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SEROPREVALENCE AND HAEMATO-BIOCHEMICAL INSIGHTS INTO LISTERIOSIS IN SHEEP OF PUNJAB, PAKISTAN

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Abstract: This study was planned to explore the seroprevalence, haemato-biochemical effects, and risk factors relevant to the prevalence of *listeriosis* in sheep at different private and public sector veterinary hospitals, farms, and small households from eleven districts of Punjab. A total of 192 sheep's sera were assessed by an enzyme-linked immunosorbent assay. Overall, 22.91% (44/192) sheep were identified as seropositive. Haematological analyses revealed that seropositive animals had higher levels of WBCs and plasma fibrinogen than seronegative animals. Increased WBCs were observed to have a significant relation to *listeriosis* in sheep. The other haematological parameters measured were not significantly related with *listeriosis*. Biochemical data revealed that ALT and AST levels were significantly associated to *listeriosis*. A significant association of breed, age, sex, presence of ticks, diet, water source, abortion history, mastitis, rearing source, grazing system, and health stats of seropositive sheep with *listeriosis* was also noted. No significant difference was observed in breed, sex, body health, ticks, diet, water source, mastitis, rearing source, or grazing system, while animals with an age between one and three years had the highest prevalence as compared to other age groups. Furthermore, a significant difference was observed between females with abortion history and females with no abortion history. This study revealed that*listeriosis* in sheep can result in anomalies related to internal homeostasis and liver function, which should be taken into account when developing a treatment plan. In conclusion, disease existed moderately prevalent in different districts of Punjab, Pakistan.

Keywords: Listeriosis, sheep, prevalence, biochemistry, hematology

Introduction

Listeriosis is a serious zoonotic and life-threatening disease, also called circling disease, meningeoencephalitis, or silage sickness [1]. Listeria species are gram-positive and facultative anaerobes. The genus Listeria contains ten species: *L. monocytogenes, L. ivanovii, L. grayi, L. innocua, L. seeligeri, L. welshimeri, L. marthii, and L. rocourtiae* [2, 3], as well as two newly discovered species, *L. fleischmannii and L. weihenstephanensis* [4, 5]. Among them, L. *ivanovii* and L. *monocytogenes* are dangerous to both people and animals [6]. L. *monocytogenes* is most important

as it has been found in many animal species and humans worldwide and has occurred in sporadic or epidemic outbreaks [7, 8]. The majority of infections in animals are asymptomatic, but serious cases may occur [9]. Although this disease is uncommon, its significant mortality rate (20-30%) renders it a major public health concern [10]. This disease has a seasonal occurrence in the northern hemisphere, probably due to the seasonal feeding of silage, with its peak in December [11, 12].

Punjab has the largest livestock population, and the majority of the population lives in rural areas. Livestock is the main source of income there, and listeria may cause significant economic losses in the dairy. The prominent pathogen involved is *L. monocytogenes*, which is of zoonotic importance. It can cause abortions and deaths in severe cases. *L. monocytogenes* has an internal life cycle and is capable of moving from cell to cell without being released, so it can easily cross the blood-brain barrier and placental barrier [13]. Listeriosis is clinically manifested as encephalitis, abortion, and septicemia [14, 15] in addition to mastitis [16] and keratoconjunctivitis[17].

Listeria affects many species, including sheep, goats, cattle, buffaloes, horses, canines, wild animals, birds, and humans. *L. encephalitis* is found more commonly in small ruminants, especially sheep and goats [1]. It has been separated from the flesh and milk of sheep, goats, cattle, and poultry [18-20]. This disease is ubiquitous in the environment and can live in water, soil, and decomposing plants, where it can contaminate food for humans and animals [11]. This organism resists thawing and freezing and can even survive in feces and dry fodder for long periods of time. It can tolerate a wide variety of temperatures (4-45 °C) and pH values of 5.5-9.6 [1].

