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Research Article

Advancing wildlife management: Pioneering non-invasive Urinalysis protocol for captive ungulates in Pakistan

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Abstract

Ungulates held in captivity often face challenges in their health and well-being due to the constraints of their artificial environments. Traditional diagnostic methods, such as blood sampling, can induce stress and discomfort in these animals, potentially affecting the accuracy of results and overall welfare. This study explores the protocol establishment of urinalysis as a stress-free alternative diagnostic technique for assessing health and stress levels in captive ungulates. Ethical considerations were followed throughout the study as samples of captive animals, including Zebra, Mouflon, Punjab Urial, and cattle, were collected from Lahore Zoological Garden, Lahore Safari, and Jallo Forest and Wildlife Park. Urinalysis utilized non-invasive techniques to determine the urinary standard values in ungulates, including the urinary proteins. Results indicated that urinalysis provided reliable indicators of health and stress levels in captive ungulates without causing additional stress. The reference standards for urine proteins, specific gravity, creatinine, and urine protein to creatinine ratios have been provided as 13.39 mg/dL,1.009, 57.89 mg/dL, and 0.23. Bradford Assay revealed a concentration of proteins in cattle, Punjab Urial, Mouflons, and zebra with a mean value of 58.56 µg/ml, 40.81 µg/ml, 48.39 µg/ml, and 40.34 µg/ml, respectively. It has been hypothesized that the presence of these concentrations of proteins in captive animals might be responsible for renal dysfunction. Furthermore, urinalysis may be an effective instrument for assessing renal activity and alterations in physiological conditions in ungulates.

Keywords: Non-invasive, Bradford Assay, Lahore Zoological Garden, captivity, Punjab Urial

Introduction

Pakistan is a country that contains an extensive array of plants and animals, with distinct species living in different types of environments, such as fertile plains and high mountains. Nevertheless, this variety of living organisms faces severe challenges, including habitat degradation, excessive hunting, and ecological imbalance. Globally, conservationists are combining resources to counteract the decrease in species and ecosystems, emphasizing the pressing requirement for inventive methods to record and oversee biodiversity (Molur, 2003). Ungulates in Pakistan symbolize resilience and adaptability among the country's varied wildlife. There are 19 cetartiodactyls, including the endangered Punjab Urial (Vaughan *et al*., 2013). These creatures can be found in many habitats, such as mountains, deserts, plains, and aquatic settings. However, their solitary and often inaccessible lifestyles pose challenges for study and conservation (Feldhamer *et al*., 2020). The Punjab Urial is currently on the brink of endangerment as it confronts various perils, including habitat degradation, poaching, illicit hunting, and parasitic infections (Molur, 2003). Captive breeding has emerged as a vital conservation strategy to combat the decline of these remarkable species. In the face of anthropogenic impacts and the alarming rate of species loss globally, breeding animals in captivity has become a lifeline for endangered species (Ahmed, 2007). However, the confined conditions of breeding facilities challenge these animals' well-being and natural behaviors, necessitating a delicate balance between preservation and ethical considerations (Khattak *et al*., 2021).

Ensuring the viability of captive breeding programs necessitates rigorous attention to animal health. Traditional diagnostic techniques frequently require invasive procedures, which might induce stress and pose possible risks to the confined ungulates (Williams & Hoffman, 2009). Within this framework, urinalysis is a promising and straightforward diagnostic method that can revolutionize how we monitor and protect the well-being of captive ungulates. Urinalysis, examining urine samples, offers a non-intrusive and cost-efficient alternative to conventional diagnostic techniques. This technique has become vital for veterinary medicine in evaluating animals' physiological and metabolic conditions. The importance of urinalysis in regular health examinations is its simplicity, convenience, and capacity to offer renal and systemic well-being data (Milani & Jialal, 2023).

This study seeks to explore the utility of urinalysis as a non-invasive diagnostic tool in captive ungulates, with a specific emphasis on the endangered Punjab Urial species. The primary objectives include establishing a non-invasive urine sampling technique (urinalysis) within captive settings, evaluating the effectiveness of proteinuria as a diagnostic indicator for diseases in ungulates, and

examining the factors influencing ungulates in captivity. Through these aims, the research aims to contribute valuable insights into the health monitoring and overall well-being of ungulates in captivity, providing a foundation for informed management practices and conservation measures regarding this endangered animal.

Material and methods

Animals and urine collection

The fresh twenty-three urine samples were collected from Lahore Safari, Jallo Forest, and Lahore Zoological Garden. The samples were collected from March 2023 to August 2023. On March 10, 2023, six urine samples from the soil were taken from Lahore Safari animals: four zebra and two mouflons. On May 28, 2023, nine urine samples from Punjab Urial were collected at Jallo Park. On August 10, 2023, four urine samples were collected from Lahore Zoological Garden: 3 Punjab Urial and one zebra. Three fresh free-catch urine samples from cattle (cow and sheep) were also collected to compare the findings of the domestic and wild research (Fig. 1). All experimental procedures done in this study were carried out after acquiring permission from the Bio-ethical Committee of the University of Punjab. The non-invasive sampling in this work enables collecting samples (urine samples) without the need to disturb or contact the animal.

