South African Journal of Animal Science 2023, 53 (No. 1)

Serological and haemato-biochemical insights into bovine leukosis in dairy cattle in D.I. Khan, Pakistan

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(Submitted 20 June 2022; Accepted 16 December 2022; Published 6 April 2023)

Abstract

Bovine leukosis is an economically important disease of dairy cattle caused by the bovine leukaemia virus (BLV). The study aimed to determine the seroprevalence, haemato-biochemical effects, and risk factors pertinent to the prevalence of bovine leukosis in Holstein–Friesian purebred dairy cattle in the D.I. Khan region of Pakistan. A total of 192 sera were assayed using an enzyme-linked immunosorbent assay. Overall, 31.3% (60/192) cattle were detected as seropositive. There was a marked increase in total leukocyte count (11.29 ± 0.48×10^3/µl), lymphocytes (5.73 ± 0.42%), monocytes (0.81 ± 0.06%), haemoglobin (11.09 ± 0.46 g/dl), red blood cells (7.23 ± 0.37 ×10^6/µl), and packed cell volume (31.75 ± 1.48%) in seropositive cattle. Serum biochemical parameters in seropositive cattle showed a marked increase in the liver enzymes, alanine transaminase (24.25 ± 1.03 U/l) and aspartate aminotransferase (49.33 ± 3.31 U/l), with a marked decrease in glutathione peroxidase (1365.63 ± 12.03 (U/l) and superoxide dismutase (2.14 ± 0.13 U/ml) activity. A significant association of age, pregnancy, breeding method, milk yield, and health status of seropositive animals with bovine leukosis was also recorded. The prevalence was higher in animals which were older, pregnant, artificially inseminated, low milk producers, and had a history of ailments. The current study found that bovine leukosis virus could cause changes in internal homeostasis, oxidative stress, and liver dysfunction, all of which should be considered during a control regimen. It was concluded that bovine leukosis was moderately prevalent in the D.I. Khan region in Pakistan.

Keywords: Bovine leukaemia virus, enzyme-linked immunosorbent assay, haemato-biochemical evaluation, Holstein–Friesian cows

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Introduction

Bovine leukosis is an important neoplastic disease of cattle and is caused by bovine leukaemia virus (BLV) of the genus, *Deltaretrovirus,* and family, Retroviridae (Ramiz *et al*., 2021). The disease is usually subclinical and may assume an asymptomatic path in which the infected animals act as a transmission agent without themselves showing any sign of disease. The virus infects lymphocytes and introduces its genome into the host genome, thereby instigating lifelong infection with persistent lymphocytosis and an increase in B-lymphocytes in the lymph nodes (Selim *et al*., 2020). BLV was first

URL: http://www.sasas.co.za
ISSN 0375-1589 (print), ISSN 2221-4062 (online)
Publisher: South African Society for Animal Science
http://dx.doi.org/10.4314/sajas.v53i1.05

perceived in diseased cattle by Miller *et al*. (1969) in tissue culture (Khudair et al., 2021). The disease is transferred both horizontally and vertically. Horizontal transmission of the disease occurs through contact with infected animals, infected lymphocytes, blood transfusion, rectal palpation, use of common needles, and mechanical transmission by insects (Polat *et al*., 2015); vertical transfer occurs through ingestion of colostrum and also the affected placenta (*Selim et al.,* 2020). The oncogenic properties of the virus may produce pathogenicity in farm workers drinking unpasteurized milk (Buehring *et al*., 2014). This illness is listed as a disease of economic importance to international trade by the World Organization for Animal Health (OIE).

The disease results in increased heifer replacement costs, decreased milk production, reduced reproductive performance, premature culling of animals, as well as international trade restrictions (Khan *et al*., 2019). It also enacts a restriction on the import of exotic semen and cattle (Nishimori *et al*., 2017). According to the OIE, bovine leukosis has a global spread, infecting large numbers of susceptible host animals. The reported global prevalence differs widely. Variable prevalence has been reported globally: in Canada, 26%; United States, 84% (Bartlett *et al.,* 2013);Turkey, 48.3%; Iran, 64.7% (Rodriguez *et al*., 2011); China, 21% (Ma *et al*., 2016); Egypt, 20.8% (Selim et al., 2020); South Korea, 10.2% (Kim *et al*., 2017); Philippines, 4.8% (Polat *et al*., 2015); and Japan, 28.6% (Murakami *et al*., 2011). The occurrence of bovine leukosis has been reported to be highly associated with various risk factors including gender, age, pregnancy, breed, milk yield, herd size, breeding, health status, and country of import. The existence of the disease is higher in older, pregnant animals, exotic breeds, low milk-producing animals, herd sizes >200, artificially inseminated cows, and in imported animals (Norby*et al*., 2016; Khan *et al*., 2019; Ramiz *et al*., 2021).