Listeria infections are a serious public health hazard due to poor inspection procedures during food preparation and transportation. Listeria infections in ready-to-eat food have been reported by many workers from all over the world [21]. Food from animal origins ingested before being processed, such as raw milk and plant-based items tainted by manure from diseased or shedding animals, may indicate a direct link between human infection and the agricultural environment. The disease is acute and highly fatal, and the clinical signs of the disease are similar to those of other livestock diseases like Gid (*coenurosis*), which makes the study of the prevalence of Listeria monocytogenes necessary for the betterment of livestock [22, 23].

To the best of our knowledge, no research has been carried out to assess the distribution of infection in different districts of Punjab, Pakistan, despite being highly prevalent around the world. Therefore, this study was explored to investigate, for the first time, the seroprevalence, blood biochemical effects, and risk factors of sheep Listeriosis in different districts of Punjab. It is speculated that the disease is present in sheep breeds in the context of the trend of small ruminant entrepreneurs in the region.

2. Materials and Methods

2.1. Ethical statement

All animal treatments were carried out in compliance with established animal welfare rules, which were authorized by the Ethical Review Committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan, vide number DR/463.

2.2. Study animals

The study was conducted in eleven districts of Punjab in different private and public sector veterinary hospitals, farms, and small households. Samples were collected from Sargodha, Khushab, Bhukar, Dera Ghazi Khan, Lodhran, Bahawalpur, Khanewal, Lahore, Kasur, Jhelum, and Hafizabad districts. The summer season (April to September) is mostly dry and hot, whereas the winter months (December, January, and February) are cold. These districts have a large livestock population, including sheep. A total of 192 blood (sera harvested) samples from sheep, irrespective of age, breed, and sex, were collected. Animals showing one or more clinical signs among unilateral paralysis, encephalitis, dropping ears, deviated muzzle, lacerated lips, circling toward the affected side, abortion history, and mastitis were included in the study. Animals without any clinical signs listed above in the inclusion criteria were excluded.

2.3. Collection and processing of samples

A total of 3 mL of blood was aseptically collected from the jugular vein with two distinct types of vacutainers, EDTA and Gel/Clot activator tubes. The blood samples were transferred to the laboratory (Medicine Lab, Veterinary and Animal Sciences, Lahore) for analysis. All serum samples underwent serological testing to detect antibodies against *L. monocytogenes* using a sheep *Listeria* ELISA kit methodology (Abbexa, UK). The obtained blood samples have been placed in gel and clot activator tubes and spun at 2000 rpm for 10 minutes to separate out the serum. After being moved to Eppendorf tubes, sera were kept at -18 °C until they were processed further for ELISA. A vortexer was used to homogenize the reagents after they had been brought to room temperature (21 ± 5 °C) for the test. As directed by the manufacturer, 80 µL of dilution buffer was applied to each well of a 96-well plate. Then, 20 µL of both positive control (PC) and negative control (NC) were introduced to the double wells, followed by 20 µL of each sample in the rest of the wells. Wrap the surface of the dish with aluminum foil and incubated at 21 °C (± 5 °C) for 45 minutes. Following the incubation period, each well was rinsed three times with a 300 µL cleaning solution. After cleaning, 100 µL of conjugate was added to each well and incubated for 30 minutes at 21°C (± 5°C). Then 100 µL of substrate solution was added to each well of the plate and incubated for 15 min at 21 °C (± 5 °C). When immunological complexes are present, peroxidase transforms the substrate into a blue chemical. Then, pour 100 L of stop solution into each well, which will become yellow after it has been blocked. The optical density (OD) of the generated color was measured at 450 nm using an ELISA device (Biobase-EL10A, ELISA reader), and the degree of the color and the number of antibodies present in serum samples were computed.

The assessment was validated when the average value of the NC optical density (OD) was over 0.7 (ODNC > 0.7) and the average value of the PC optical density was < 30% of the NC optical density, i.e., ODPC < 30% of ODNC; ODPC/ODNC < 0.3 [24].