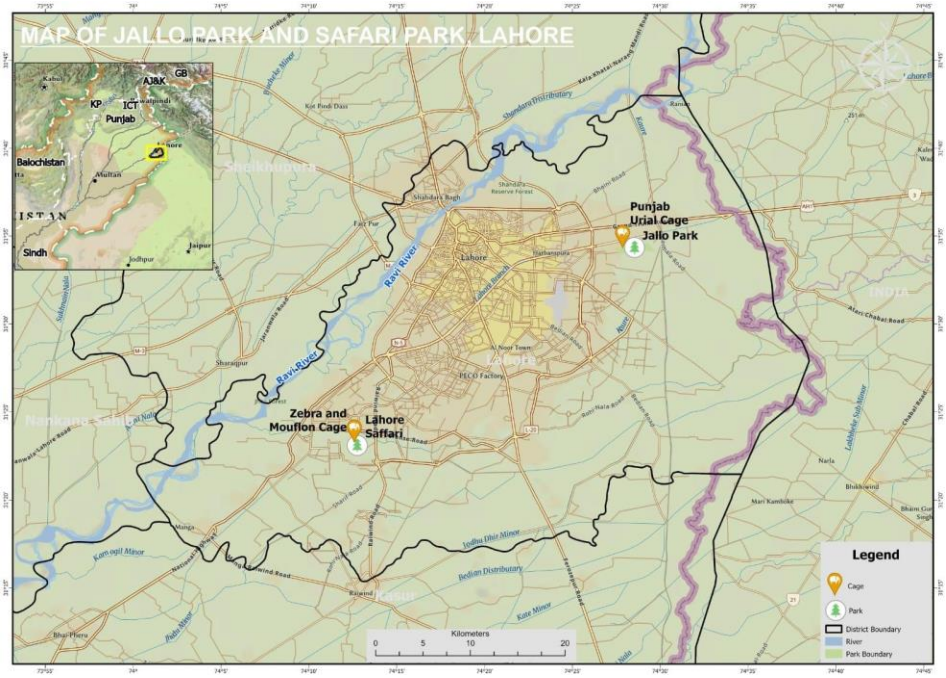

Figure 1. The map shows the location of the Lahore Safari and Jallo Wildlife Park. (Source: WWF-Pakistan)

Assessment of urine analysis and urine creatinine-to-protein ratio:

The urine collection from animals is carried out as part of routine maintenance; therefore, there was no disruption to the animals' administration (Fig. 2). Under standardized settings, the collection was

placed within 5 hours after meals, specifically between 7 and 12 noon. Following spontaneous voiding, a syringe collected 5 milliliters of urine separately from the zoo and safari grounds. The urine supernatant was taken using techniques similar to those in earlier studies (Fasoli *et al*., 2021; Zhu *et al*., 2020). Urine samples were kept in separate containers following the collection and examined independently. Following transfer by syringe into a cup, each sample received physical and chemical investigation, with color and turbidity assessed according to guidelines specific to dogs and cats (Piech & Wycislo, 2019). An analytical dipstick test was conducted to determine the chemical examination and urine-specific gravity using Combostick-10 Urine Strips. The samples underwent centrifugation at 1500 g for 10 minutes. (Ferlizza *et al*., 2015). The urine supernatants were separated and kept at -20 ◦C. The urine samples were sent to the BioMed clinical laboratory (Molecular et al. Center) to evaluate uTP, uCr, and uTPCR. The urine protein to creatinine ratio (uTPCR) was determined using the formula:

$$\textbf{UP: C} \quad \frac{\textbf{Protein concentration (mg/dL)}}{\textbf{Creatinine concentration (mg/dL)}}$$

SDS PAGE

Each sample's protein concentration was assessed using spectrophotometry, explicitly employing Bio-Rad microassay for protein based on the Bradford method (Bonjoch & Tamayo, 2001). The Laemmli approach was followed by a one-dimensional analysis utilizing a 10% SDS-polyacrylamide gel electrophoresis (PAGE) technique (Laemmli, 1970). The polyacrylamide gels were incubated with a Coomassie Brilliant Blue R-250 stain for 15 minutes. Subsequently, the distillation procedure was conducted utilizing a blend of methanol and acetic acid, forming a gel.

Statistical Analysis

A paired comparative plot utilizing TUKEY'S was constructed using OriginPro 2022 to analyze the urinalysis variables for the samples site-wise. The dipstick parameters were examined by calculating the percentages from all samples, and a pie chart was created. A polar heat map with a Dendogram was created, and Pearson Correlation was used to analyze the individual factors.

