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Original Article

Effects of *Pasturella Multocida* B:2 and its immunogens (LPS and OMP) on reproductive hormones in Nili-Ravi Buffaloes

Efeitos de *Pasteurella multocida* B:2 e seus imunógenos (LPS e OMP) em hormônios reprodutivos em búfalos Nili-Ravi

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Abstract

Livestock is a fundamental part of the agriculture industry in Pakistan and contributes more than 11.53% to GDP. Among livestock species, the buffaloes are regarded as the black gold of Pakistan. Being the highest milk producers globally, Nili-Ravi buffaloes are the most famous ones. Buffaloes are affected by many endemic diseases, and "Hemorrhagic septicemia" (HS) is one of them. This study was designed to ascertain 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 reproductive hormonal profiles in Nili-Ravi buffaloes. Repeated serum samples were collected from the jugular vein of experimental animals for 21 days (0, 02, 04, 08, 12, 16, 20, 24, 36, 48, 72, 120, 168, 216, 264, 360, 456 and 504 hours). Hormonal assays to determine the serum concentrations of Gonadotropin-releasing hormone (GnRH), Follicle-stimulating hormone (FSH), Luteinizing hormone (LH), Estrogen (E_2) and progesterone (P_4) were performed using (MyBioSource) commercial Elisa kits. The hormonal profile of all treatment groups of the buffalo heifers exhibited significant ($P<0.05$) variations as compared to the control group (G-1). These results indicate suppression in Nili-Ravi buffaloes' reproductive hormonal profile on exposure to *P. multocida* B:2 and its immunogens. This influence warrants that exposure to H.S may be a possible reason for delayed puberty and poor reproduction performance in Nili-Ravi buffaloes.

Keywords: Nili-Ravi Buffaloes, hormonal variation, *Pasteurella multocida* B:2, Lipo Poly Saccharide (LPS), Outer Membrane Proteins (OMP).

Resumo

A pecuária é uma parte fundamental da indústria agrícola no Paquistão e contribui com 11,53% do PIB nacional. Entre as espécies de gado, os búfalos são considerados o ouro negro do Paquistão. Sendo os maiores produtores de leite em todo o mundo, os búfalos Nili-Ravi são os mais famosos. Os búfalos são afetados por muitas doenças endêmicas, entre as quais a "septicemia hemorrágica" (SH). Este estudo busca verificar os efeitos da exposição experimental de *P. multocida* B:2 (oral) e seus imunógenos, ou seja, LPS (oral e intravenoso) e OMP (oral e subcutâneo), nos perfis hormonais reprodutivos em búfalos Nili-Ravi. Amostras de soro repetidas foram coletadas da veia jugular de animais experimentais por 21 dias (0, 2, 4, 8, 12, 16, 20, 24, 36, 48, 72, 120, 168, 216, 264, 360, 456 e 504 horas). Os ensaios hormonais para determinar as concentrações séricas do hormônio liberador de gonadotrofina (GnRH), hormônio foliculoestimulante (FSH), hormônio luteinizante (LH), estrogênio (E2) e progesterona (P4) foram realizados usando kits comerciais Elisa (MyBioSource). O perfil hormonal de todos os grupos de tratamento das novilhas bubalinas apresentou variações significativas ($P < 0,05$) em relação ao grupo controle (G-1). Esses resultados indicam supressão no perfil hormonal reprodutivo de búfalos Nili-Ravi na exposição a *P. multocida* B:2 e seus imunógenos. Essa influência garante que a exposição à SH possa ser uma possível razão para o atraso da puberdade e o baixo desempenho reprodutivo em búfalos Nili-Ravi.

Palavras-chave: Búfalos Nili-Ravi, variação hormonal, *Pasteurella multocida* B:2, lipopolissacarídeo (LPS), Proteínas de Membrana Externa (OMP).

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1. Introduction

Pakistan is an agricultural economy, and a large proportion of its population is directly or indirectly involved in the agriculture industry. Livestock is a fundamental part of the agriculture industry (Shahid et al., 2020). Livestock contributes more than 11.53% to GDP and contributes 60.07% to the agriculture sector (Pakistan, 2020). Among livestock species, the buffaloes are regarded as the black gold of Pakistan (Sikandar et al., 2020). There are four major buffalo breeds in Pakistan, and among 7sure source of income for poor livestock farmers of Pakistan. This animal has excellent potential to produce high nutritional value having milk and beef from low nutritive value having roughages (Bibi et al., 2018). Despite the high economic importance of this animal, the reproduction performance of this animal is slower. Delayed puberty, prolonged calving interval, poor estrus expression and strong mother-offspring bonding (Budiarto et al., 2021) are significant concerns about buffalo reproduction.