Bovine leukosis has been described as affecting the haemato-biochemical parameters of the host, including increased blood cell counts, leukocytosis, lymphocytosis, neutropenia, and monocytopenia (Nikolay *et al*., 2013). The serum aspartate aminotransferase concentrations were found to be decreased in BLV-infected cows (Akalin *et al*., 2015). Furthermore, increased creatinine levels, decreased superoxide dismutase and glutathione peroxidase activity (Souza *et al*., 2011), and a marked decrease in calcium level are also associated with this disease (Ali *et al*., 2019). The test of choice for the diagnosis of bovine leukosis is agar gel immuno-diffusion (AGID) but in recent years it is replaced by enzyme-linked immunosorbent assay (ELISA) because AGID is time-consuming and requires skilled technicians. ELISA has an excellent ability to detect specific antibodies against BLV (mainly the glycoprotein, gp51) (Giuseppe *et al*., 2004). Polymerase chain reaction (PCR) is also used for the diagnosis of BLV because PCR enables detection of the proviral genome integrated in the host genome (Villalobos-Cortes *et al*., 2017).

Despite being highly prevalent around the world, no study has ever been conducted to determine the distribution of infection in the vast region of Dera Ismail Khan (D.I. Khan), a southern, livestock district of Khyber Pakhtunkhwa, Pakistan. Therefore, the present study was aimed to investigate the seroprevalence, haemato-biochemical effects, and risk factors of bovine leukosis in dairy cattle in this area for the first time. It is hypothesized that the disease exists in the Holstein–Friesian breed of dairy cattle in the context of imported dairy cattle entrepreneurs trending in the area.

Materials and Methods

The study was conducted in accordance with the principles of good practice and approved by the Ethical Committee of University of Veterinary and Animal Sciences Lahore, sub campus Jhang.

The study was conducted in different areas of D.I. Khan, Khyber Pakhtunkhwa, situated close to the River Indus in a north-east to south-west direction. The summer season (April to September) is mainly dry and hot while December, January, and February are cold (winter) months. The district is highly populated with livestock, serving as the main source of income in rural areas. Commercial dairy farming has experienced a rising trend of importing dairy cattle of high yielding milk breeds such as Holstein–Friesians and Jerseys in the study area. Dairy cattle (Holstein, Friesian pure breed) of both sexes and different age groups (>2, >4, >6 years) were randomly selected for this study. A total of one hundred and ninety-two (n = 192) blood (sera harvested) samples from Holstein–Friesian (n = 192) dairy cattle were collected. A total volume of 3 ml of blood was collected aseptically from the jugular or coccygeal veins in two types of vacutainers i.e., EDTA and Gel/Clot activator tubes. The samples were transferred to the laboratory (Medicine Lab, College of Veterinary and Animal Sciences, sub campus, Jhang) for further analyses.

All the serum samples were examined serologically using an ELISA kit (Anti-BLV gp51 antibody detection, ID Vet; Garbels, France) to detect the antibodies against BLV. The collected blood samples were transferred into gel and clot activator tubes and centrifuged at 2000 rpm for 10 min to separate

serum. The sera were transferred into Eppendorf tubes and stored at -18 ˚C till further ELISA processing. At the time of assay procedure, all the reagents were allowed to come to room temperature (21 ± 5 ˚C) before use and then homogenized using a Vortex. As per the manufacturer's instructions, 80µl of dilution buffer was added to each well of a 96-well plate. Then, 20 µl of positive (PC) and negative controls (NC) were added into duplicate wells; 20 µl of each sample was added to the remaining wells. The plates were covered with aluminium foil and incubated for 45 min at 21 ˚C (± 5 ˚C). After incubation, three washings were carried out with 300 µl of wash solution in each well. After washing, 100 µl of conjugate was added to each well and was incubated for 30 min at 21 ˚C (± 5 ˚C). After washing, 100 µl of substrate solution was added to each well of the plate and incubated for 15 min at 21 ˚C (± 5 ˚C). If the immune complex was present, the peroxidase transformed the substrate into a blue-coloured compound. Then, 100 µl of stop solution was added to each well, making it yellow after blocking. The optical density of the colour development was read with the help of an ELISA reader at 450 nm (Biobase-EL10A, ELISA reader). The measurement of the intensity of colour and the number of antibodies present in serum samples were calculated.