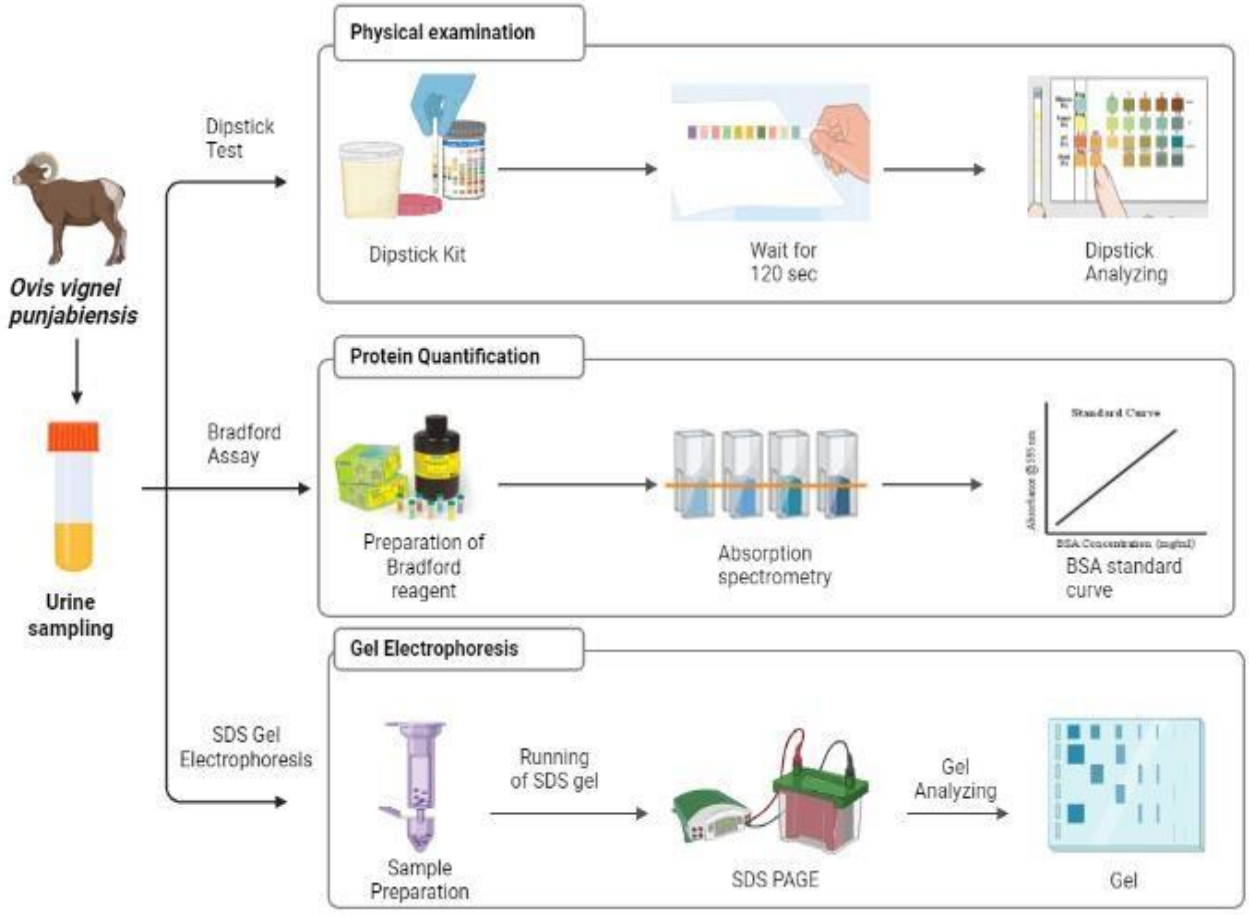

Figure 2. Summary of methodology

Results

Factors affecting ungulates in captivity

Ungulates, including Punjab Urial, Mouflon, and zebras, may experience stress in captivity due to various factors. Inadequate enclosures, such as smaller cages at Jallo Wildlife Park compared to Lahore Safari, contribute to stress, potentially caused by overcrowding and the absence of suitable resting spots. Social interactions are impacted by enclosure size, affecting the ability of animals to establish hierarchies and relationships. Limited space in smaller enclosures leads to higher resource competition and potential aggressiveness. Larger enclosures allow for more physical activity, promoting normal behaviors, while inadequate space may result in animals, like the Punjab Urial, exhibiting anomalous behaviors such as limping. Competition and social dynamics during feeding schedules, influenced by uneven sex distribution, can lead to stress and aggression. Overcrowding increases the risk of disease transmission among ungulates, and the temperature and climate in captivity, especially in Lahore's subtropical conditions, can cause physical discomfort, heat stress, and reproductive challenges, impacting the overall well-being of captive ungulates (Fig. 3).

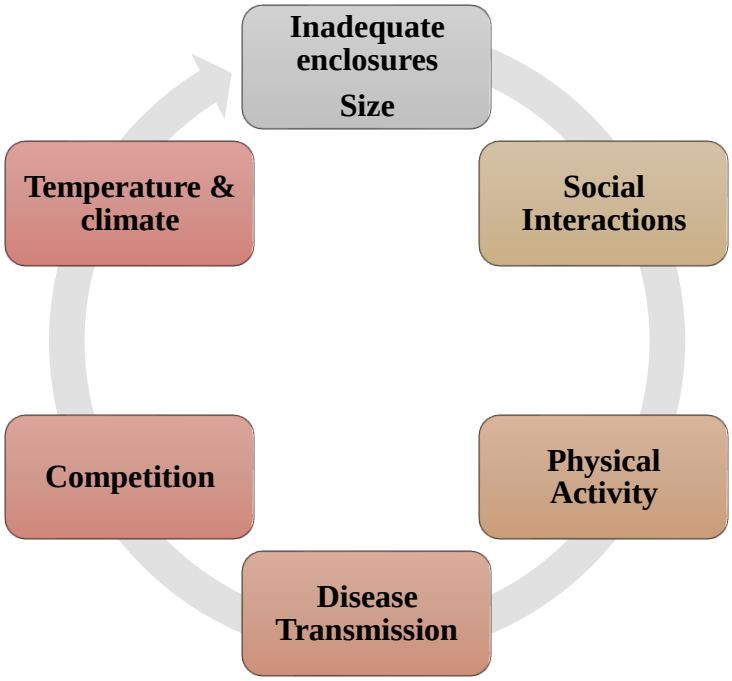

Figure 3. Factors affecting the ungulates in captivity

Dipstick Analysis

A dipstick test, a traditional urinalysis method, was performed on cattle and ungulates from Jallo and Lahore Safari and Lahore Zoo to examine the physicochemical properties of urine samples. The dipstick analysis includes certain components such as Specific Gravity (SG), pH, Protein, Leukocytes, Nitrites, and Blood.

Samples from Lahore Safari

The specific gravity values ranged from 1.005 to 1.025, indicating the concentration of solutes in the urine. MZ1 has the highest SG at 1.025, suggesting a more concentrated urine sample. The urine samples were highly basic with a pH of 9 except pH of MZ1, which was neutral at 7.5. An alkaline pH is relatively common in herbivorous animals (Fig. 3.2-a). The protein values are consistent across all groups, with a value of 30. This suggests the presence of protein in the urine samples. Most groups show negative results for leucocytes. However, FZ2 has a value of 25, suggesting the presence of white blood cells. Most groups showed negative results for blood, indicating the absence of blood in the urine. However, MF2 shows traces of blood, suggesting a small presence. All samples were negative for ketones except for FZ2, where traces of ketones were found. The MZ1, MZ2, and FZ2 were positive for bilirubin and Urobilinogen. Besides these parameters, all samples

were negative for nitrites and glucose. No turbidity was present in all urine samples. However, the color of most of the urine samples was amber except MZ1 and FZ1, which were colorless.