Different studies have described fodder availability, traditional disease control methods, availability of vaccines and environmental stress as significant concerns for buffalo farmers (Ul-Haq et al., 2016). For efficient production from buffaloes, a faster growth rate, early age of maturity, and fertile reproductive cyclicity are essential (Madan and Prakash, 2007). This reproductive efficiency of young and mature buffaloes is controlled by endocrine hormones (Hafez and Hafez, 2013). The endocrine hormones which control the reproductive cyclicity in buffaloes and other domestic species include Gonadotropin-releasing hormone (GnRH), Follicle-stimulating hormone (FSH), Luteinizing hormone (LH), Estrogen (E_2) and progesterone (P_4). These hormones are regulated by the hypothalamic-pituitary and gonadal axis (Bhimte et al., 2021). The level of circulating reproductive hormones affects the reproductive performance of cattle and buffaloes (Ramadan, 2017). The regulation and secretory pattern of these hormones are affected by different disease outbreaks. In hot and humid countries (like Pakistan), Hemorrhagic septicemia (H.S) is one of the major diseases that affect buffalos and other bovines. Despite regular vaccination programs, outbreaks of H.S do occur. The research to understand the pathological pathways, the interaction of this disease with different body systems is ongoing.

H.S is caused by *Pasturella multocida* (*P.multocida*). The pathogenicity of *P.multocida* is driven by its structural components, which include lipopolysaccharides (LPS), outer membrane protein (OMP), *P. multocida* toxins (PMT) and its capsule (Kubatzky, 2012). These structural components can activate the systemic immune response by the host species. *P.multocida* is a gram-negative bacteria, and LPS is an integral structural component. Based on the composition of LPS, *P.multocida* has been further divided into 16 types (Harper et al., 2011). Similarly, five serogroups (A to F) of *P.multocida* have been described based on differences in its capsular OMPs (Wilson and Ho, 2013).

Specific serotypes of *P. multocida* are predominant in particular geographic zones. Serotype B:2 is dominant in Asian countries, while serotype E:2 is prevailing in African countries (Chung et al., 2015; Zaman et al., 2021). The effects of experimental exposure to H.S have been studied in Malaysian buffaloes recently (Jesse et al., 2017). This study has revealed the negative impacts of H.S on reproductive hormones and reproductive organs of pre-pubertal Malaysian buffaloes. However, no such research has been reported for Nili-Ravi buffaloes. In our previous study, it has been reported that *P.multocida* type B:2 and its immunogens (LPS and OMP) produce significant changes in clinical, haematological and serum biochemical parameters in Nili-Ravi buffaloes. The current study was aimed to ascertain 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 reproductive hormonal profiles in Nili-Ravi buffaloes.

2. Material and Methods

2.1. Experimental design

The sample population of this study consisted of pre-pubertal female buffaloes (N=18) of Nili- Ravi breed having10-12 months of age with good health conditions. All the animals were kept in individual pens. The detailed design was given in our previous report (Zaman et al., 2021).

2.2. Inoculum preparation

Inoculum of *P. multocida* type B:2 (used in this study) was prepared as described by in our earlier report (Zaman et al., 2021).

Lipo Polysaccharide (LPS) Extraction from *P. multocida* type B: 2 LPS extract of *P. multocida* type B2 for this study was obtained by a commercial LPS-extraction kit (MAK 339 Sigma-Aldrich USA). The extraction was done as per the manufacturer›s instructions already described in our previous study (Zaman et al., 2021).

Outer-Membrane Proteins (OMP) Extraction from *P. multocida* type B: 2 As described in our previous study (Zaman et al., 2021), OMP extract was obtained by freezing the freshly harvested cell pellets for 24 hours before starting the OMP extraction procedure. Afterwards, the cell pellets were thawed for 15 minutes on ice before re-suspension in native lysis buffer (10 ml). Later on, the suspension was incubated at ice for half an hour. Finally, it was centrifuged at 14,000 rpm for 30 minutes at 39.2 °F to obtain the OMP extract.

