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Original article

Seroprevalence and molecular characterization of lumpy skin disease virus in Bahawalpur district of South Punjab, Pakistan

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Abstract

Lumpy skin disease (LSD) is a viral infectious disease that affects cattle and causes significant economic losses, particularly in low and medium income countries, where livestock is often the main source of income for small-scale farmers and rural communities. In the last few years, the disease has become widespread in several countries in Asia, including Pakistan. The present cross-sectional study aimed to determine the seroprevalence and molecular characteristics of LSD virus (LSDV) among cattle in the Bahawalpur district (Pakistan), while identifying potential associated risk factors. A total of 400 serum samples were collected from cattle and analysed using a commercial ELISA kit to determine seroprevalence. Additionally, 12 skin scrapping samples of cattle were collected from sick animals to detect and characterize the currently potentially circulating LSDV strains using PCR, targeting the P32 gene. The overall LSD seroprevalence among cattle was found to be 38.0%, with significant variation observed between different geographical areas of the Bahawalpur district, showing the highest prevalence of 46.2% in Yazman Tehsil. Age and feeding system were identified as significant risk factors for LSD occurrence in cattle. The genetic analysis revealed a high genomic similarity between the LSDV strain sequences reported in Asian and Middle Eastern countries. The P32 gene phylogenetic analysis further confirmed the close relationship between LSDV sequences from Pakistan and vaccine strains of sheep and goat pox viruses. The present study provides important baseline information for an understanding of the epidemiology and characterisation of LSDV enzootic strains in Pakistan, and highlights the need for effective disease control strategies, including vaccination campaigns, particularly in disease-endemic regions.

Keywords: Cattle, lumpy skin disease, molecular characterization, p32 gene, phylogenetic analysis, seroprevalence

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Introduction

Lumpy skin disease (LSD) is considered both an emerging and re-emerging viral disease that affects cattle and causes substantial economic losses in the livestock industry (*Bos taurus, Bos indicus, Bubalus bubalis*) worldwide in many developing countries (Tuppurainen and Oura 2012). The disease is caused by a DNA virus known as lumpy skin disease virus (LSDV), a member of the *Capripoxvirus* genus within the *Poxviridae* family (McInnes et al. 2023). LSDV is closely related to goat pox virus and sheep pox virus with a high nucleotide similarity of 96% to 97% genome identity. LSDV is primarily a vector transmitted disease, transmitted by biting insects (flies and mosquitoes) and ticks from infected animals to susceptible animals (Tuppurainen et al. 2013). Moreover, a recent study has suggested that LSDV can also be spread in the absence of a vector through direct contact transmission (Aleksandr et al. 2020**)**. The disease is characterized by persistent fever, swollen lymph nodes, excessive lacrimation, excessive drooling, pneumonia, generalized or localized skin nodules, and the formation of pox type lesions in the mucous membranes of the digestive and respiratory systems. Over time, the nodules may either regress or necrotize, leaving distinct, hard, elevated regions known as "sit-fasts." Eventually, these areas exfoliate, resulting in slow-healing ulcers and scarring (Tuppurainen and Oura 2012). The morbidity rate of lumpy skin disease ranges from 15% to 25%, while the mortality rate is generally low, typically ranging from 2% to 5% (Tuppurainen and Oura 2012). Case fatality rates ranged from 2-10% (Mathivanan et al. 2023).

Owing to the ability of the virus to transmit to disease-free regions and occurrence of widespread epidemics, the World Organization for Animal Health (WOAH) has classified LSD as a notifiable disease (WOAH 2024). Various clinical and laboratory diagnostic methods can be used to detect LSDV infection in animals. One of the most reliable techniques is the genome-based polymerase chain reaction (PCR), which allows for the identification of different capripox viruses in a particular geographic region. Previous investigations have effectively employed the p32 (Envelop protein) antigen gene-based PCR techniques to detect and identify different capripox viruses, including LSDV (Adedeji et al. 2019).

