	20	93.87	47.75	20	87.22	45.44	20	85.06	28.05	20	86.39	24.2
Triglyceride	20	16.09	5.39	20	15.71	5.77	20	17.95	7.31	20	20.57	7.54
Uric acid	20	1.28	0.23	20	1.38	0.34	20	1.22	0.28	20	1.41	0.45

Magnesium	20	3.06	0.43	20	3.04	0.29	20	3.08	0.31	20	3.08	0.46
Calcium	20	8.58	1.17	20	9.15	1.21	20	8.78	1.16	20	9.17	1.21
Creatinine	20	1.67	0.63	20	1.77	0.43	20	1.72	0.46	20	1.61	0.4

Table3.2: Testing using Non parametric Kruskal- Wallis test

Serum Biochemistry	Test Statistic	d.f	P-value	Decision
Albumin	6.085	3	0.108	Accept Ho
Glucose	8.4	3	**0.038***	**Reject Ho**
Potassium	0.648	3	0.885	Accept Ho
Phosphorus	2.508	3	0.474	Accept Ho
Sodium	2.726	3	0.436	Accept Ho
Protein	1.401	3	0.705	Accept Ho
Cholesterol	0.283	3	0.963	Accept Ho
Triglyceride	5.505	3	0.163	Accept Ho
Uric acid	3.11	3	0.375	Accept Ho

Table 3.3: Post Hoc multiple comparisons for Glucose

Groups	p-value
Vaccinated with ISA 206 - vaccinated with ISA 50	0.816
Vaccinated with ISA 206 - vaccinated with LP LA	**0.039***
Vaccinated with ISA 206 - Unvaccinated	0.184
Vaccinated with ISA 50 - Unvaccinated	1
Vaccinated with ISA 50 - vaccinated with LP LA	1
Vaccinated with LP LA - Unvaccinated	1

Table3.4: Testing using ANOVA Technique

Serum Biochemistry	Test Statistic	d.f	P-value	Decision
Magnesium	0.058	3	0.982	Accept Ho
Calcium	1.183	3	0.322	Accept Ho
Creatinine	0.353	3	0.787	Accept Ho

Statistical Data Analysis and Results

Normal reference values were taken by following [12] and Merck Manual 2015.

The data were tested for normality and homogeneity of variance. Normality was tested through Shapiro Shapiro-wilk test ($p > 0.05$, data is normal) and homogeneity of variance was tested through Levene's test ($p > 0.05$, variances are homogeneous). Out of all serum biochemical markers evaluated; Magnesium, calcium, and creatinine satisfied both assumptions of normality and homogeneity so ANOVA technique was applied and found that all the p-values erewere greater than 0.05 so the accepted null hypothesis i.e. there is no effect of vaccination on the serum biochemistry profile of the vaccinated along with unvaccinated groups of animals (Table 4). On the other hand, the rest of the factors didn't meet the assumptions so a nonparametric Kruskal-Wallis test was used to test the hypothesis. It was found that except for Glucose all the p-values were greater than 0.05 so null hypothesis was accepted i.e. there is no effect of vaccination on the serum biochemistry profile of the vaccinated along with unvaccinated groups of animals (Table 2). Glucose showed a significant result ($p<0.05$). Now to find which group differs significantly, post hoc multiple comparisons were carried out and found that the animal group vaccinated with ISA 206 vs vaccinated with LP+LA was statistically significant (Table3).

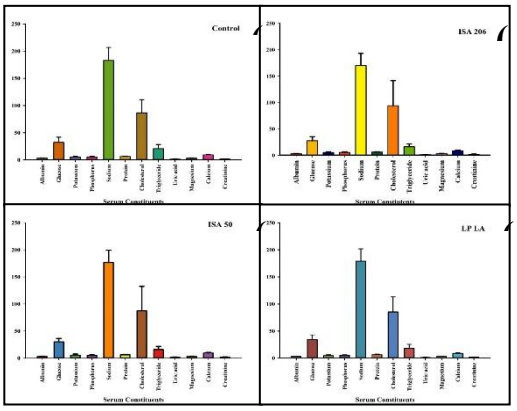

Fig. 1. Serum biochemistry of blood in response to treatment with Control (a) ISA 206 (b) ISA 50 (c) and LP LA (d).