2.3. Serum sample collection

The repeated blood samples were collected from the jugular vein of experimental animals for 21 days (0, 02, 04, 08, 12, 16, 20, 24, 36, 48, 72, 120, 168, 216, 264, 360, 456 and 504 hours) after experimental exposure to *P.multocida* and its immunogens. The collected blood samples were transferred into gel clot vacutainers and adequately labelled. The blood containing vacutainers were kept at room temperature for 2-3 hours to ensure blood clot formation. The vacutainers were then centrifuged at 1000 x g for 15 minutes. The serum was collected from the vacutainers and stored at – 20 °C in 2 ml centrifugation tubes. The serum samples were adequately labelled, and a record was maintained accordingly. The hormonal assay of these serum samples was performed in batches.

2.4. Hormonal assay

Hormonal assays to determine the serum concentrations of GnRH, FSH, LH, E_2 and P4 were performed using (MyBioSource) commercial Elisa kits. The kit instructions were followed for each assay. Briefly speaking, GnRH, P4 and FSH Elisa kits were based on a competitive enzyme immunoassay. The assay plates were for GnRH estimation were coated with an anti-GnRH antibody. GnRH and horse reddish peroxidase (HRP) conjugate, serum samples/ controls and substrates were incubated in the 96 well Elisa plate. The optical density was measured at 450 nm after adding the stop solution. A standard curve was worked out to ascertain the values of GnRH in the collected serum samples. Quantitative Sandwich ELISA was performed for measuring E_2 levels in serum samples, and the kits used to measure the quantity of LH hormone were based on double antibody sandwich ELISA.

2.5. Statistical analysis

The normality of the collected data was checked by the Shapiro-Wilk test. The normality of data was confirmed. The results obtained were statistically analyzed by One-Way ANOVA using SPSS version 24.0. The variations in different parameters with a 95% confidence interval were considered statistically significant. The graphs for the obtained results were generated using graph prismpad version 9.0.

3. Results

3.1. Clinical signs and haematology / serum biochemistry

As described in our previous study (Zaman et al., 2021), noticeable clinical signs were observed in the buffaloes of treatment groups (G-2 to G-6) which included respiratory distress, high rectal temperature, excessive salivation, congestion of mucous membranes and lowered rumen motility. The death of animals also occurred in G-6 animals. The haematological and serum biochemistry profiles (total protein, Albumin and creatinine) for all treatment groups were also altered (*P<0.05*).

3.2. Effects on serum GnRH profile

The differences in mean serum GnRH values among the different groups at "0" hours were statistically non-significant (*P>0.05*). As these buffaloes were pre-pubertal and non-cyclic, hence only the pulsatile pattern of GnRH release (from tonic centre) was observed among all the groups. After exposure to *P. multocida* type B2 and its immunogens, the mean value of circulating GnRH dropped significantly (*P<0.05*) among all treatment groups after two hours compared to the control. This value remained substantially lower (*P<0.05*) by the end of 21 days period (estrous cycle) in G2 to G5. G-6 animals showed a similar trend, but their results could not be calculated beyond three days. The animals of this group expired within 72 hours after subcutaneous exposure to OMP extract of *P. multocida* type B:2 Graphical illustration of these results has been presented in Figure 1.

3.3. Effects on serum FSH/LH profile

The differences in mean serum FSH / LH values among the different groups at "0" hours were statistically non-significant (*P>0.05*). As these buffaloes were pre-pubertal and non-cyclic, only a baseline FSH release pattern was observed among all the groups. After exposure to *Pasturella multocida* type B2 and its immunogens, the mean value of circulating FSH / LH dropped significantly (*P<0.05*) among all treatment groups after two hours compared to the control. This value remained substantially lower (*P<0.05*) by the end of 21 days period (estrous cycle) in G2 to G5. G-6 animals showed a similar trend, but their results could not be calculated beyond three days. The animals of this group expired within 72 hours after subcutaneous exposure to OMP extract of *Pasturella multocida* type B:2 Graphical illustration of these results has been presented in Figure 2 and Figure 3.