LSD was historically restricted to sub-Saharan Africa since its first report in Zambia in 1929. However, in recent years, it has expanded its geographical distribution to encompass countries in the Middle East, Southeast Europe, and Asia (Tuppurainen and Oura 2012). From 2019 to 2021, LSD continued to spread throughout continental Asia, resulting in outbreaks in cattle populations. Since the first outbreak in late 2021, LSD has progressively spread to various regions of Pakistan (Khatri et al. 2023, Ul-Rahman et al. 2023). Unfortunately, there is a lack of information on the epidemiological situation in affected regions of Pakistan. This study aims to provide a foundation of LSDV (seroprevalence) in the study site, identify the strains of LSDV in circulation and through vaccination campaign in this disease endemic area to establish disease control.

Materials and Methods

Study area

This current cross-sectional study was conducted at various farms located in six administrative regions (tehsils) of the Bahawalpur district in Punjab (29.3544° N, 71.6911°E), Pakistan including: Bahawalpur city; Bahawalpur Sadar; Hasilpur; Khairpur Tamewali; Ahmadpur East; and Yazman (Figure 1). The Bahawalpur district is one of the largest districts by area in Punjab, covering approximately 24,830 square kilometres and having substantial livestock population making it a vital area for studying infectious diseases such as LSD. It is bordered by India to its south and southeast and local neighbouring districts in the other directions. The selected study areas are known for their rainy season, which facilitates animal grazing and nomadic movements across different sub-regions, enhancing the risk of transboundary animal diseases. The western border of the district adjoins Sindh Province, where the first case of LSD in the country was reported during the recent outbreak of its kind in Pakistan. It is also a trade route for animals between Punjab and Sindh province. There was no report of research on LSDV in Bahawalpur, and its selection as a study area fills a critical gap in the epidemiological data necessary for effective disease control and prevention strategies.

Sample collection

The study animals were raised in both urban and rural areas of the Bahawalpur district, under natural and semi-controlled climatic conditions. Vaccination against LSDV was not practiced in the study area, as Pakistan was previously considered as an LSD-free country until November 2021. Samples were collected from 1st June to 13th September 2022. To ensure a statistically significant sample size, a minimum of seven serum samples were collected from each cattle herd. This sample size was calculated using a previously described formula (Thrusfield 2008), to achieve a 95% chance