The findings were read out as follows: when the S/N% = or < 50%, the animal was categorized positive for Listeria monocytogenes antibodies; when the S/N% > 50% or < 60%, the animal was categorized to be uncertain; and when the S/N% = or > 60%, the sample was categorized as negative. S/N (%) was calculated using the following formula:

S/N % = OD sample/ODNC × 100

2.4. Haemato-biochemical evaluation

A 3ml blood sample was collected from the jugular vein of each animal and poured into a blood vacutainer that had been treated with anticoagulant (K2 EDTA). The collected blood sample was sent to Medicine Laboratory at the University of Veterinary and Animal Sciences (UVAS), Lahore, for hematology. The blood samples were spun at 3000 rpm for 15 minutes at 4 °C in a centrifuge machine (Harrier 18/80). The serum was isolated and kept at -20 °C for future use. Hematological parameters including red blood cells (RBCs), hemoglobin (Hb), packed cell volume (PCV), and white blood cells (WBCs), lymphocytes, neutrophils, monocytes, eosinophils, plasma proteins, and plasma fibrinogen were analyzed during the study using a VET hematology analyzer (Model No. DW3680/DW36 (China)). A chemistry analyzer (Sphera Edif®) was used to analyze the biochemical parameters, including aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN), and creatinine.

2.5. Statistical analysis

The SPSS software program (IBM SPSS Statistics, version 21) was used to analyze the data obtained during the study. A student *t*-test was employed for haemato-biochemical parameters and Pearson's chi-square test was used to evaluate the prevalence and risk factors (sex, age, breed, food, ticks, water source, abortion, mastitis, raising system, grazing system, and health status) of *listeriosi*s in sheep. The results were deemed statistically significant with a probability of 5% ($p<0.05$).

3. Results

3.1. Seroprevalence of Listeriosis in Sheeps in selected districts of Punjab with its association of risk factors

The overall seroprevalence of *listeriosis* in this study was 22.91% (44/192) in sheep. The different sheep breeds like Kajli, Thali, Lohi, Mundri, Fat Tail, and Sipli were included in the study. The maximum prevalence was recorded in Mundri (33.33%), followed by Fat Tail and Lohi with 22.85 and 22.58 percent prevalence, respectively. Among sheep, females were more prevalent (25.50%) as compared to males (11.42%). The emaciated sheep were found to be most prevalent (43.33%), followed by apparently normal sheep (20.61%), while thin sheep were least prevalent (16.92%). In the case of sheep, the maximum percentage (26.08%) was recorded in animals feeding on silage, followed by dry/green mix food (24.35%).

Sheep used different sources of water, and the maximum prevalence (26.41%) was recorded in those having a pond as a water source, followed by canal water at 25%. Out of ten, three sheep were also found to be positive and had abortion history. Twelve sheep were screened for *listeriosis* after having mastitis; among them, 25% of the sheep tested positive, while among the normal goats and sheep, 19.77 percent and 22.77 percent were positive for *listeriosis*, respectively. Animals were categorized based on rearing source, where 26.15% of sheep were found positive for *listeriosis* with grazing sources. Based on the grazing system, 25.67% of animals were found positive for the mixed gazing system for sheep, respectively. It is the system where these animals can graze alone as well as with bovines.

Eleven factors, including breed, age, sex, body health, presence of ticks, diet, water source, abortion history, mastitis, rearing source, and grazing system, were selected to find out their association with risk factors in sheep. No significant difference was observed in breed, sex, body health, ticks, diet, water source, mastitis, rearing source, or grazing system ($p>0.05$), whereas there was a statistically significant difference between age groups ($p<0.05$). Animals with an age between one and three years had the highest prevalence as compared to other age groups. Furthermore, a significant difference was observed between females with abortion history and females with no abortion history ($p<0.05$) (**Table 1**).

3.2. Haemato-biochemical profile

Haematological analyses revealed that seropositive animals had higher levels of WBCs and plasma fibrinogen than seronegative animals. Increased WBCs were observed to have a significant ($p<0.01$) relation to *listeriosis* in sheep. The other haematological parameters measured were not significantly related with *listeriosis* ($p>0.05$) (**Table 2**). Biochemical data revealed that ALT and AST levels were significantly ($p<0.01$) associated to listeriosis (**Table 3**).