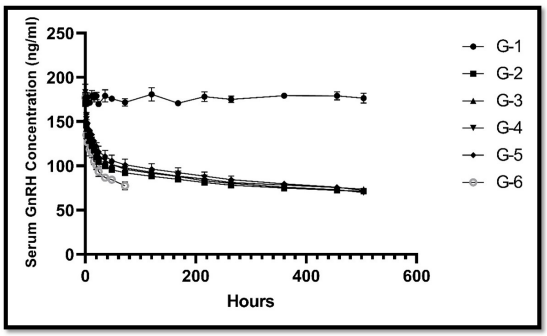

Figure 1. Changes in Serum GnRH Concentration of Nili-Ravi buffaloes inoculated with *P. multocida* B:2 and its Immunogens (LPS and OMP) through various routes.Note: G-1 (Negative control), G-2 (Bacterial culture PO), G-3 (LPS extract PO), G-4 (LPS extract IV route), G-5 (OMP extract PO) and G-6 (OMP-extract Subcutaneous route). At any given point, variations in serum hormonal concentration were considered significantly different with a 95% confidence interval (*P<0.05*).

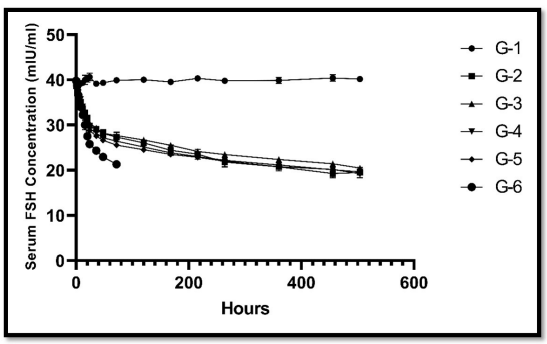

Figure 2. Changes in Serum FSH Concentration of Nili-Ravi buffaloes inoculated with *P. multocida* B:2 and its Immunogens (LPS and OMP) through various routes.Note: G-1 (Negative control), G-2 (Bacterial culture PO), G-3 (LPS extract PO), G-4 (LPS extract IV route), G-5 (OMP extract PO) and G-6 (OMP-extract Subcutaneous route). At any given point, variations in serum hormonal concentration were considered significantly different with a 95% confidence interval (*P<0.05*).

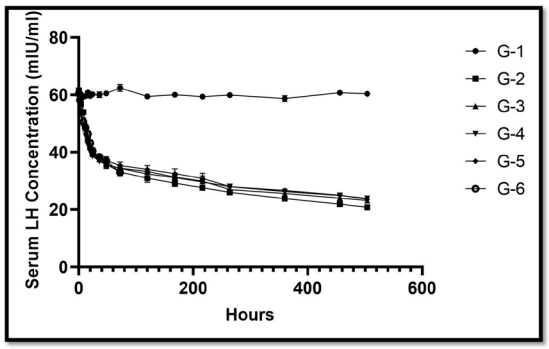

Figure 3. Changes in Serum LH Concentration of Nili-Ravi buffaloes inoculated with *P. multocida* B:2 and its Immunogens (LPS and OMP) through various routes.Note: G-1 (Negative control), G-2 (Bacterial culture PO), G-3 (LPS extract PO), G-4 (LPS extract IV route), G-5 (OMP extract PO) and G-6 (OMP-extract Subcutaneous route). At any given point, variations in serum hormonal concentration were considered significantly different with a 95% confidence interval (*P<0.05*).

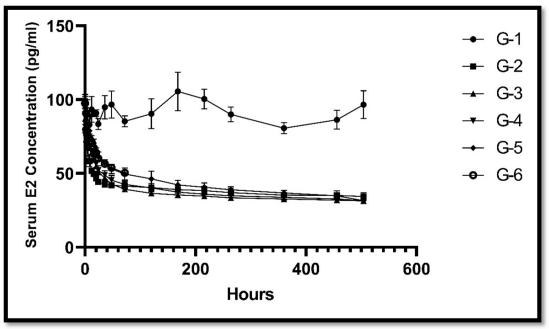

Figure 4. Changes in Serum Estrogen (E_2) Concentration of Nili-Ravi buffaloes inoculated with *P. multocida* B:2 and its Immunogens (LPS and OMP) through various routes.Note: G-1 (Negative control), G-2 (Bacterial culture PO), G-3 (LPS extract PO), G-4 (LPS extract IV route), G-5 (OMP extract PO) and G-6 (OMP-extract Subcutaneous route). At any given point, variations in serum hormonal concentration were considered significantly different with a 95% confidence interval (*P<0.05*).