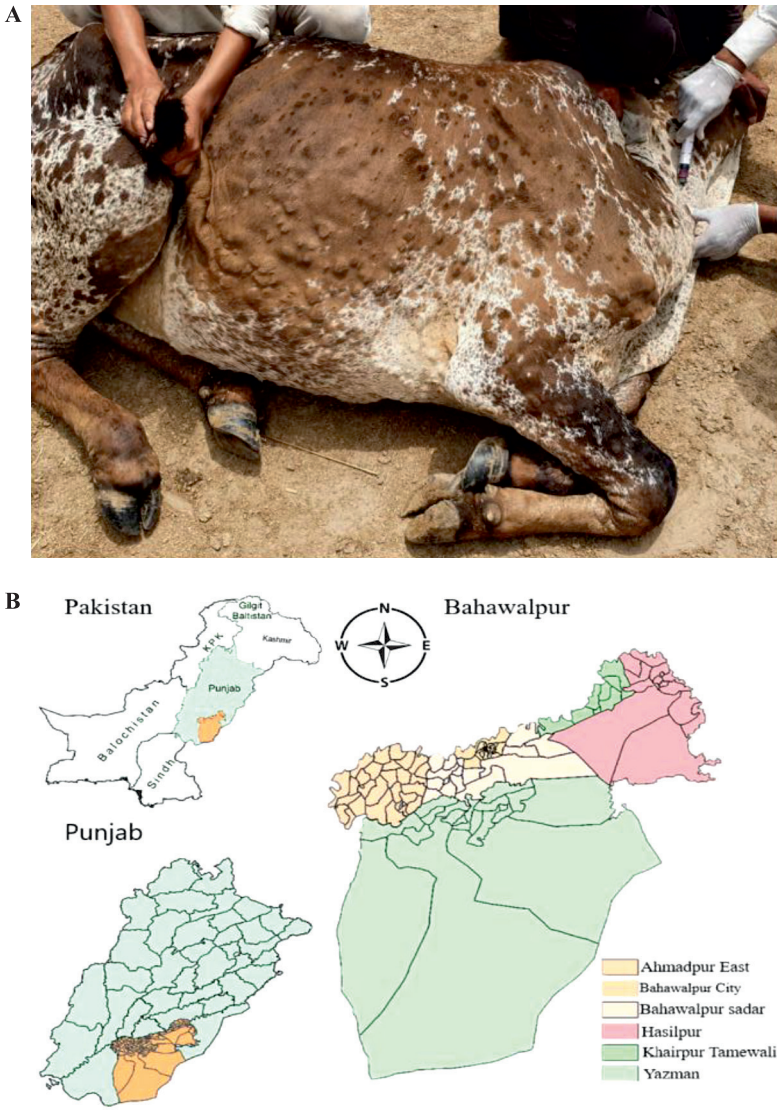

Fig. 1. A – blood sampling from a cow naturally infected with LSD virus presenting multiple typical skin nodules, B – the maps on the left show the country map (above) of Pakistan and the map of Punjab province (below), the map on the right represents the study district of Bahawalpur with its six administrative regions (tehsils) located in the south of Punjab province

of observing 1% seropositivity with 15% precision at 95% confidence interval. A total of 400 blood samples (3ml each) were aseptically collected from the jugular vein into Bolton® gel and clot activator tubes. The collected samples were allowed to clot at indoor or outdoor temperature (25 to 35°C) and then centrifuged at 3,000 g for 5 minutes to collect the sera, and then stored at -20°C until serological analysis. For LSDV molecular identification, a total of 12 skin-scraping samples were collected from sick animals presenting clinical symptoms, such as nodules and respiratory distress (Figure 1A).

Laboratory analysis

All serum samples were tested for the presence of antibodies against LSDV using a commercial ELISA kit (ID Screen® Capripox Double Antigen Multi-species ELISA, Montpellier, France) following the manufacturer's instructions. The optical density (OD) of the samples was measured at 450 nm, and the results were calculated by dividing the corrected OD value of the tested serum by the corrected OD value of the positive control. Serum samples with an OD value of percentage transmission ≥ 10% were considered positive. For molecular characterization of LSDV, genomic DNA extraction was performed using a genomic extraction kit (GeneJET, Thermo Scientific, Waltham, MA) following the manufacturer's protocol. Following genomic extraction, PCR amplification targeting the 759 bp fragment of the p32 gene of LSDV was conducted using previously described primers (LSDV-F: 5`-TCGTTGGTCGCGAAATTTCAG-3` and LSDV-R: 5`-GAGCCATCCATTTTCCAACTCT-3`) (Sameea et al. 2018).

For the PCR reaction, a mixture of 25 µl was prepared, which included 14 µl of PCR master mix (Dream Taq™ Green PCR Master Mix, Waltham, MA), 2 µl of each forward and reverse primer, and 7 µl of extracted viral DNA. The PCR reactions were conducted using a Bio-Rad T100™ thermal cycler (Hercules, CA) with the following thermal conditions: an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 45 seconds, primer annealing at 56°C for 45 seconds, and strand extension at 72°C for 65 seconds. The reaction concluded with a final strand extension step at 72°C for 5 minutes. The resulting PCR products were separated by electrophoresis on a 1.5% (w/v) agarose gel at 75 V for 1 hour and visualized using a UV transilluminator. Following amplification, the PCR products were purified using Wizard SV gel and the PCR clean-up system. Subsequently, the purified products (5) were sequenced bidirectionally using the same primers on the ABI PRISM Genetic Analyzer 3130x1 version (Applied Biosystems, USA). To assess the genetic diversity of the strains, the obtained sequences were aligned with LSDV strains from different geographic regions. The ClustalW method in BioEdit® version 5.0.6 (Hall, 1999) was used for sequence alignment. Phylogenetic analysis based on the p32 gene was performed using the maximum likelihood statistical method with the maximum composite likelihood model. This analysis included 1000 replication bootstrap values and was conducted using MEGA 11® software (Kumar et al. 2018).

Statistical analysis

Descriptive statistical analysis was performed to determine the percentages of all variables including sex, breed, age, and feeding systems of the animals using IBM SPSS Statistics version 29 (IBM Corp: Armonk, NY). To assess the association between disease status (seropositive versus seronegative) and categorical variables (risk factors), Pearson's Chi-Square test was performed. If the necessary assumption of the Chi-Square test was violated, the Fisher exact test was used as an alternative approach. A p-value of ≤ 0.05 was considered statistically significant for seroprevalence.

