Research Article

Effect of Exposure to *Pasteurella multocida* B:2 and its Immunogens on Clinical, Hematological and Serum Biochemical Parameters in Neeli Ravi Buffaloes

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Abstract | Buffaloes and other bovines suffer from a fatal disease, "Hemorrhagic septicemia" (HS), which is produced by a gram-negative bacterium "*Pasteurella multocida.*" Existing literature has established bacteria's pathogenesis, but still, the literature about the role of bacteria and its immunogens on the demonstration of clinical signs, hematology and serum biochemistry in Nili Ravi buffaloes under subtropics is lacking. This study was designed to fulfill this gap by analyzing the effects of exposure to the bacterial culture of *Pasturella multocida (P. multocida) type* B:2 and its immunogens, i.e., lipopolysaccharide (LPS) and outer membrane protein (OMP) on Nili Ravi buffaloes. Prepubertal Nili Ravi female buffaloes (N=18) of 10-12 months age in good health condition were divided into five treatment groups (n=3 each) and a control group (n=3). Treatment groups' animals were exposed to *P. multocida* bacterial culture and its immunogens, i.e., LPS (oral and intravenous) and OMP (oral and subcutaneous). Animals were analyzed for the development of clinical signs, hematology and serum biochemistry. All treatment groups exhibited clinical signs (ranging from mild to acute) like increased temperature, variation in rumen motility, mucous membrane, salivation, edema, dullness, depression, nasal discharge, respiratory distress and recumbence or death. A significant change in complete blood count (CBC) including packed cell volume (PCV), hemoglobin (Hgb) concentration, red blood cells (RBCs) count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) platelets count, mean platelet volume (MPV), white blood cells (WBCs) count, monocytes count, lymphocytes count and granulocytes count. Serum biochemistry profiles (total protein, Albumin and creatinine) of treatment group animals were also changed significantly. Cumulatively it was revealed that *P. multocida* B: 2 and its Immunogens (LPS and OMP) affect the hematology and serum biochemistry profiles of Neeli-Ravi buffaloes. These findings prove that OMP and LPS from *P. multocida* B: 2 may be considered for vaccine development.

Introduction

Buffalo is considered the main dairy animal in Pakistan. Almost 76% of total buffaloes are found in Punjab and the remaining 24% of the buffaloes are found in other provinces (Anonymous, 2019-20). The total number of buffaloes in Pakistan is 41.2 Million, while 73% of the national milk production

is contributed by Punjab, out of which 60% of the milk is obtained from buffaloes. In Pakistan, buffaloes are kept in a traditional small farming system and the farming is also integrated with the production of crops. It is believed that 85% of the farmers are keeping a herd of one to five animals. 70% of milk production is contributed by 0.5 Million landless farmers in Pakistan (Chung et al., 2016).

HS is considered endemic in Pakistan and costs heavy economic losses to the livestock farmers every year. HS has been reported globally, excluding UK, Australia and various other European countries (Othman et al., 2016). HS is an extraordinarily catastrophic disease of bovines in humid states. Specific serotypes of P. multocida are predominant in peculiar geographic zones. Serotype B:2 is predominant in Asian countries, while serotype E:2 is prevailing in African countries (Saad et al., 2013).

HS mostly occurs in acute form; however, sub-acute and chronic forms are also not very infrequent. Initially, there is an increase in rectal temperature, which often goes undetected. Afterward, respiratory distress, excessive salivation, fatal septicemia, and eventually recumbence result in the animal's death. The usual incubation period of this disease is 1–3 days, but sudden death without any observable clinical signs may also happen (De Alwis, 1999). The acute form of HS onsets all of a sudden and causes death within 24 hours. The animals exhibit an unusual increase in rectal temperature (105–107°F), cyanosis of visible mucus membranes and weak pulse coupled with dyspnea (Benkirane and De Alwis, 2002). The sub-acute form of HS is described by bronchopneumonia and edema at the throat/brisket region. The progression of HS infection takes a longer time in the chronic form. The exhibited signs include dyspnea and mucopurulent/bloody nasal discharge (OIE, 2009).