Samples from Jallo Forest and Safari Park

The specific gravity values in urine samples collected from Jallo Forest and Safari Park ranged from 1.010 to 1.020, indicating the concentration of solutes in the urine. F1 has the highest SG at 1.020, suggesting a more concentrated urine sample. The urine samples were highly basic with a pH of 8 except for the pH of M2, which was slightly acidic 6 (Fig. 4-b). The protein values are consistent across all groups, with a value of 30. This suggests the presence of protein in the urinary samples. The nitrite levels in F2, F3, and F4 were positive, indicating microbial urinary tract infections. All samples had blood traces, and hemolysis was present in the urine except for F1. All samples were negative for ketones, glucose, leukocytes, bilirubin, and Urobilinogen. F3 and F4 urine samples were turbid. The samples gathered from Jallo Park revealed a variety of colors. M5 and F1 had straw-colored urine, F2 had amber urine, and F3 and F4 had brown urine.

Samples of Cattle

The dipstick analysis of urine samples from cattle is shown in (Fig. 4-c). Specific gravity ranged from 1.005 to 1.025, indicating the concentration of solutes in urine. The protein values were consistent in all three samples with +30. The pH of all urine was alkaline 8. Traces of ketones and urobilinogen were found in C1. All samples showed negative leukocytes, glucose, nitrites, bilirubin, and blood results. There was no turbidity in urine samples. Samples C1 and S1 were colorless, while S2 was yellow, indicating healthy urine.

Samples of Lahore Zoo

The dipstick analysis of urine samples from cattle is shown in (Fig. 3.2-d). The S.G values ranged from 1.005 to 1.020, indicating the concentration of solutes in urine. The protein values were consistent in all three samples with +30. The pH of all urine was alkaline 9. Traces of ketones were found in ZM3 and ZZ4. Traces of urobilinogen were found in ZM1. All samples showed negative leukocytes, glucose, nitrites, bilirubin, and blood results. There was no turbidity in urine samples. All samples were yellow, indicating healthy urine.

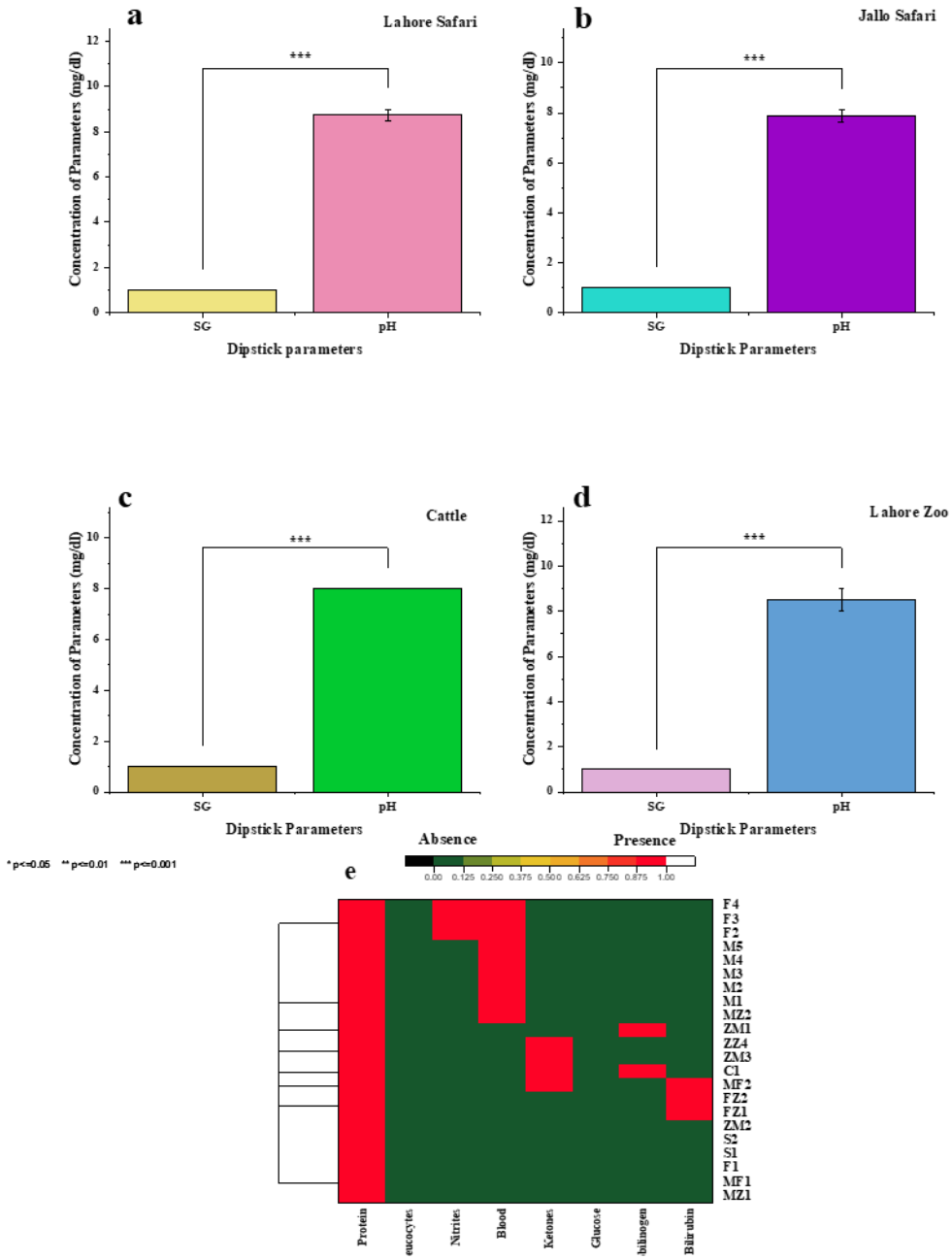

Figure 4. (a-d) Paired comparison plot with Tukey's test on specific gravity and pH of urine samples collected from Lahore Safari, Jallo Safari, Cattle, and Lahore Zoo. Asterisks showed a significant difference among the displayed parameters. (e) Heat map showing the presence (red) and absence (green) of other dipstick parameters (Protein etc) in all urine samples taken from different sites. The dendrogram showed the correlation between the samples. On the Y-axis, the sample key is displayed.