4. Discussion

Listeriosis, a highly prevalent disease with zoonotic consequences, is caused by *L. monocytogenes* [25]. *L. monocytogenes* is widely distributed in nature and has been detected in soil, vegetation, sediments, farm animals, exotic and domestic animals, animal waste, and animal surroundings [26]. *L. monocytogenes* infections exist in sporadic or epidemic forms all throughout the world, with various outbreaks described [27]. In Pakistan, no study has yet been undertaken to identify the spread of infection in different districts of Punjab. As a result, the current study sought to evaluate the seroprevalence, haemato-biochemical consequences, and risk elements of Listeriosis in sheeps in different areas of Punjab for the first time.

The present study revealed that *listeria* was more prevalent in sheep (22.91%). Similar results were reported by Vasava *et al*., [28], with 18.75 percent prevalence in sheep. Another study reported that *listeria* was more prevalent in 10/58 seropositive sheep [29]. In the current study, no significant differences were observed in breed, sex, body health, ticks, diet, water source, mastitis, rearing source, or grazing system, while there were significant differences in different age groups. Animals aged between one and three years had the highest prevalence as compared to other age groups. Furthermore,

a significant difference was observed between females with an abortion history and females without an abortion. Yadav and Roy from India similarly reported *listeria* seroprevalence in sheep [30]. Among sheep, females were more prevalent (25.50%) as compared to males (11.42%). Among 67 animals (45 sheep and 22 goats) tested positive for *listeriosis*, 58 were females (86.5%) and nine were males (13.5%) in a study conducted by [31]. In the case of sheep, the maximum percentage (26.08%) recorded in animals feeding on silage was followed by dry or green mix food (24.35%). Listeriosis is often called "silage disease" [32], being considered a primary source of *listeriosis* [32, 33]. Sheep used different sources of water, and the highest prevalence (26.41%) was recorded in those with ponds as water sources, followed by canal water (25%). Another study also suggested that the main route of *L. monocytogenes* transmission is oral, either through food or water [34]. An investigation on the bacterial etiology of miscarriages in over 203 flocks of sheep and goats revealed 5–6% infections with *L. monocyotgenes*[35]. Ten sheep having an abortion were screened for *listeriosis*, and three were found positive [36]. Clark et aldescribed that mastitis is infrequently documented, while gastro-intestinal illnesses are often encountered in sheep [37]. This disparity in occurrence could be attributed to changes in population density, housing systems, animal husbandry practices, and geographic changes.

In the present study, haemato-biochemical examination revealed that leukocytes, plasma fibrinogen, ALT, and AST levels were enhanced in *listeriosis*, while BUN and creatinine levels were lowered. These findings are consistent with those of a previous study that found elevated levels of leukocytes, AST, and reduced BUN values in bovine *listeriosis*[38]. The minor variations in plasma fibrinogen could be the result of inflammation (bacterial/traumatic/chemical) or cell damage. On the contrary, higher levels of WBCs, RBCs, neutrophils, and eosinophils have been seen in *listeriosis* relative to seronergative livestock in Turkey [39]. The haemato-biochemical study wasn't mandatory for the diagnosis of *listeriosis*, but it can only serve as a guide for fluid infusion [40]. In Brazil, no significant association was discovered between bovine listeriosis and hematobiochemical marker [41]. Such differences in findings could be attributed to varied physiological conditions, infection stages (encephalitis, septicemic, and reproductive), and the extent of infection in ruminants.