Ethics Approval

This study was approved by the Directorate of Advance Studies, University of Veterinary and Animal Sciences, Lahore (DAS no: 1920 A) and the Ethical Review Committee, College of Veterinary and Animal Sciences, Jhang (ERS no: 104). All procedures involving animals were done in compliance with animal welfare and ethical guidelines. All animal handling was carried out under the supervision of a veterinarian. All samples from cattle were collected with the consent of their owners.

Results

In the present study, an overall seroprevalence of LSD of 38.0% (152/400; 95% CI: 33.3-42.8) was observed among the cattle population in the Bahawalpur district. In our study, the overall prevalence in the herds (n=45) investigated was 51.1% (n=23), and within herds, the prevalence ranged from 30.8% to 54.5%. Although female animals showed a higher seroprevalence (41.2%; 95% CI: 34.9-47.5) compared to male animals (33.5%; 95%CI: 26.3-40.7), there was no significant association (p=0.119) observed between disease occurrence and sex of the animal. The disease was comparably prevalent among all the cattle breeds included in the study, with no statistically significant differences observed (p=0.829). The highest seroprevalence (41.8%: 95%CI: 28.8-54.8) of LSD was observed in crossbred animals, followed by 39.7% (95%CI: 31.3-48.1) in the Cholistani breed and 36.4% (95%CI: 27.4-45.4) in the Sahiwal breed. The seroprevalence of LSD was found to be significantly higher (p=0.047) in the adult age group (>3 Years) compared to younger (<1 year) and intermediate-age (>1 year – 3 years) animals. Adult animals showed a high seroprevalence of 44.5% (95%CI: 37.3-51.7), followed by intermediate-age animals with a seroprevalence of 33.6% (95%CI: 25.3-41.9) and young animals with a seroprevalence of 31.2% (95%CI: 21.8-40.6). The seroprevalence of LSD was also significantly higher (p=0.002) in animals being grazed compared to those fed in a stall-feeding system. Grazed animals had a seroprevalence of 45.9% (95%CI: 38.8-53.1), while animals in stall feeding systems had a seroprevalence of 31.2% (95%CI: 25-37.4). No significant (p=0.647) association was observed based on geographical distribution. Animals from Yazman Tehsil showed the highest seroprevalence of 46.2% (95%CI: 35.1-57.2), followed by Bahawalpur city (37.7%; 95%CI: 24.7-50.8), Bahawalpur Sadar (37.5%; 95%CI: 25.6-49.4), Khairpur Tamewali (37%; 95%CI: 24.2-49.9), Ahmadpur East (36.9%; 95%CI: 25.2-48.7), and Hasilpur (32.6%; 95%CI: 22.7-42.5) (Table 1).

PCR testing

Using PCR, all 12 clinical samples were found positive for the presence of LDSV. Of these, five randomly selected samples were processed for sequences and subsequent phylogenetic analysis. The obtained sequences of p32 genes were submitted to the National

Table 1. Prevalence and risk factors associated with Lumpy skin disease (LSD) in cattle.

Risk Factors		Total / Positive (%)	95% C.I.	P value
Sex	Female	233 / 96 (41.2)	34.9 – 47.5	0.119
	Male	167/ 56 (33.5)	26.3 – 40.7	
Breed	Crossbred	55 / 23 (41.8)	28.8 – 54.8	0.829
	Cholistani	131 / 52 (39.7)	31.3 – 48.1	
	Nondescript	104 / 37 (35.6)	26.4 – 44.8	
	Sahiwal	110 / 40 (36.4)	27.4 – 45.4	
Age	Neonates – 1-year-old (Young)	93 / 29 (31.2)	21.8 - 40.6	0.047
	>1 year – 3 years old (Intermediate)	125 / 42 (33.6)	25.3 - 41.9	
	>3 years (Adult)	182 / 81 (44.5)	37.3 - 51.7	
Feeding system	Grazing	185 / 85 (45.9)	38.8 – 53.1	0.002
	Stall feeding	215 / 67 (31.2)	25 – 37.4	
Geographical distribution	Bahawalpur City	53 / 20 (37.7)	24.7 – 50.8	0.647
	Bahawalpur Sadar	64 / 24 (37.5)	25.6 – 49.4	
	Ahmadpur East	65 / 24 (36.9)	25.2 – 48.7	
	Yazman	78 / 36 (46.2)	35.1 – 57.2	
	Khairpur Tamewali	54 / 20 (37.0)	24.2 – 49.9	
	Hasilpur	86 / 28 (32.6)	22.7 – 42.5	
TOTAL		400 / 152 (38.0)	33.3 – 42.8	

Values with $p>0.05$ indicate a non-significant difference, while $p<0.05$ indicate a significant difference.