The test was validated when the mean value of the NC optical density (OD) was greater than 0.7 (OD_{NC} > 0.7); the mean value of the PC optical density was <30% of the negative control optical density i.e.,

OD_{PC} <30% of OD_{NC}; OD_{PC}/OD_{NC} <0.3 (Kuczewski *et al*., 2018).

The results were interpreted according to the recommendations as: when the S/N % was equal to or less than 50%, the animal was regarded positive for BLV antibodies; when the S/N % of the sample was greater than 50% and less than 60%, the sample was declared doubtful; and when the S/N % was equal to or greater than 60%, the sample was graded as negative.

The formula used to calculate S/N (%) was:

$$S/N\ \% = OD\ sample/OD_{NC} \times 100$$

Haematological parameters (total leukocyte count, lymphocytes, monocytes, haemoglobin, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, red blood cells, mean corpuscular volume, packed cell volume, and platelets) in Holstein–Friesian cattle (n = 20 seropositive and n = 10 seronegative) were measured by using an automatic haematology analyser (Exigo-H400 Boul Medical, Sweden).

Transaminases, alanine transaminase (ALT), and aspartate aminotransferase (AST) in Holstein–Friesian dairy cattle (n = 16) were assayed by using commercially available kit (Randox, IFCC, RX Daytona Plus, UK). The oxidative stress levels of Holstein–Friesian dairy cattle (n = 16) were evaluated by determining the level of glutathione peroxidase enzyme activity and superoxide dismutase enzyme activity using standard kits (Ransod, Randox, UK).

The data obtained during the study were analysed using the SPSS software program (IBM SPSS Statistics, version 21). Pearson's chi-square test was used to determine the prevalence of bovine leukosis in cattle while a single sample *t*-test was used for haemato-biochemical parameters. The risk factors (sex, age, breed, pregnancy, breeding, milk yield, and health status) were also evaluated using a chi-square test. The outcomes were declared statistically significantly different at a probability of 5% (P <0.05).

Results and Discussion

The overall seroprevalence of bovine leukosis in the current study was 31.3% (60/192) in Holstein–Friesian dairy cattle. Mean values of haematological parameters of animals are shown in Table 1.

Table 1 Haematological values (mean ± standard error) of seropositive (n = 20) and seronegative (n = 10) Holstein–Friesian cattle

	Animals		
Haematological Parameters	Seropositive	Seronegative	*P*-Value
Total leukocyte count (×10^3/µl)	11.29 ± 0.48	8.13 ± 0.59	0.001
Lymphocytes (%)	5.73 ± 0.42	3.30 ± 0.30	0.000
Monocytes (%)	0.81 ± 0.06	0.45 ± 0.06	0.049
Haemoglobin (g/dl)	11.09 ± 0.46	8.22 ± 0.64	0.000
Mean corpuscular haemoglobin (pg)	17.11 ± 0.26	15.35 ± 0.55	0.110
Mean corpuscular haemoglobin concentration (g/dl)	38.58 ± 0.22	37.12 ± 0.43	0.093
Red blood cells (×10^6/µl)	7.23 ± 0.37	5.53 ± 0.18	0.000
Mean corpuscular volume (fl)	59.76 ± 0.35	59.13 ± 0.33	0.094
Packed cell volume %	31.75 ± 1.48	23.81 ± 0.93	0.000
Platelets ×10^3/µL	799 ± 10.04	752.20 ± 24.52	0.145

where, pg = pictogram; µ = microgram; fL = femtoliter; P values of the parameters in the same column are statistically significantly different (*P* <0.05)

The values of total leukocytes, lymphocytes, monocytes, haemoglobin, red blood cells, and packed cell volume were higher in seropositive cattle than seronegative cattle (*P* <0.05). These imply the severity of viral infection in cattle. The elevated lymphocyte count is attributed to a 45-fold increase in infected CD 5^+ and a 99-fold increase in infected CD 5^- B-lymphocytes (Susan *et al*., 2016). The possible reason in this case in cattle is a shift to the left, in which the excessive demand of differentiation and maturation of the aforesaid cells occurs after the viral infection. The values of mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, mean corpuscular volume, and platelets were similar (*P* >0.05).There was no issue of malnutrition and or any injury in the cattle under study therefore the above values were found unaffected and indicate the volume/concentration in the cells/blood. The serum biochemical parameters of seropositive and seronegative Holstein–Friesian dairy cattle are shown in Table 2.