Urine Protein to Creatinine Ratio

UPCR of samples taken from Lahore Safari is given in (figure 5-a). The total urine protein raged from 6.85 to 15.6 mg/dl. The maximum creatinine was present in the urine of MZ2 and the minimum

was present in MF1. Its value ranged from 3.6 to 195.9 mg/dl. The urine Protein- Creatinine ratio was maximum in MF1 and minimum in MZ2. The total urine protein of samples taken from Jallo Safari was 8.45 to 14 mg/dl (figure 5-b). The maximum creatinine was present in the urine of F1 and the minimum was present in M4. Its value ranged from 90.4 to 0.03 mg/dl. The urine Protein-Creatinine ratio was maximum in M5 and minimum in F1. The maximum total urine protein of cattle samples was present in C1 (33.8 mg/dl) and the minimum in S2 (15.0 mg/dl) (figure 5-c). The maximum creatinine was present in the C1 and the minimum in S2. Its value ranged from 195.83 to 155.6 mg/dl. The urine Protein- Creatinine ratio was maximum in C1 and minimum in S2. The maximum total urine protein of samples taken from Lahore Zoo was present in ZZ4 (87 mg/dl) and the minimum in ZM1 (10.0 mg/dl) (figure 5-d). The maximum creatinine was present in the ZZ4 and minimum was present ZM1. Its value ranged from 313.7 to 147.5mg/dl. The urine Protein-Creatinine ratio was maximum in C1 and minimum in S2. The higher the creatinine value the lower the UPCR.