Center for Biotechnology Information (NCBI) and assigned accession numbers OQ319115, OQ450502, OQ588732, OQ588733, and OQ652097. Based on the phylogenetic analysis using the p32 gene, the submitted LSDV strains clustered together with strains reported from China (OL602174), the Kingdom of Saudi Arabia (MN244249), Egypt (MN418202), and India (MW452626), obtained during the years 2012-2019 (Fig. 2). The p32 gene sequences of the samples showed high sequence identity ranging from 99.92% to 99.97% with other LSDV field isolates identified in a wide geography of India, China, Iran, Egypt, and KSA. The understudied sequences also showed relatively high sequence identity with Taiwan (99.88%) as well as goat pox virus (98.86%) and sheep pox virus (98.72%).

Discussion

Since the first detection of LSD in Pakistan in 2021, the disease has become enzootic and has spread to various regions with a high density of bovine populations (Khatri et al. 2023). Control of this disease to minimize the economic losses depends upon various disease control interventions, including continual disease surveillance, rapid and accurate diagnosis, and vaccination campaigns (Hunter and Wallace 2001). Nonetheless, LSDV-specific vaccination is not practiced in many developing countries including Pakistan. However, active sero-surveillance and molecular characterization of field prevailing LSDV strains are crucial for developing a vaccine and ultimately eradicating the disease. In view of this, ELISA and PCR techniques were considered reliable methods for sero-surveillance, identification of clinical cases, and determining the geographical distribution of transboundary diseases such as FMD, PPR and LSD worldwide (Clemmons et al. 2021). The molecular detection of LSDV along with its associated factors is crucial for early prognosis, treatment, and prophylaxis (Badhy et al. 2021). The present study is the first-ever report on the seroprevalence of LSD in the cattle population of the disease-endemic regions of Bahawalpur (Pakistan).

The results of the current study showed an overall LSD seroprevalence of 38% among the cattle population, which is consistent with previous investigations conducted in disease-endemic regions worldwide (Ochwo et al. 2019, Selim et al. 2021a). Also, the seroprevalence of LSD observed in this study was notably higher compared to the previously reported rates in Egypt (19.5%; Selim et al. 2021a), and Uganda (8.7%; Ochwo et al. 2019). These variations in seroprevalence rates could be attributed to differences in livestock production practices and sample population, sampling period, climate variations, socio-ecological factors, and density of the vectors (Abera et al.