HS is believed to be the most dangerous and there are rare chances of cattle being recovered from it once its clinical signs appear. The most probable way to control this disease in endemic countries is through preventative vaccination programs. The vaccination's effectiveness could be enhanced by immunization of animals before the disease period's onset (Shivachandra et al., 2011). There are two serotypes (B: 2 and E: 2) of Pasteurella, which cause HS. In Pakistan and other Asian countries, B:2 is prevalent (Ibrahim et al., 2016).

Essential factors which contribute to the virulence of P. multocida include lipopolysaccharides (LPS), outer membrane protein (OMP), P. multocida toxins (PMT) and its capsule (Kubatzky, 2012). All of these virulent factors can activate the systemic immune response by the host. LPS has been described as an important component of gram-negative bacteria. P. multocida is also a gram-negative bacterium and based on dissimilarities in LPS composition, its 16 types have been established (Harper et al., 2011). Based on variation in bacterial capsules' antigenicity, P. multocida has been divided into five serogroups, i.e., A, B, D, E and F (Wilson and Ho, 2013). OMPs of P. multocida have been categorized based on their structure and immunogenicity. These proteins are believed to mediate the host immune response through cytokine production (Iovane et al., 1998).

As this disease causes sudden death despite antibiotic and serum therapies, vaccination programs are the only solution to lessen this disease's impact (De Alwis, 1999). Extensive vaccination control programs are being implemented to control this disease in endemic areas. Such programs are very important to curtail the animal death rate and economic losses cause by this disease. Presently HS is being controlled by broth vaccine and oil adjuvant vaccine. Broth vaccine is used for rapid coverage in outbreak areas and oil adjuvant vaccines are used in adjacent areas. In South Asian countries, a live attenuated vaccine is suggested preceding monsoon season (Zamri-Saad and Annas, 2016). Difficulties in gathering animals for an injectable vaccine in case of small holdings and the possibility of negative effects like a sterile abscess, localized swelling, and post-vaccination shock are some important concerns related to oil-adjuvant vaccines against HS (Rafidah et al., 2010).

Oral bacterial culture vaccines and injectable vaccines based on P. multocida immunogens may prove beneficial to be used in bovines. Recently investigations in Malaysia have stated the stimulation of host cell response in buffaloes against oral/subcutaneous exposure to bacterial culture (Chung et al., 2015). A similar study has reported severe distress and ultrastructural changes in the gastrointestinal, urinary tract and respiratory system of buffaloes on subcutaneous administration of 5ml of P. multocida type B:2 (10^9 CFU/ml) suspension (Annas et al., 2015).

Another study on oral and subcutaneous inoculation of OMP extract of *P. multocida* type B: 2 has resulted in significant differences in hematology and biochemistry profiles of experimental buffaloes (Chung *et al.*, 2016). In a recent study, in-vitro exposure of *P. multocida* type B:2 and its LPS endotoxins has resulted in ultrastructural changes in buffaloes' endothelial cells (Puspitasari *et al.*, 2020).

Mice have been extensively used as an experimental animal in pathogenesis studies against capsular type A or B of *P. multocida* (Harper *et al.*, 2006). Intraperitoneal inoculation of mice with a very small dose of *P. multocida* organisms (20 CFU) has caused its death within 24 hours (Ramdani *et al.*, 1990; Biswas *et al.*, 2004). The geographical distribution of *P. multocida* serovars is well established, but no differences in virulence of these serotypes have been reported (De Alwis, 1999).

A study on rabbits inoculated subcutaneously with LPS extract of *P. multocida* has established a correlation between improved antibody titer and an increased LPS antigen dose (Ashraf *et al.*, 2014). An inclusive proteome analysis study of sarcosine-insoluble membrane segments has demonstrated a substantial role of *P. multocida* OMP-based vaccine in providing cross-protective immunity (Boyce and Adler, 2006).