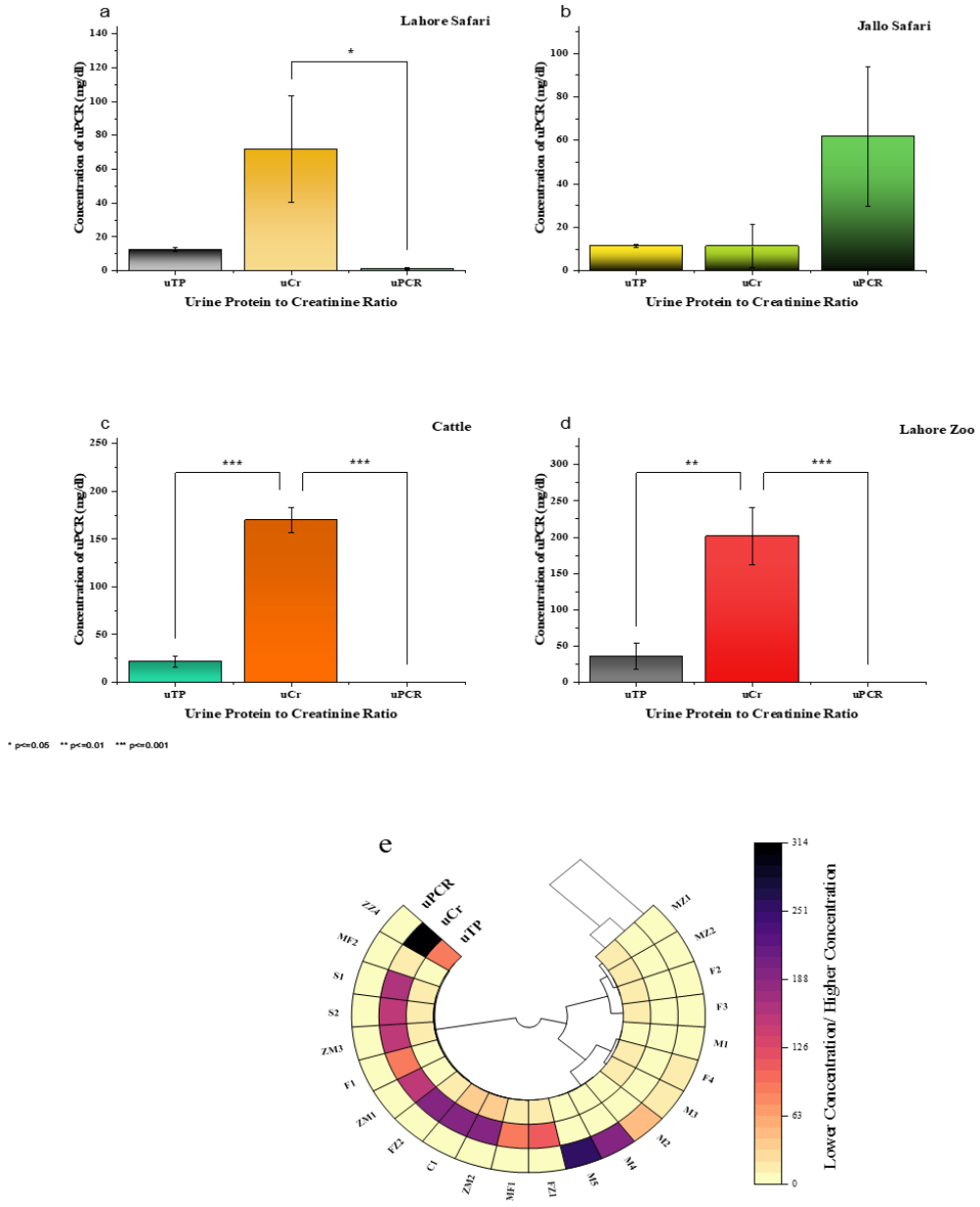

Figure 5. (a-d) A paired comparison plot utilizing the Tukey test on urine total proteins (uTP), urinary creatinine (uCr), and the urine protein to creatinine proportion (uPCR) from samples collected at Lahore Safari, Jallo Safari, Cattle, and Lahore Zoo, respectively. Asterisks indicated a substantial difference among the presented factors. (e) Polar Heat map showing the concentration of uTP, uCr, and uPCR (light green higher concentration and red lower concentration) in all urine samples taken from different sites. The dendrogram showed the correlation between the samples. Labels at outer angular orbit displayed samples key.

SDS PAGE

The urine sample showed a pattern of common protein bands that could be identified using SDS-PAGE. Only samples with a high urine-to-creatinine ratio were analyzed using SDS gel; not all samples underwent this procedure. The range for protein bands was 1 to 4. The predominant protein bands in most samples had apparent molecular masses of 32, 37, and 42 kDa. The band with lower molecular masses (14, 10, and 11 kDa) was also observed in certain studied specimens. Conversely, the band with a molecular mass above 64 kDa was observed in just a subset of samples (Figure 6).

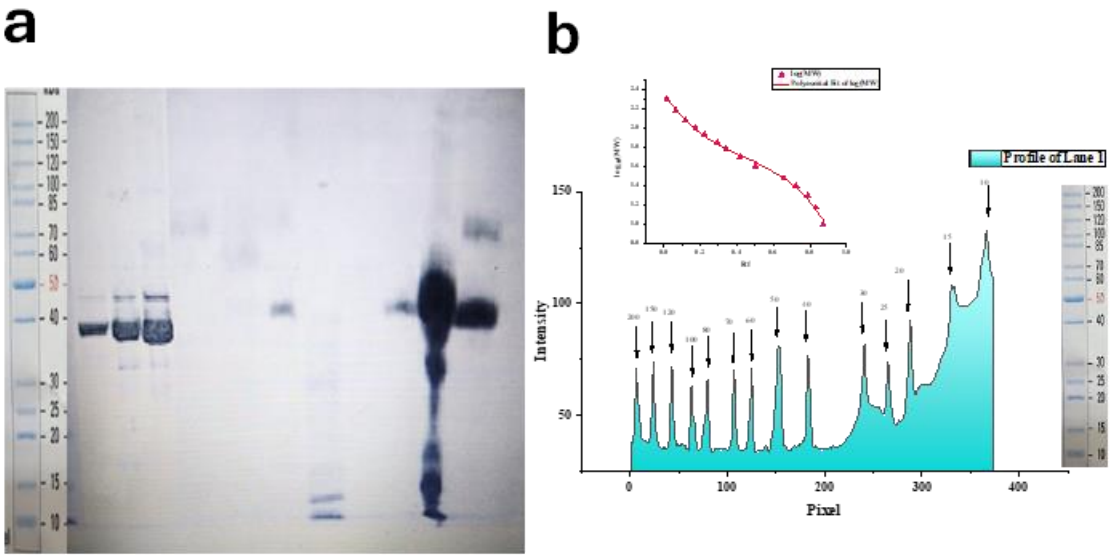

Figure 6. (a) SDS gel electrophoresis: Molecular weight indicators (left) and bands (right), (b) representative phenogram.