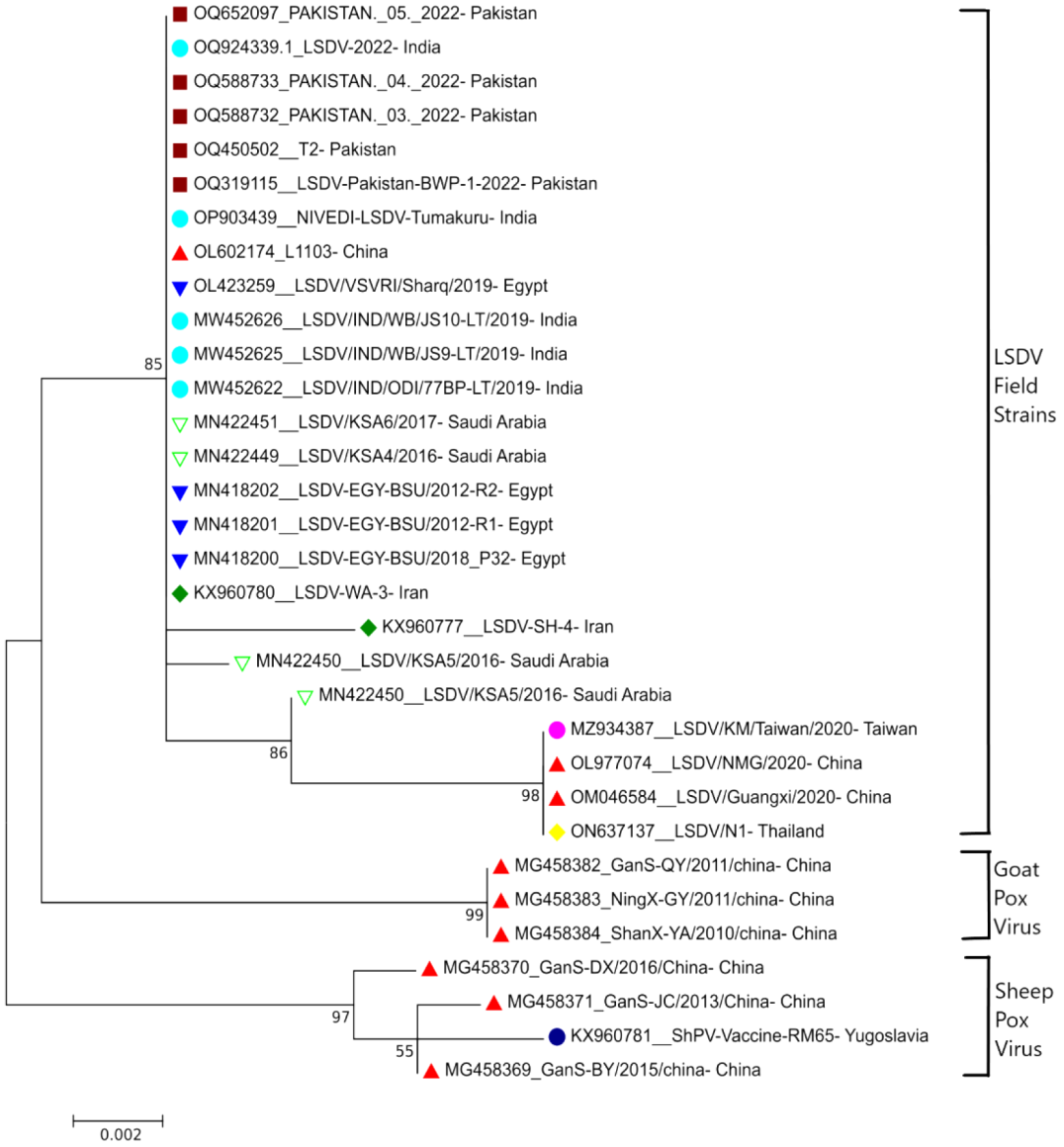

Fig. 2. Lumpy Skin Disease Phylogeny.
Phylogenetic tree constructed based on p32 gene registered to GenBank showing the relationship among LSDV strains isolated in this study with contemporary strains from neighboring Asian countries and some non-Asian countries.

2015, Selim et al. 2021a). The analysis conducted in the current study did not find any significant variation in the seroprevalence of LSD between male and female animals. However, it was more prevalent in female animals (41.2%) than in male animals (33.5%). These findings are in agreement with previous observations that female animals are typically kept on farm for longer periods as dairy herd animal for reproduction and milk production (Selim et al. 2021a). Furthermore, the stress factor during the pregnancy and lactation period may increase the chance of infection and contribute to higher seroprevalence in female animals (Tuppurainen and Oura 2012).

In terms of breed of cattle, a non-significant association was observed among breeds (Crossbred, Cholistani, Non-descript and Sahiwal), and the trend of seroprevalence of LSD is consistent with previous findings reporting high susceptibility to the disease among crossbreed cattle compared to indigenous breeds (Abera et al. 2015, Selim et al. 2021a). Similar findings have also been reported from a study in Ethiopia (Molla et al. 2023) describing similar results with a 26% seropositivity in crossbred and 25.3% among indigenous cattle. Traditionally, the number of crossbred animals on farms is generally lower compared with the large number of indigenous breeds in the study area. Moreover, crossbred and indigenous breed animals are farmed and grazed together, enhancing the possibility of infection in the indigenous animals.