P. multocida causes fowl cholera in poultry birds. Outer membrane protein gene (*ompH*) has been reported as a strong immunogen of *P. multocida* in poultry birds. A recombinant vaccine has been devised for the poultry industry using Escherichia coli plasmid-encoded with *ompH*-gene, which has induced antibody production and ensures good immunity (Khedr *et al.*, 2005). Another similar study from China (Gong *et al.*, 2013) has reported an effective immune response in poultry birds with avian *P. multocida* OMP encoded vaccine. "C terminal fragment" of PMT has successfully been used in a recombinant DNA vaccine and has produced humoral and cellular immunity in swine (Lee *et al.*, 2012). Keeping in view the importance of HS and substantial losses rendered by this disease, the present study was designed to check the effects of experimental exposure of *P. multocida* B:2 (oral) and its immunogens, i.e., LPS (oral and intravenous) and OMP (oral and subcutaneous) on clinical, hematological and serum biochemical parameters in Neeli-Ravi buffaloes.

Materials and Methods

Experimental design

In this study, the sample size consisted of prepubertal Nili Ravi female buffaloes (N=18) of 10-12 months of age with good health conditions. They were divided into six groups from Group-1 to Group-6 (G-1 to G-6) and each group consisted of three buffaloes. G-1 animals (negative control) were treated with 10 ml sterile phosphate buffer saline (PBS) orally. On the other hand, 10 ml of 10^{12} colony-forming unit (CFU) of *P. multocida* B: 2 was given orally to G-2 animals (Chung *et al.*, 2015). Furthermore, 10 ml bacterial LPS extract was given to G-3 animals orally and G-4 animals intravenously (Jesse *et al.*, 2017). At last, 10 ml bacterial OMPs extract was inoculated subcutaneously and orally to the remaining animals of G-5 and G-6, respectively. All the experiments were conducted as per guidelines given in the animal handling manual and were approved by the ethical committee of the Department of Zoology, University of the Punjab, Lahore.

Inoculum preparation

Stock culture of *P. multocida* B:2 (used in this study) was taken from a Veterinary Research Institute, Lahore. Culturing of bacteria was done on blood agar plates with an incubation period of 24 hours at 98.6 °F. After the culture's growth, a loopful was inoculated in nutrient broth with continuous shaking at 150 rpm for 24 hours at 98.6 °F. Broth cultures were pooled and the purity was confirmed and the concentration was adjusted to 1×10^{12} colony forming units (CFU) per ml as described by (Goldman and Green, 2015).

Lipo poly saccharide (LPS) extraction from P. multocida type B: 2

Lipopolysaccharide (LPS) isolation kit (MAK 339 Sigma-Aldrich USA) was used to extract LPS from the bacterial culture of *P. multocida* type B2. The kit contained LPS isolation buffer and proteinase K. Isolated bacterial culture was grown on LB medium plate at 98.6 °F for overnight. The next day, 12 ml of cold PBS was added to a (pre-weighed) 15 ml conical tube. Bacterial culture was suspended in PBS as per kit instructions and centrifuged at 2500 x g for 10 minutes. The supernatant was discarded, while centrifugation was repeated. The supernatant was completely removed and Lysis buffer (in volume 10X to pellet weight) was added after weighing the pellet. The mixture was sonicated thrice for 30 x seconds to

disrupt the aggregates of bacteria while keeping the tube on the ice during the procedure. The tube was incubated on ice for 10 minutes. The mixture was then centrifuged at 39.2 °F for 10 minutes at 2500 g. The lysate was transferred to a clean 1.5ml centrifuge tube and proteinase K was added. The tube was incubated at 140 °F for an hour. Afterward, it was centrifuged at 39.2 °F for 10 minutes at 2500 x g and the supernatant was shifted to a fresh Eppendorf tube.