The mean values of ALT and aspartate aminotransferase were higher in seropositive cattle than seronegative cattle (*P* <0.05). The mean values of glutathione peroxidase and superoxide dismutase were lower in seropositive cattle. The seroprevalence of bovine leukosis was higher in older females that were pregnant or artificially inseminated, and in animals with prior disease history with reduced milk production (*P* <0.05) (Figure 1).

The seroprevalence (31.3%) observed in the current study is close to the prevalence (32.5%) reported in the UAE (25.7%; Hassan *et al*., 2020) and in Korea (35%; Suh *et al*., 2005). The current results do not concur with the findings of the research conducted in Japan (44.8%; Meas *et al*., 2000), Argentina (84%; Gutierrez *et al*., 2011), Canada (20.8%; Nekouei *et al*., 2015), Egypt (21.5%; Hamada *et al*., 2020), and China (21%; Ma *et al*., 2016). This difference in prevalence may be due to differences in housing systems, population density, animal raising practices, and geo-climatic variations.

Regarding the haematological parameters in Holstein–Friesian dairy cattle, the total leukocyte count was markedly higher in ELISA-positive cattle than in ELISA-negative cattle, as stated earlier (Yang *et al*., 2016).This increase in leukocyte count in all the positive cattle is a sign of mounting a competent immune response against the viral infection (Juliarena *et al*., 2007).The current outcome of total leukocyte count was not in accordance with Ali *et al*. (2019), who showed a non-significant association of total leukocyte count in seropositive and seronegative cattle. The mean value of lymphocytes was higher (*P* <0.05) in seropositive cattle; this was consistent with a study (Mekata *et al*., 2018) reporting higher level of lymphocytes in Japanese black (JB) adult cattle affected with bovine leukosis. The current outcome stands in disagreement with another study (Ali *et al*., 2019), in which a non-significant (*P* >0.05) association was present regarding lymphocytes.

This virus basically affects the genome of B-lymphocytes, which consequently stimulates further cell division, resulting in an increased number of peripheral blood lymphocytes (Ali *et al.,* 2019). This effect in cattle may be due to breed differences, feed quality, and geo-biological variation. In the current study, the value of monocytes was markedly higher in seropositive cattle than in seronegative cattle, and was in agreement with a similar study (Notsu *et al*., 2018). The monocyte result was not in accordance with an earlier study (Yang *et al*., 2016), which showed a significant decrease in the level

of monocytes in affected animals. In general, the monocytes are the components of the white blood cells that are generally elevated in the certain conditions of the body involving infections, chronic inflammation, and allergic reactions (Sikandar *et al*., 2012).

The mean value of haemoglobin was higher (P <0.05) in seropositive cattle, which is in accordance with the findings reported previously (Yang *et al*., 2016). An increased haemoglobin value reflects the abnormal morphology of red blood cells resulting from macrocytosis associated with viral infection. The severe stress mediated by the detrimental effects of the virus may even lead to death of the red blood cells. The outcome suggests that the cattle under investigation were experiencing a severe stage of viral infection. The mean value of red blood cells was higher in seropositive dairy cattle, which is not in accordance with the study of Notsu *et al*. (2018), who showed non-significant decreases in red blood cells; this difference may be due to the severity of infection in seropositive and seronegative cattle. The packed cell volume was markedly higher in seropositive cattle than in seronegative cattle; Yang *et al*. (2016) also reported significant increases in packed cell volume.

In terms of liver enzymes, seropositive cattle had higher ALT activity than seronegative animals (Table 2). The increased ALT indicates that the virus has invaded the liver via portal circulation and resides within the hepatocytes. Consequently, the disease has affected the liver parenchyma, leading to the higher enzyme concentration in the blood. The findings are consistent with those of Ali *et al*. (2019), who reported similar findings. Leukaemic lymphocytic cell infiltration in hepatic tissues may cause the liver function abnormalities that are manifested in the early stages of leukosis (Nikolay *et al*., 2013). Another study, which contradicted the findings of the current study, found that the level of ALT in seropositive animals remained unaltered (Akalin *et al*., 2015). The activity of another liver enzyme, AST, was considerably lower in BLV-infected animals than seronegative cattle. Another study (Nikolay *et al*., 2013) also reported decreased AST activity in seropositive cattle. The current findings differ from those of Ali *et al*. (2019), who found a substantial rise in AST enzyme activity in BLV-infected cattle. The possible metabolic cause of decreased AST levels in their study may be attributed to vitamin B6 deficiency, certain drugs, anorexia, and severe weight loss (Rossouw *et al*., 1978; Schwarz 1996).