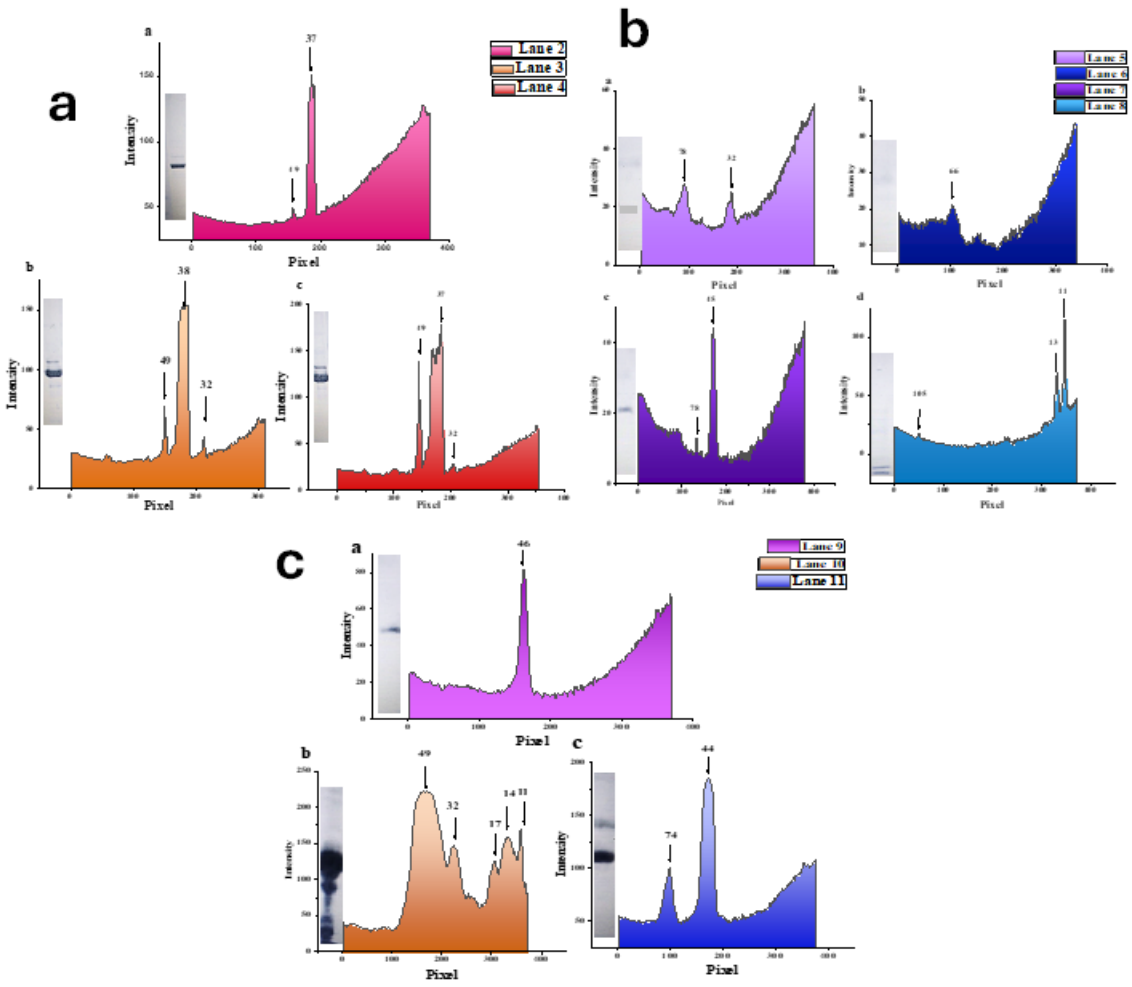

Figure 7. A phenogram with bands of urine samples in some studied animals, Samples from Cattle; a: S1 (49, 37 kDa), b: S2 (49, 38, 32 kDa), and c: C1 (49, 37, 32 kDa); Samples of Zebra; a: MZ1 (78, 32 kDa), b: FZ1 (66 kDa), c: MZ2 (78, 45 kDa) and d: FZ2 (105, 13, 11 kDa); Samples of Punjab Urial; a: M2 (46 kDa), b: F3 (49, 32, 17, 14, 11 kDa) c: F4 (74, 44 kDa)

Discussion

Research on felids in zoos has expanded from a focus on taxonomy and anatomy to include ethology, genetics, physiology, and pathophysiology, focusing on protecting wild felids (Hosey *et al*., 2013). The welfare of animals in zoos, emphasizing the reduction of human-induced stress, is protected by national laws that require veterinarians to conduct clinical evaluations. Evaluating the health of zoo animals poses difficulties, thus highlighting the importance of non-invasive techniques such as urinalysis. Urine samples, easily collected from cage floors or snow, offer insight into endocrine, reproductive functions, and overall health (Bechert, 2011). Urinalysis is crucial in monitoring the health of captive ungulates as it provides valuable information about metabolic conditions, early detection of diseases, kidney function, and hydration levels without causing stress. The reliability of urinalysis results in captive ungulates, which relies on developing standardized

protocols and training for personnel. Obtaining consistent and representative samples can be challenging, particularly in large enclosures. A *urinalysis* is a valuable tool that helps make informed decisions and provides optimal care for captive ungulates. Urinalysis can be conducted using a dipstick test, while protein quantification can be achieved using UP: UC and the Bradford Assay. The current study's analysis of urine, both physically and chemically, confirmed findings from previous scientific literature. The alkaline pH, which has been found in cows (Ferlizza et al., 2020; Herman et al., 2019) and giraffes (Fasoli et al., 2021; Sullivan et al., 2010), was specifically confirmed, and the presence of proteins indicated by positive results on the dipstick test. These protein levels could be attributed to the alkaline pH of the urine and the method of sample collection (Jones *et al*., 2012; Sink & Weinstein, 2012). However, it is recommended to employ a quantitative analytical rather than a semi-quantitative method to ascertain urine total proteins, given the significant prevalence of false positive results in the dipstick test.

The USG results in the study were within the usual range of specific gravity, which is 1.005 to 1.025. Clinically healthy goats have been found to have low USG values, such as 1.003 (Belknap & Pugh, 2002). The USG values for Punjab Urail, sheep, and cows were lower than those reported in domestic animals, such as cows (E Ferlizza *et al*., 2020), sheep, and goats. The USG values in zebra were also lower than reported in captive rhinoceros (Haffey *et al*., 2008) and horses (Yuan *et al*., 2017). Considering that water consumption and urine concentration might vary depending on exercise, nutrition, etc., healthy animals can have a wide range of USG (1.001-1.065). However, the results of this variable should be interpreted carefully, and USG should be evaluated repeatedly since a single sample is not indicative of urine concentrating ability. Some bacteria can convert nitrate to nitrite. Although nitrite test pads are available on some dipsticks, nitrite is not a reliable marker of bacteriuria in small animals (Reine & Langston, 2005). In our study, samples of female Punjab Urals from Jallo Park tested positively for nitrites, indicating a bacterial disease.

In our investigation (MF2 and C1), 11% of samples showed ketone traces (15 mg/dL), aligning with findings by (Ferlizza *et al*., 2020). Unlike captive rhinoceros studies (Haffey *et al*., 2008), cows and heifers exhibited ketone traces. Acetoaetic acid, acetone, and β-hydroxybutyric acid, originating from fatty acid metabolism, are primary ketones in small mammals. Ketonuria's common causes include poorly controlled diabetes, malnutrition, high-protein/low-carb diet, and certain medications (Reine & Langston, 2005). In Jallo Park samples, 50% exhibited hematuria, possibly indicating upper (kidney, ureter) or lower (urethra, prostate, vagina, penis, bladder) urinary system diseases. Nitrites in F2, F3, and F4 suggest bacterial infection, common in parasitic infections among wild ungulates, with elevated color, pH, and USG values.