Outer membrane proteins (OMP) extraction from P. multocida type B: 2

The first step involved freezing the freshly harvested cell pellets for 24 h before extraction. The cell pellets were then thawed for 15 minutes on ice and re-suspended in 10 ml of native lysis buffer. After that, incubation was done on ice for 30 minutes, followed by centrifugation at 14,000 rpm for 30 minutes at 39.2 °F.

Hematology and blood chemistry

The blood samples were taken from the calves' jugular vein after each treatment and serum was taken after centrifugation at 10000 rpm for 10 minutes.

Serum protein, Albumin and creatinine were measured by BioChem SA Semi-Auto Chemistry Analyzer by (High Technology, Inc Massachusetts USA) using the commercial kits (MTD diagnostics Srl, Maddaloni Italy). In contrast, RBCs, WBCs, lymphocytes, monocytes, granulocytes, PCV, Hgb, MPV, MCH and MCHC were measured by URIT-2900 Vet Plus Automated Hematology Analyzer (URIT Medical Electronics, China) using reagents (URIT Medical Electronics, Guangxi541001, PR China).

Clinical study

The clinical signs such as temperature, rumen motility, respiratory rate, serous nasal discharge, recumbence, mucous membrane, salivation, edema, dullness, depression and death were checked in all the treatment groups.

Statistical analysis

The collected data was statistically analyzed by One Way ANOVA using SPSS version 24.0. The variations in different parameters with 95% confidence interval were considered statistically significant.

Results and Discussion

Clinical signs and symptoms

The clinical signs and symptoms observed in treatment groups (G-2 to G-6) were noticeable compared to the control group (G-1). The incubation periods for prepubertal buffalo calves varied according to the type and route of infection. Exposure to the bacterial culture, LPS and OMP through oral route (G-2, G-3 and G-5, respectively) caused a high rise of body temperature up to 3 days, but after that, it remained in a constant range of 100°F-102°F. The rumen motility was also slowed down, but the mucous membrane remained normal in these three groups. There was a high rise in body temperature for five days in animals treated with LPS through an intravenous route (G-4). Afterward, the rectal temperature of G-4 animals remained constant between the ranges of 100°F-102°F. The mucous membrane of these animals was congested slightly and indigestion was also observed. Among OMP through subcutaneous route animals (G-6), the temperature noted after 2 hours was 106.5°F. It remained constant at 104°F for 12 hours. These animals exhibited respiratory distress, absence of rumen motility and submandibular edema after 24 hours of inoculation. These animals died after 60 hours on average. The serous nasal discharge was seen among animals after 4 to 6 hours of immunogenic treatment of all groups except G-4.

Mild dyspnea and normal posture were observed in all treatment groups except G-6 as the illness was acute in these animals. There were no observed clinical signs in control group G-1. The major clinical signs and symptoms observed in prepubertal female buffalo calves following inoculation with *P. multocida* B:2 and its immunogens (LPS and OMP) are summarized in Table 1.

Hematology and serum biochemistry

The mean value of RBCs, WBCs, lymphocytes, monocytes and granulocytes were significantly higher among all treatment groups as compared to the control. PCV, Hgb, MPV and MCH also exhibited a similar trend. MCHC was also higher in treatment groups, but the difference among G-1, G-5 and control were not statistically significant ((P>0.05). Platelet count was significantly decreased (P<0.05) among all treatment groups compared to the control. OMP treatment groups (G-4 and G-5) showed the highest increase in WBCs count than

other treatment groups and control. These differences were statistically significant. The change in Hgb was significantly greater in the LPS-IV (G-4) treated animals compared to all other groups. The details of changes in hematological parameters of treatment groups have been illustrated in Table 2.