Table 2 Serum biochemical parameters in seropositive and seronegative Holstein–Friesian cattle (Mean ± standard error)

	Animals		
Serum biochemical parameters	Seropositive	Seronegative	*P*-value
ALT (U/l)	24.25 ± 1.03	18.80 ± 1.02	0.002
AST (U/l)	49.33 ± 3.31	46.42 ± 2.50	0.00
GPx (U/l)	1365.63 ± 12.03	2016.25 ± 10.54	0.00
SOD (U/ml)	2.14 ± 0.13	3.39 ± 0.22	0.00

ALT = alanine transaminase, AST = aspartate aminotransferase, GPx = glutathione peroxidase, SOD = superoxide dismutase

Oxidative stress is a central issue in the transformation or death of living cells. In the current study, the activity of the antioxidant enzyme, GPx, was markedly lower in BLV-infected cattle than in seronegative cattle. It may be due to the viral infection, which can alter the oxidative status either by increasing the formation of nitric oxide or by inhibiting the synthesis of enzymes involved in the oxidative defence within the host cell. The result of reduced glutathione activity is not in agreement with the outcome of another study (Ali *et al*., 2019) who found non-significant changes in GPx activity. The current finding is consistent with the results of earlier investigations (Souza *et al*., 2011; Nikolay et al., 2013; Akalin *et al*., 2015) describing the oxidative status and the markers of oxidative stress in BLV-infected dairy cattle and showing a marked decrease in glutathione peroxidase activity. The activity of superoxide dismutase enzyme in the seropositive cattle was also lower in this study; reduced superoxide dismutase activity was different from another study (Souza *et al*., 2011), which showed non-significant (P >0.05) changes in superoxide dismutase activity in bovine leukosis.

In the current investigation, a relationship between the animal's sex and the prevalence of BLV infection was evidenced (P <0.05) (Figure 1). Another study found a substantial relationship between animal sex and disease incidence, with female animals manifesting higher prevalence than male animals (Khudhair *et al*., 2016). The most plausible reason for a higher incidence in females may be the importation of unscreened heifers (for fresh stock or replacement heifers) and a variety of stress-inducing cyclic physiological processes (such as pregnancy, lactation, breeding, oestrous) that females experience throughout their life span. Khan *et al*. (2019) found that the prevalence was higher in male

animals than in females, which may be due to the fact that the referred study evaluated a larger sample size of male animals than the current study, which have led to the gender-based prevalence outcome. It could also be associated with the environmental variations in the study areas. The occurrence of BLV was also found to be linked to the age of animals. Seropositivity was higher in animals above the age of six years (P <0.05). This finding is consistent with several earlier studies (Murakami *et al*., 2011; Ramiz *et al*., 2021). Several reasons may contribute to the greater frequency of BLV in elderly animals. The longer an animal lives, the more chances it has of being exposed to and interacting with BLV-infected animals. Furthermore, elderly animals are more prone to infections (Khudhair *et al*., 2016). A negative relationship was observed between the ages of seropositive animals in another investigation (Erskine *et al*., 2012), implying that the animals might be infected at any age.

The current study revealed a link between pregnancy and BLV infection. Pregnant animals had a higher prevalence than non-pregnant animals. In buffalo, similar outcomes were obtained (Selim *et al*., 2020) and this could be due to pregnancy, which acts as a stressor on animals, lowering the immunological competency of the dam and raising the risk of BLV infection. This results differs from Selim *et al.* (2020), who reported non-significant (P >0.05) associations between seropositivity and pregnancy of animals. This could be due to the fact that the disease is mainly transmitted horizontally.