Table Legends

Table 1. Results of variables selected for seroprevalence in sheep

Variable	**Variable Levels**	**Number of Animals Screened**	**Positive**	**Negative**	**%age**	**Univariable Analysis**	
						95% CI	**p-value**
	Kajli	45	10	35	22.22	0.314-2.158	0.524
	Thalli	50	11	39	22.00	0.202-2.319	0.481
Breed	Lohi	31	7	24	22.58	0.157-2.621	0.265
	Mundri	15	5	10	33.33	0.1852-2.257	0.489
	Fat tail	35	8	27	22.86	0.127-2.935	0.624
	Sipli	16	3	13	18.75	1	

	Less than 1						
Age	year	23	5	18	21.74	0.0034-0.258	0.000
	1-3 year	97	24	73	24.74	0.126-2.899	0.251
	Above 3 years	72	15	57	20.83	1	
Sex	Male	70	8	62	11.43	0.898-6.841	0.052
	Female	122	36	86	29.51	1	
Body health	Normal	97	20	77	20.62	0.254-1.597	0.158
	Emaciated	30	13	17	43.33	0.142-0.818	0.051
	Thin	65	11	54	16.92	1	
Ticks	Present	52	9	43	17.31	0.114-1.256	0.235
	Absent	140	35	105	25.00	1	
Diet	Dry	9	2	7	22.22	0.219-2.119	0.424
	Silage	23	6	17	26.09	0.293-1.597	0.411
	Green Fodder	82	17	65	20.73	0.248-2.125	0.326
	Dry /Green Mix	78	19	59	24.36	1	
Water Source	Fresh	95	19	76	20.00	0.123-1.254	0.273
	Pond	53	14	39	26.42	0.142-1.398	0.359
	Canal	44	11	33	25.00	1	
Abortion	Yes	10	3	7	30.00	1.024-1.547	0.002
	No	182	41	141	22.53	1	
Mastitis	Yes	12	3	9	25.00	0.751-9.256	0.052
	No	180	41	139	22.78	1	

Rearing	Grazing	130	34	96	26.15	0.164-1.861	0.614
	Confined	15	2	13	13.33	0.131-1.259	0.527
Source							
	Both	47	8	39	17.02	1	
Grazing	With Bovine	13	2	11	15.38	0.149-1.258	0.322
	Alone	105	23	82	21.90	0.216-1.374	0.281
system							
	Mix	74	19	55	25.68	1	
			50				

Table 2.Hematological parameters in seropositive and seronegative sheep

Hematological profile	SI Units	Seropositive	Seronegative	p-value
RBC	x 1012/L	7.44 ± 0.73	7.22 ± 0.69	0.331
WBC	x 109/L	23.55 ± 2.28	17.30 ± 2.10	<0.01**
PCV	%	28.65 ± 3.01	28.55 ± 2.96	0.916
HB	g/dL	99.25 ± 7.25	99.80 ± 6.77	0.805
Lymphocyte	x 109/L	57.00 ± 4.37	56.75 ± 5.428	0.873
Monocyte	x 109/L	0.46 ± 0.05	0.48 ± 0.06	0.902
Eosinophil	x 109/L	0.48 ± 0.07	0.50 ± 0.05	0.892
Neutrophil	x 109/L	6.22 ± 1.69	6.24 ± 1.28	0.958
Plasma Protein	g/L	64.25 ± 5.59	63.75 ± 5.79	0.787
Plasma Fibrinogen	g/L	7.35 ± 0.90	5.77± 0.64	<0.01**

Table 3.Biochemical parameters in seropositive and seronegative sheep

Biochemical Profile	SI Units	Seropositive	Seronegative	p-value
ALT	units/L	51.60 ±5.79	41.70 ±2.96	<0.01**
AST	units/L	291.6 ± 17.94	240.9 ± 33.80	<0.01**
BUN	µmol/L	14.75 ± 4.13	13.25 ± 3.09	0.200
Creatinine	mmol/L	1.48 ± 0.30	1.36 ± 0.23	0.179

5. Conclusion

The current study's findings demonstrated that sheep in several districts of Punjab, Pakistan, were affected by the disease. In the presence of many pathological abnormalities, it is observed that the disease impairs the haemato-biochemical indicators of infected animals, generating physiological distress and liver function challenges in the host. Following the screening of all animals at dairy farms, the adoption of excellent husbandry techniques, segregation, and the elimination of contaminated animals, more focus is needed to build efficient preventative and control strategies. In the interest of public safety, the data from the current study may be useful to dairy farmers and veterinarians.

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Statement of conflict of interest
The authors declare no conflict of interest.

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