Table 1: *Clinical signs observed in Neeli–Ravi buffaloes inoculated with P. multocida B:2 and its Immunogens (LPS and OMP) through various routes.*

Clinical Parameters	Control G-1	Bacterial culture	LPS		OMP	
		Oral route G-2	Oral route G-3	Intravenous route G-4	Oral route G-5	Subcutaneous route G-6
Death	Survived for 21 days	Alive up to 21 days after infection	Alive up to 21 days after infection.	Alive up to 21 days after infection.	Live for 21 days after infection.	Alive for 60 hours only.
Temperature (Temp)	Normal	High rise of rectal temp for initial 3 days but afterwards, it lowered down to 100-102°F.	High rise of rectal temp for initial 3 days but afterward lowered down to 100 -102°F.	High rise of rectal temp for the initial 5 days but afterward lowered down to 100 -102°F.	High rise of rectal temp for initial 3 days but afterwards, it lowered down to 100-102°F	Temp noted after 2 hours was 106.5°F. The temperature remained constant at 104°F for 12 hours.
Rumen motility	Normal	Rumen motility slight slowdown.	Rumen motility slight slowdown.	Indigestion.	Rumen motility slight slowdown.	Absence of rumen motility after 24 hours of infection.
Respiration	Normal Range	Mild dyspnea.	Mild dyspnea.	Mild dyspnea.	Mild dyspnea.	Severe dyspnea started after 24 hours of infection.
Mucous membrane (MM), Salivation Edema and Dullness	Normal	Normal MM.	Normal MM.	Slightly congested MM.	Normal MM.	MM was congested after 6 hours of infection, Submandibular edema extending up to the brisket region and dullness seen after 24 hours of infection.
Serous nasal discharge	No nasal discharge.	For the initial 4 days of infection only.	For the initial 4 days of infection only.	For the initial 5 days of infection only.	No nasal discharge.	Seen after 6 hours of infection.
Recmbency	None.	Normal posture	Normal posture.	Normal posture	Normal posture	Animals collapsed after 36 hours.

Table 2: *Hematological profile of Neeli–Ravi buffaloes inoculated with P. multocida B:2 and its Immunogens (LPS and OMP) through various routes.*

Treatment	Control	Culture	LPS		OMP	
Parameter/ Group	PBS	PO	PO	IV	PO	S/C
PCV (%)	34.33 ± 0.88^a	53.60 ± 0.92^b	47.77 ± 1.94^c	51.67 ± 1.60^{bc}	47.93 ± 1.90^c	52.03 ± 1.25^{bc}
Hgb (g/L)	10.67 ± 0.24^a	13.63 ± 0.38^b	14.03 ± 0.29^{bde}	15.70 ± 0.65^c	13.90 ± 0.57^d	15.37 ± 0.46^{ce}
RBCs (x10^{12}/L)	8.10 ± 0.35^a	$10.77\pm0.22_b$	11.33 ± 0.13^{bc}	11.47 ± 0.37^{bc}	11.90 ± 0.06^c	12.33 ± 0.47^c
MCV (fl)	43.33 ± 2.90^a	35.50 ± 1.80^b	34.43 ± 0.82^b	35.77 ± 0.96^b	37.60 ± 1.25^b	35.33 ± 0.77^b
MCH (pg)	13.53 ± 0.35^a	16.80 ± 0.26^b	17.63 ± 0.79^b	17.10 ± 0.17^b	17.63 ± 0.26^b	18.16 ± 0.49^b
MCHC (g/L)	32.50 ± 2.18^a	35.33 ± 3.05^{ab}	37.00 ± 1.73^b	36.83 ± 2.75^b	36.57 ± 2.10^b	35.30 ± 1.10^{ab}
Platelets (x10^9/L)	526.00 ± 51.03^a	294.33 ± 24.36^b	312.67 ± 26.99^b	367.67 ± 17.97^b	358.00 ± 17.43^b	289.33 ± 49.64^b
MPV (fl)	5.10 ± 0.49^a	3.47 ± 0.23^b	3.33 ± 0.20^b	3.50 ± 0.34^b	3.13 ± 0.22^b	3.30 ± 0.30^b
WBCs (x10^9/L)	10.63 ± 0.50^a	14.80 ± 0.85^b	15.40 ± 1.01^b	15.43 ± 0.62^b	17.50 ± 0.50^c	17.67 ± 0.23^c
Monocytes (%)	5.40 ± 0.70^a	8.17 ± 0.18^b	8.67 ± 0.39^b	8.60 ± 0.30^b	10.43 ± 0.84^c	9.70 ± 0.77^{bc}
Lymphocytes (x10^9/L)	5.17 ± 0.58^a	8.27 ± 0.37^b	8.70 ± 0.46^b	8.77 ± 0.23^b	8.60 ± 0.38^b	8.57 ± 0.66^b
Granulocytes (x10^9/L)	6.97 ± 0.30^a	11.00 ± 0.61^b	11.44 ± 0.39^b	12.27 ± 0.98^b	11.47 ± 0.42^b	11.73 ± 0.14^b