Degraded hemoglobin yields bilirubin, excreted in bile. Lahore Safari samples (FZ1, FZ2, MF2) showed bilirubin traces without blood, suggesting cirrhosis or liver diseases. Urobilinogen, a common finding, was in only 5% of C1 samples, poorly correlating with hepatobiliary disease (Reine & Langston, 2005).In our study, protein traces (30mg/dL) were found in all samples. Positivity to proteins (30-100 mg/dL) was also recorded in (E Ferlizza *et al*., 2020; Haffey *et al*., 2008). Proteinuria can be estimated semi-quantitatively using urine dipsticks. Most urine protein is albumin, which is more sensitive to the color indicator (tetra bromophenol blue) than globulins. Results from urine protein analysis must always be considered in conjunction with specific gravity. Therefore, quantitative proteinuria testing is suggested since the animals' alkaline urine pH may have produced false positive results (Herman et al., 2019). However, the presence of proteins in bovine urine has been documented in prior research (Herman *et al*., 2019; Ihedioha *et al*., 2019; Mokbul *et al*., 2016). The conventional first-line screening test for proteinuria-albuminuria is a urine dipstick colorimetric test. Proteinuria found by urine dipstick is thought to be of renal origin and is frequently confirmed and quantified by the UTP: C (Lyon *et al*., 2010).

Limited information is available on the use of urinary proteomics in captive animals. However, urine standard values and identifying the predominant proteins in captive giraffes were documented by Fasoli et al. (2020). Based on data already published for domestic animals, the study revealed standard values for uTP, uCr, and UPC in giraffes. The research also found that giraffe urine did not contain many total proteins, just like the urine of other healthy ruminants (Fasoli et al., 2020). The standard levels for urine total proteins (uTP), urine creatinine (Cr), and urine protein: creatinine ratio (UPC) were 17.58 (4.54–35.31) mg/dL for uTP, 154.62 (39.59–357.95) mg/dL for Cr, and 0.11 (0.07–0.16) mg/dL for UPC (Fasoli et al., 2020). In our study, C1 exhibited the greatest urinary total protein (uTP) level at 33.8 mg/dL, while MF2 demonstrated the lowest at 6.85 mg/dL. The highest and lowest uCr concentrations were found in FZ2 (195.9 mg/dL and 0.03 mg/dL, respectively). The UPC levels were highest in MF5 (261.6 mg/dL) and lowest in FZ2 (0.07 mg/dL). Our investigation showed a positive correlation between urine creatinine levels and urine total protein. In our study, the average values for urine total proteins (uTP), urine creatinine (uCr), and urine protein: creatinine ratio (UPC) were 13.39 mg/dL, 57.89 mg/dL, and 0.23 mg/dL, respectively. The animals included in our study might be classified as proteinuric because these levels were much higher than those reported in cows (E Ferlizza et al., 2020) as well as in giraffes (Fasoli et al., 2020). In our study elevated UCr findings may be due to a USG-creatinine urine concentration association. According to prior research (Herman et al., 2019), the USG value can be influenced by the creatinine concentration. Dehydration, diabetes, and excessive muscular tone are all indicated by an increase

in urine creatinine levels above normal levels. The UP: UC ratio changes when the creatinine value changes. High protein levels in the urine could be a sign of an infection, blood, damaged red blood cells, muscle proteins, or albumin. The presence of protein may occasionally be a sign of a problem with the kidney's glomerulus (the filtering system), which results in protein loss from the body into the urine. (Enea Ferlizza *et al*., 2020). The results obtained from SDS gel electrophoresis reveal distinct protein profiles in samples from different species. S1 exhibits bands at 49 and 37 kDa in cattle samples, while S2 displays bands at 49, 38, and 32 kDa. Similarly, bands at 78 and 45 kDa for MZ2 in zebra samples, bands at 105, 13, and 11 kDa for FZ2, and bands at 66 kDa for MZ1. M2 is 46 kDa, F3 is 49, 32, 17, 14, and 11 kDa, and F4 is 74 and 44 kDa; the protein bands in the Punjab Ural samples were different.

The observed molecular weights suggest the presence of specific proteins in each sample. For instance, in cattle, the 49 kDa band may correspond to a particular protein, and in zebra samples, the 78 kDa band could represent another distinct protein. The variability in molecular weights across species highlights the diversity of proteins present, possibly reflecting species-specific physiological or functional differences. Further protein identification techniques, such as mass spectrometry, would be essential for more precisely characterizing the proteins associated with these molecular weights. Both wild and captive ungulates are susceptible to various diseases, including zoonotic ones like Q fever, salmonellosis, chlamydiosis, leptospirosis, and campylobacteriosis in largehorn sheep and wild cervids (Ramanzin *et al*., 2010). Chronic wasting disease (CWD) and bovine tuberculosis (TB) are notable diseases found in both wild and captive ungulates (Ramanujam & Palaniyandi, 2023). A European survey on disease transmission between wild and domestic ungulates revealed the susceptibility of wild ungulates to various infectious agents (Pires *et al*., 2023). However, information on disease prevalence in captive ungulates is limited. Implementing practical diagnostic approaches benefits individual animals and supports broader conservation and management efforts, ensuring the health and sustainability of ungulate populations in captivity.

Conclusion

Urinalysis, a non-invasive diagnostic method, should be frequently employed for large animal clinical assessments. Recognizing urine abnormalities necessitates a thorough comprehension of the characteristics of normal urine. This study introduced the first standards for measuring urine chemistry in ungulates, especially in the Punjab Urial. The data presented in this study may indicate captive animals and confirm findings already published in the literature. The Punjab Urial exhibited greater stress levels than the zebra and Mouflon, as validated by urine total protein analysis, Bradford Assay, and SDS PAGE. It might be due to small enclosures, overcrowding, insufficient

diet, and competition. Nonetheless, additional research is necessary to enhance understanding of the ungulate urine proteome.

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