3.4. Effects on serum estrogen hormone's concentration

The differences in mean serum estrogen (E_2) values among the different groups at "0" hours were statistically non-significant (*P>0.05*). As these buffaloes were pre-pubertal and non-cyclic, hence only the baseline pattern of E_2 release was observed among all the groups. After exposure to *Pasturella multocida* type B2 and its immunogens, the mean value of circulating E_2 dropped significantly (*p<0.05*) among all treatment groups after two hours compared to the control. A fluctuating trend in E_2 secretion was observed in the control group over 21 days; however, the results were statistically non-significant (*p>0.05*). This value remained substantially lower (*p<0.05*) by the end of 21 days period (estrous cycle) in G2 to G5. G-6 animals showed a similar trend, but their results could not be calculated beyond three days. The animals of this group expired within 72 hours after subcutaneous exposure to OMP extract of *Pasturella multocida* type B:2 Graphical illustration of these results has been presented in Figure 4.

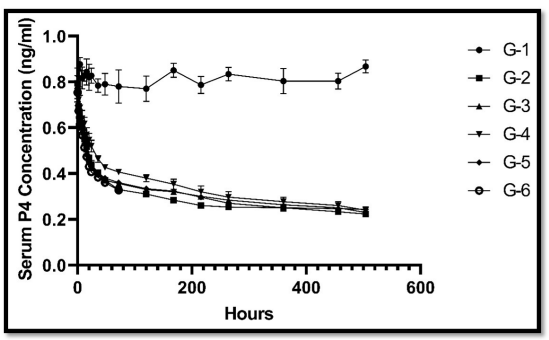

Figure 5. Changes in Serum Progesterone (P4) Concentration of Nili-Ravi buffaloes inoculated with *P. multocida* B:2 and its Immunogens (LPS and OMP) through various routes.Note: G-1 (Negative control), G-2 (Bacterial culture PO), G-3 (LPS extract PO), G-4 (LPS extract IV route), G-5 (OMP extract PO) and G-6 (OMP-extract Subcutaneous route). At any given point, variations in serum hormonal concentration were considered significantly different with a 95% confidence interval (*P<0.05*).

3.5. Effects on serum progesterone (P4) profile

The differences in mean serum P4 values among the different groups at "0" hours were statistically non-significant (*P>0.05*). As these buffaloes were pre-pubertal and non-cyclic, hence the serum p4 value among all the groups was below one ng/ml. After exposure to *Pasturella multocida* type B2 and its immunogens, the mean value of circulating P4 dropped significantly (*P<0.05*) among all treatment groups after two hours period as compared to the control. This value remained substantially lower (*P<0.05*) by the end of 21 days period (estrous cycle) in G2 to G5. G-6 animals showed a similar trend, but their results could not be calculated beyond three days. The animals of this group expired within 72 hours after subcutaneous exposure to OMP extract of *Pasturella multocida* type B:2 Graphical illustration of these results has been presented in Figure 5.

4. Discussion

In this study, significant changes in the reproductive hormones of Nili-Ravi buffaloes due to experimental exposure of *P. multocida* B:2 bacterial culture (orally) and its immunogens, i.e., LPS-extract (orally and intravenously) and OMP-extract (orally and subcutaneously) have been reported for the very first time. Although *P. multocida* B:2 affects both cattle and buffaloes, the latter is more susceptible to H.S(Zamri-Saad and Annas, 2016). During *P. multocida* infection, the pathogen enters through the oral or intranasal route and causes acute infection in the lungs. Then it crosses blood capillaries through endothelial cells and produces septicemia (Puspitasari et al., 2020). This disease causes very critical respiratory and systemic signs (Habib et al., 2019).

According to the results section of our study, all treatment groups of the buffaloes exhibited variations in their reproductive hormonal profile compared to the control group. These results indicate suppression in Nili-Ravi buffaloes' reproductive hormonal profile on exposure to *P. multocida* B:2 and its immunogens.

The estrous cycle of a buffalo ranges between 17-22 (average 21) days. During the estrous cycle, buffaloes undergo different physiological and behavioural changes (Harun-Or-Rashid et al., 2019). These changes are controlled through the sequential release of reproductive hormones. The hypothalamic-pituitary-gonadal axis regulates these hormonal variations through negative and positive feedback mechanisms (Pereira and Hartmann, 2018). GnRH is a neuropeptide hormone that acts on the anterior lobe of the pituitary to regulate the secretion of FSH and LH hormones. The hypothalamus secretes it from the tonic and surge centres. The surge centre develops during fetal life in the females only and is triggered by the E_2 peak during estrus. In our study, GnRH was reduced in all treatment groups as time elapsed. A similar response has been reported from a study on Malaysian buffaloes (Jesse et al., 2017). This study on Malaysian buffaloes has reported a decrease in plasma concentration of GnRH, FSH, LH, E_2 and P4 hormones due to significant pathological changes in the hypothalamic-pituitary and gonadal axis.