Note: Parameters in a row with different superscripts vary significantly from each other (P<0.05).

Table 3:. *Blood Chemistry Profile of Neeli–Ravi buffaloes inoculated with Pasturella. multocida B:2 and its Immunogens (LPS and OMP) through various routes.*

Culture	LPS		OMP			
Parameter	PBS	PO	PO	IV	PO	S/C
Serum Protein (g/L)	7.00±0.15[a]	8.47±0.18[b]	8.63±0.23[bc]	9.03±0.29[b]	8.97±0.62[b]	8.07±0.14[c]
Serum Albumin (g/dL)	3.40±0.11[a]	4.43±0.20[b]	4.70±0.23[bc]	4.97±0.46[bc]	5.00±0.30[bc]	5.37±0.18[c]
Serum Creatinine (mg/dL)	1.93±0.18[a]	3.60±0.26[b]	3.67±0.27[b]	4.10±0.36[bc]	4.67±0.53[c]	4.80±0.38[c]

Note: Parameters in a row with different superscripts vary significantly from each other (P<0.05).

Blood biochemistry parameters were also significantly disturbed among all treatment groups as compared to the control. The highest serum protein value (9.03±0.29) was observed in the LPS-Intravenous group (G-3). The mean value of serum protein remained significantly higher in treatment groups as compared to control. The highest serum albumin and creatinine value were 5.37±0.18 and 4.80±0.38, respectively, observed in the OMP-S/C group (G-6). Cumulatively, serum albumin and creatinine's mean values were also significantly (P<0.05) higher among treatment groups than the control. The detailed summary of blood biochemistry results has been illustrated in Table 3.

In this study, changes in clinical, hematological and serum biochemical parameters in Neeli-Ravi buffaloes due to experimental exposure of *P. multocida* B:2 culture (orally) and its immunogens, i.e., LPS (orally and intravenous) and OMP (orally and subcutaneous) were documented for the first time in Pakistan.

Although *P. multocida* B:2 affects both cattle and buffaloes, but later are more susceptible to HS compared to cattle (Zamri-Saad and Annas, 2016). During natural infection, the pathogen enters through the oral or intranasal route. It causes acute infection in the lungs and then crosses blood capillaries through endothelial cells to produce septicemia (Puspitasari *et al.*, 2020). This disease causes very acute respiratory and systemic signs (De Alwis, 1999).

According to our study, all treatment groups of buffaloes exhibited characteristic clinical signs of HS. These signs were ranging from mild to very acute in severity. The rise in rectal temperature lowered rumen motility and distress to the respiratory system were common observations among all the treatment groups. However, clinic signs' exhibition remained considerably higher in LPS-intravenous (G-4) and OMP-subcutaneous (G-6) animals. Animals from these two groups exhibited congestion of mucous membrane as well. In the latter group (OMP-subcutaneous), the development of submandibular edema and even one animal's death within 60 hours was also witnessed.