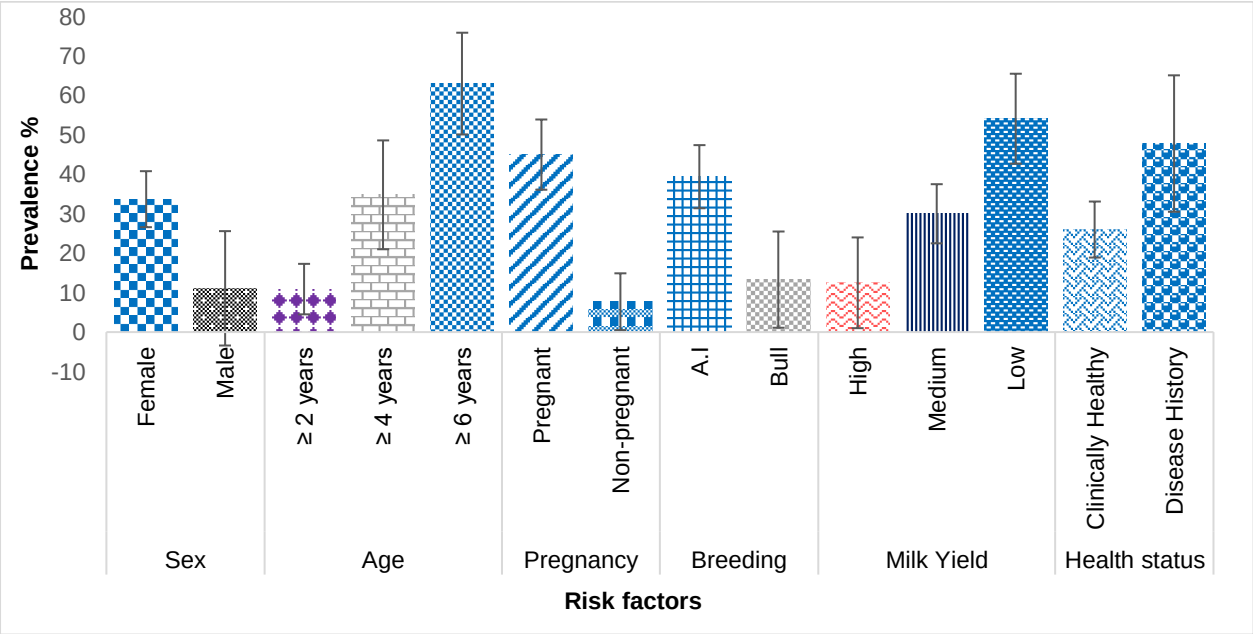

Figure 1 Association of various risk factors with the prevalence of bovine leukosis in Holstein–Friesian dairy cattle

The current investigation found an association between BLV seropositivity and breeding strategy. In comparison to natural service, animals bred by artificial insemination (AI) showed higher seropositivity, in agreement with Ramiz *et al*. (2021). On farms, it is common practice to use a single glove for AI of multiple animals; the blood-contaminated glove increases the risk of spreading BLV-infected lymphocytes among individual animals in the herd.

In the present study, a marked decrease in milk production in dairy animals was associated with the incidence of BLV compared to seronegative animals (P <0.05). This could be attributed to BLV-infected animal frailty and decreased feed intake. Similar findings have also been observed in another study (Nekouei *et al*., 2015). This could be due to the animal's health declining over time, weight loss, or other factors, resulting in a steady decline in milk yield. Our findings differ from those of Jacobs *et al*. (1991) and Heald *et al*. (1992), who found no significant link between milk production and BLV. This could also be attributed to differences in geographical location, feeding techniques, management aspects, and knowledge of contemporary husbandry practices pivotal for disease control and prevention.

We found a significant correlation of animal health history with BLV in seropositive animals. The prevalence was higher in those animals that had any history of previous disease including

abortions, repeat breeding, mastitis, or infectious diseases. This may be ascribed to the down-regulated immune status of the host as a result of previous diseases increasing susceptibility to BLV.

Conclusion

The results of the current study confirmed that the disease was prevalent in the Holstein–Friesian dairy cattle in different areas of the Dera Ismail Khan region of Pakistan. It has been observed that the disease affects the haematological and biochemical parameters of infected animals in the presence of varied pathological alterations, leading to physiological stress and liver function disorders in the host. A greater degree of attention is required to establish effective prevention and control measures subsequent to the screening of all animals at dairy farms and adopting good husbandry practices, segregation, and eliminating infected animals.

Acknowledgement

The authors would like to thank all the facilitators for their support in sample collection and processing, and the staff of the Department of Clinical Sciences for their technical assistance.

Authors' contribution

AN, MK, and MS conceived the idea and facilitated the execution of research activities. HU, AR, and MUF performed the sampling and testing. FU helped in the statistical analysis and AS equally contributed in the manuscript write-up.

Conflict of Interest

The authors declare that there is no conflict of interest related to this article.

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