According to their biological classification, FSH and LH are glycoproteins and are secreted from the anterior lobe of the pituitary. In this study, the serum concentration of FSH and LH was reduced significantly in all treatment groups. This decrease may be explained based on the decrease in GnRH as described earlier. Another reason for the decrease in FSH / LH serum concentration in Nili-Ravi buffaloes exposed to *P.multocida* B:2 and its immunogens may be due to the pathological changes in the pituitary glands as described in previous studies on mice and buffaloes (Ibrahim et al., 2016; Marza et al., 2015). FSH and LH act on the male and female gonads. In females, FSH is responsible for follicular development, and LH is responsible for ovulation of the dominant follicle, dominant follicles and secretion of P4 from the corpus luteum (CL). E_2 is secreted from the granulosal cells of the growing ovarian follicles, and it is responsible for the sexual behaviour (lordosis) of ruminants during the estrus phase of the heat cycle (Liu et al., 2017). In our study, E_2 secretion was decreased significantly among all treatment groups as compared to the control. A study on male and female mice as an animal model has reported that exposure to *P.multocida* type B:2 and its LPS extract results in reproductive hormones and causes pathological changes in the reproductive organs (Abdullah et al., 2015; Sahu et al., 2018). Some studies have reported a two-fold increase in sex hormones (E_2 and testosterone) after experimental exposure to bacterial endotoxins (Khuder, 2012). The possible reason for the increase in circulating sex hormones was a decrease in immune function.

In buffaloes, the cut-off value of serum P4 concentration between a cyclic and non-cyclic buffalo is 1 ng/ml (El-Razek et al., 2019). The serum P4 concentration was below 1 ng /ml for all the groups at 0 hours in our study. The circulating P4 concentration dropped significantly in the treatment groups. A similar trend was seen in a study on variations in reproductive hormones of Malaysian buffaloes due to exposure to *P.multocida* B:2 and its immunogens (Jesse et al., 2017). However, the reported plasma P4 concentration in that study was above 1 ng/ml in the control group during the entire study period. Various studies on mice, ewes, goats, cows, heifers and monkeys have reported impaired reproductive functions and even fetal death due to bacterial endotoxemia. (Bidne et al., 2018). The study on Malaysian buffaloes has reported the most severe adverse effects on the reproductive hormones due to exposure with OMP of *P.multocida* type B:2. Similarly, the most severe response in our study was from G-6 (OMP via subcutaneous route).

The onset of puberty in the buffalo heifers is affected by genetics, nutritional status, managemental practices, seasonal breeding pattern, environmental stress, immunity status, and exposure to various diseases. Disease outbreaks of any type may cause a drop in production and temporary infertility in the buffaloes (Mohyuddin et al., 2019; Plansky and Dimitrov, 2020; Sreedhar et al., 2017). In our study, Nili-Ravi buffaloes' altered reproductive hormonal profile on experimental exposure to *P.multocida* type B:2 and its immunogens (LPS and OMP) through various routes can be used to assess the immune status of buffalo populations at risk. This viewpoint is further augmented with the results of our previous study on clinical and haematological changes in Nili-Ravi buffaloes due to exposure to *P.multocida* type B:2 and its immunogens (Zaman et al., 2021). This disease is endemic in many African and Asian countries, including Pakistan. The mortality rate for this disease may be very high if initial treatment is delayed (Moffatt et al., 2010). A review study on *Mannheimia haemolytica* and its immunogens has affirmed an association between *P.multocida* and the reproductive performance of female animals (Jesse et al. 2020). Some other studies have reported a correlation between C*orynebacterium pseudotuberculosis* / *Brucella mellitensis* and variations in reproductive hormones of mice and goats (Jesse et al., 2016; Othman et al., 2016; Othman et al., 2014). Hence, H.S may be considered as a contributing factor to delayed puberty and infertility in Nili-Ravi buffaloes.

5. Conclusion

Hemorrhagic septicemia is an endemic disease of buffaloes in tropical countries, and it is caused by *P. multocida* type B:2. It can be inferred from this study 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), significantly lowers the serum concentration of reproductive hormones Nili-Ravi buffaloes. This influence warrants exposure to H.S may be a possible reason for delayed puberty and poor reproduction performance in Nili-Ravi buffaloes.

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