Although there is no constant pattern of hematological response by the host towards an experimental exposure of a gram-negative bacteria and its immunogens, however significant changes in hematological parameters of experimental buffaloes are suggestive of reaction by the immune system of buffaloes towards experimental exposure of *P. multocida* and its immunogens (LPS and OMP).

WBCs are involved in the defense mechanism against infectious agents. WBCs include neutrophils, lymphocytes, monocytes, eosinophils and granulocytes. A significant increase in circulating WBCs was observed. Previous studies also showed that change in WBCs was due to bacterial infection (Steven, 2000; Todar, 2008). RBCs are involved in Oxygen transport and the term "polycythemia" is used for the increased number of red blood cells. In treatment groups, significant polycythemia was observed as compared to the control group. However, the mean RBC value for all treatment groups was not too high compared with the normal range of RBCs in buffaloes. Hence no specific conclusion may be associated with this polycythemia. Platelet count and MPV were decreased in experiment groups. It was indicative of thrombocytopenia due to experimental exposure to *P. multocida* and its immunogens. However, this change was within the acceptable (100 to 800 x 10^9/L) range (Roland *et al.*, 2014).

Serum protein, Albumin and creatinine have high value in disease diagnosis. The serum protein and Albumin of all treatment groups were on the higher side (P<0.05) compared to control. High serum protein indicates inflammatory reactions or some infection, while the increased serum albumin values indicate an immunogenic response by the animals,

as observed in case of acute infections. A high level of creatinine generally indicates dehydration or some kidney disease. Although serum creatinine values were significantly higher among treatment groups than control ($P<0.05$), they can only be correlated to systemic inflammatory response due to immunogenic treatment. Other studies also suggested that creatinine increases during pasteurellosis (Seleim *et al.*, 2003; Kamal, 2010).

P. multocida B:2 inoculation (10 ml of 10^{12} CFU/ml) through subcutaneous and oral routes on buffaloes (Chung *et al.*, 2015) has been correlated with clinical signs, changes in hematology and histopathology. These changes are consistent with results exhibited by G-2 (*P. multocida* oral inoculation group) animals of our study. Endotoxin properties of *P. multocida* B:2 LPS extract were confirmed by its intravenous exposure in buffaloes (Horadagoda *et al.*, 2002). A similar study has affirmed changes in clinical signs, hematology and histopathology on experimental exposure to LPS-extract of *P. multocida* B:2 via the oral and intravenous route in buffaloes (Chung *et al.*, 2016). Immunogenicity studies of OMP of *P. multocida* in buffalo calves have suggested a positive immune response (Pati *et al.*, 1996).

Intranasal and subcutaneous exposure of rabbits to *P. multocida* resulted in HS's clinical signs (Leghari *et al.*, 2016). Immunogenic response on experimental exposure to LPS of *P. multocida* in rabbits has also been reported from Pakistan (Ashraf *et al.*, 2014). Recently a recombinant OMP based intranasal vaccine has been successfully experimented in swamp buffaloes against HS (Muenthaisong *et al.*, 2020).

Conclusions and Recommendations

Hemorrhagic septicemia is a very lethal disease of bovines in tropical countries caused by *P. multocida* type B:2. This study provided clear evidence that experimental exposure of *P. multocida* type B:2 culture (orally) and its immunogens, i.e., LPS (orally and intravenous) and OMP (orally and subcutaneous), produces significant changes in clinical, hematological and serum biochemical parameters in Neeli-Ravi buffaloes. This influence warrants further investigation to use LPS and OMP of *P. multocida* B: 2 as an alternate option for vaccine production against HS in bovines.

Novelty Statement

The study provides clear evidence of immunogenic response in Nili Ravi buffaloes by LPS and OMP extract of P.multocida B:2 and hints about their use as an alternate source of vaccination.

Author's Contribution

Ali Zaman: Conducted the experiments.
Nabila Rohi: Supervised the study.
Muhammad Irfan: Interpreted the data and wrote the manuscript.

Conflict of interest
The authors have declared no conflict of interest.

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