Discussion

HS, a common bacterial disease caused by *Pasteurella multocida*, is cosmopolitan in distribution and is considered one of the major and economically important diseases of buffaloes and cattle in Pakistan. Animals of all age groups and both sexes are susceptible to this disease [11]. This organism is gram-negative, coccobacillus, and capsular organism which is -non-hemolytic, non-motile, non-spore-forming and is a natural inhabitant of the respiratory tract of its host [14]. It can be isolated from the saliva, bloodstream, and bone marrow of the affected animals [11]. In all countries where HS is prevalent, vaccination is the most accepted method for the control of this disease. several vaccines are produced for this purpose. Most countries use broth bacterin or alum precipitated vaccine while a few use oil adjuvant vaccines. Vaccines prepared in different countries differ in the strain of organism used, culture media and the method of cultivation. Various culture methods have been adopted i.e., simple static broth cultures and aerated dense cultures using complex fermenters etc. Broth culture may be used for the production of simple bacterin or alum precipitated vaccine [11].

Vaccines were prepared according to standard protocols and procedures listed by [15]. The organism *P. multocida* serotype B:2 was cultured on Brain Heart Infusion (BHI) media using vortexing and aeration to get maximum growth with optimized conditions of temperature and pH. The animals used in current studies including both calves and adults were kept at LPRI Bahadurnagar Okara. Vaccines were injected via intramuscular route as done in routine vaccination and a booster was given at 90 days interval from primary dosing. After vaccination, no anaphylactic shock was recorded in any of the animals [11].

Serum biochemical analysis is very important regarding disease diagnosis, treatment, and vaccine development. The main objective of current studies was to check the effect of vaccines especially hemorrhagic septiceamia oil adjuvant vaccines on the serum biochemistry of cattle and buffaloes after vaccine inoculation as Serum biochemical levels of HS vaccinated animals are not known. Three different oil adjuvant vaccines were used for the studies and were compared with each other as well as with a negative control animal group.

For this study, the normal parameters for the animals were used according to [12] in which serum biochemicals and hematological reference intervals of water buffalo heifers were discussed. The body temperature was measured during the whole study period which is following the current studies where the body temperature observed was 38.5^{o}C. This reference value also matches that of the Food and Agriculture Organization. The serum albumin measurement was following [12] regarding reference values. A study on camels [3] found no significant difference in the serum albumin and total protein concentration of vaccinated as well as unvaccinated animals after using polyvalent Pasteurella vaccine which was following our findings on cattle and buffaloes. Non-significant differences in the serum protein levels after vaccination in different diseases like FMD, brucellosis, and West Nile Virus had also been reported [16],[17, 18]. A decrease in the total serum albumin level and an increase in globulin concentration after Pasteurellosis infection was observed [9]. Limited data is available on an estimation of biochemical parameters post vaccination which was addressed in our current study.

In this study, it was observed that after vaccination there were no significant differences in mineral electrolytes like sodium, potassium, phosphorus, magnesium, and calcium. In HS and pasteurellosis infection, a decrease in sodium level and an increase in potassium level were observed [19], [1]. It can be said that during clinical disease the levels of sodium and potassium may be deceased but after vaccination, there was no significant difference in vaccinated as well as un-vaccinated animals.

Regarding glucose levels; the observations in this study that the blood glucose level in the un-vaccinated control group was less than the normal range of blood glucose was following [12]. The reason behind this natural decrease in the blood glucose level is still unknown and can be due to environmental factors or the feeding habits of the animals in the area.

Conclusion

From the current studies, it can be concluded that there is no significant difference in the blood biochemistry profile of the cattle and buffalo after Hemorrhagic Septicemia vaccination as compared to the control group. So HS vaccine does not disturb any biochemical and mineral parameters in cattle and buffalo.

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