Regarding the age groups, a statistically significant association between age and disease occurrence ($p<0.05$) was observed in the present study, which

is comparable to observations reported in previous investigations (Ochwo et al. 2019, Selim et al. 2021a). However, it is important to note that previous studies have claimed a high seroprevalence of LSD in calves (Abera et al. 2015). The possible reason for the difference in findings could be the transfer of passive maternal immunity and the lower frequency of exposure in young animals, leading to a lower seroprevalence. Furthermore, the current study found a higher prevalence of LSD in grazed animals (45.9%) compared to stall-fed animals (31.2%). This finding is consistent with the understanding that LSD is primarily transmitted through vectors, such as mosquitoes and ticks, which are more prevalent in grazing areas (Tuppurainen et al. 2013). This finding is not unusual as several studies have reported that LSD is a transboundary disease that can rapidly spread under extensive systems. The movement of animals for grazing is considered a significant factor in the spread of the disease from one area to another. There is a high possibility of vector feeding and virus transmission occurring in free-range conditions, leading to the infection of healthy animals (Machado et al. 2019, Kiplagat et al. 2020).

On the geographical basis of the study site, Yazman Tehsil areas, often referred as the entrance to the Cholistan desert manifested the highest prevalence of LSD. The livestock in this area is mostly raised through grazing on the grasslands, away from the croplands in the uncultivated desert areas, using a single water source for household use and different grazing animal species in rural regions. Moreover, this area lies in the lands bordering India and there is a good possibility of transboundary transmission. This seems to be the most plausible reason for animals becoming infected with viruses more often, as they share water sources and also experience stress, particularly during drought seasons.

All clinical samples examined in the study tested positive for LSD, confirming the presence of the disease among the cattle population. The PCR results were consistent with the field-based diagnosis of typical clinical symptoms of LSD, including pyrexia, respiratory distress and skin nodules. The detection of LSDV in skin scrapings from nodules using PCR indicated that skin nodules are the most reliable source for virus detection compared to other samples such as blood, semen, and milk. Skin nodules contain a higher concentration of viral particles due to the tissue tropism of LSDV (Selim et al. 2021b). The observed substantial similarity in the genomic makeup of all identified LSDV strains indicates a widespread presence of either identical or closely related variants across the country. This discovery strongly implies that these strains, as evidenced by previous studies examining the partial p32 gene, might be the underlying cause of numerous LSDV outbreaks in Pakistan (Mansour 2017, Mafirakureva et al. 2017). The genetic analysis of the p32 gene revealed that the LSDV strains identified in clinical cases showed significant genomic similarity to LSDV strains reported in various Asian and Middle Eastern countries, such as Egypt, the Kingdom of Saudi Arabia, and India. This suggests that the same LSDV strains are responsible for outbreaks that occur across different borders.

Similar findings have been observed in other epidemiological investigations conducted in different regions, further supporting the widespread distribution of these strains (Sevik et al. 2017, Ma et al. 2022). Although an earlier study has shown that there is high genetic similarity between LSDV strains isolated from cattle and other poxviruses found in sheep and goats (Tulman et al. 2001), our findings indicated that despite genetic similarity among different poxviruses, the p32 gene sequence phylogeny shows different clades for LSDV, goat pox viruses and sheep pox viruses. Although the p32 gene serves as a valuable marker for genetic characterization and epidemiological tracking these observed differences are partly explanatory and in agreement with the findings of a previous study showing that most promising results at the level of cell-mediated response as well as humoral immune response were obtained when using homologous virus vaccine as compared to heterologous vaccines (Norian et al. 2019), whereas, among heterologous vaccines, goat-pox virus-based vaccine showed better results when compared to sheep-pox virus-based vaccine (Klement et al. 2020). Therefore, it is suggested that these vaccines should be used for controlling LSDV outbreaks, particularly in disease-endemic and high-risk regions. The government of Punjab is currently using Lumpyvac, a live attenuated Neethling strain vaccine against the lumpy skin disease virus in the study area (Muhammad Umer Iqbal, Deputy Director Livestock, personal communication, February 2023). Continuous monitoring and evaluation of vaccine performance are essential for refining control strategies and addressing the evolving challenges of LSDV control.

Conclusion

The present study provides the evidence of lumpy skin disease spread in susceptible cattle populations in the southern and border district of Punjab province, Pakistan. The phylogenetic analysis has shown that the p32 gene sequences from viruses isolated in Pakistan

are comparable to those from around neighboring countries including India and Iran. These findings reflect the possible cross-border transmission potential of lumpy skin disease virus. The present study also identified grazed adult cattle as high-risk group animals. Appropriate animal husbandry practices along with vaccination against lumpy skin disease virus are key to successful control of the disease in the region.

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