



RESEARCH ARTICLE

Clinico-Hemato-Biochemical and Molecular Diagnostic Investigations of Peste des Petits Ruminants in Goats

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ARTICLE HISTORY (19-430)

Received: September 19, 2019
Revised: December 22, 2019
Accepted: December 28, 2019
Published online: February 03, 2020

Key words:

Enteritis
Goats
Lymphopenia
Pakistan
Peste des petits ruminants virus
Pneumonia
RT-PCR

ABSTRACT

Peste des petits ruminants (PPR) is an acute, infectious and devastating disease of small ruminants, especially for goats. Recently, an outbreak of PPR occurred at a goat farm in Nankana District, Punjab province, Pakistan with 100% (n=105) morbidity and 24% (25/105) mortality. The goats showed characteristic signs of PPR including high temperature, oculo-nasal discharges, diarrhea and ulcerative lesions in the oral cavity. The clinical signs, pathological lesions, hematological values, and serum biochemistry were studied. On postmortem examination, severe pneumonia and enteritis were observed in infected animals. There was marked lymphopenia, decreased erythrocytes level with increased mean corpuscular hemoglobin volume (MCV). The release of albumin and pus cells in urine indicated the kidney damage. Clinical outcome, gross lesions, and histopathological findings were suggestive of Peste des petits ruminants virus (PPRV) infection, which was confirmed by the application of antigenically conserved N gene-based RT-PCR. More than 70% of clinically infected animals were found positive for PPR virus (PPRV) using the RT-PCR. Further investigations carried out to understand the phylogenetic relationship revealed lineage IV PPR viruses involved in the outbreak having more than 90% similarity with isolates previously reported from Pakistan. Pakistan is still in the endemic state for PPR as various outbreaks have been reported from various regions of the country. Regular monitoring of PPR disease and viruses spread are essential for the implementation of appropriate control actions and to know the risk assessment.

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To Cite This Article: Khaliq SA, Mohiuddin M, Habib M, Hussain R, Abbas M, Du X, Nasir AA, Din AMU, Khan A and Bayi J, 2020. Clinico-hemato-biochemical and molecular diagnostic investigations of Peste des petits ruminants in goats. Pak Vet J. <http://dx.doi.org/10.29261/pakvetj/2020.013>

INTRODUCTION

Peste des petits ruminants (PPR) is an acute, and transmissible infectious disease of small ruminants, mostly of goats and less commonly of sheep caused by morbilliviruses of family paramyxoviridae (Latif *et al.*, 2018). Infected animals usually exhibit severe pyrexia, conjunctivitis, oculonasal discharge, stomatitis, enteritis,

diarrhea and pneumonia followed by either death or recovery of the animal (Lefèvre *et al.* 1991). PPR is included in List-A diseases of the OIE and is endemic in Pakistan where half of the small ruminant population (48.3-48.5%) is seropositive (Abubakar *et al.*, 2017). Many PPR outbreaks have been reported from various parts of the country, but only a few have been documented and the diagnosis is mostly based on clinic-epidemiological observations (Khan *et al.*, 2018). It causes great economic losses as the morbidity rate in

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severe cases may reach up to 100% and the mortality rate may increase 50-90% (Ali *et al.*, 2013). The PPR is prevalent in Africa, the Middle East, and Asia (Hailat *et al.*, 2018; Souley *et al.*, 2019). Recently, it has been estimated that 63% population of small ruminants in Southern Africa, Asia, and Europe is at risk of PPR as determined by the Food and Agriculture Organization (Libeau *et al.*, 2014).

The PPR virus (PPRV) is lymphotropic as well as epitheliotropic that induces characteristic lesions in organs have plenty of lymphoid and epithelial tissues. The PPRV enters the body via the respiratory system and replicates in the regional lymph nodes as well as tonsils (Truong *et al.*, 2014). Before the appearance of the clinical sign, usually, viremia develops 2-3 days post-infection, then virus disseminates to the mucosa of the gastrointestinal tract and the respiratory system as well as spleen and bone marrow (Scott, 1990).

In the recent few years, there are more outbreaks of PPR in Pakistan reporting higher morbidity and mortality particularly in goats (Khan *et al.*, 2018). For the initiation of proper disease control strategies, it is important to understand the genetic relationship of PPRVs isolated or prevailing in various regions of the world. Therefore, after observing signs and symptoms of infected animals, laboratory investigations were carried out. For genetic characterization, a worldwide standard diagnostic test such as RT-PCR was carried out using the nucleocapsid (N) protein gene (Niamat *et al.*, 2019). The sequencing and phylogenetic analysis were carried out based on the N gene to understand the genetic relatedness of the viruses isolated from Pakistan and also with those from other parts of the world.

MATERIALS AND METHODS

Description of the outbreak: In this study, the PPR disease outbreak in a goat farm at district Nankana (Pakistan) was studied. The farm consisted of 105 male goats from six months to one-year age, weighing 30-50 kg and reared for the transitory period to be sold for slaughter at Eid-ul-Azha. The animals were purchased from Dera Ghazi Khan (n=85) and Bahawalnagar (n=20) districts. After 3 days of the introduction of 20 animals from Bahawalnagar district, the outbreak started. Up to the 5th day of the start of the disease, 25 animals (24%) died.

Animals monitoring, Necropsy, and histopathology:

All the animals were monitored for clinical signs and rectal temperature noted. Postmortem of eight freshly dead goats was performed. Gross lesions were observed, and morbid organs were collected for histopathology (Khan *et al.*, 2018) and virus isolation. Collected organs were placed for fixation in 10% neutral buffered formalin. Tissues were then washed, dehydrated in ascending grades of alcohol, cleared in xylene, impregnated, and embedded in paraffin tissues. Sections of 4-5µm thick were cut and stained with H & E staining techniques. The urine samples were also collected for analysis.

Hemato-biochemistry parameters: Blood samples with anticoagulant (EDTA) were collected from the diseased (n=10) and healthy animals (n=5). The complete blood

count, hemoglobin (Hb) concentration, MCV, mean corpuscular hemoglobin concentration (MCHC) and platelet counts were determined following the standard procedures (Barger and Macneill, 2015). Similarly, blood samples without anticoagulant were also collected from diseased and healthy animals. These samples were subjected to serum extraction and stored at -20°C until analysis. Sera were subjected to biochemical analysis including phosphorous (P), sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), uric acid, bilirubin, creatinine, Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) by using the automatic analyzer. Urine samples were also checked for proteinuria.

Molecular diagnosis of PPRV by RT-PCR: Total RNA was extracted from the samples collected from goats using GF-1 Viral Nucleic Acid Extraction Kit (Vivantis Technologies, Malaysia) following the instructions provided by the manufacturer. Briefly, total RNA was extracted in elution buffer. Complementary DNA (cDNA) was synthesized using the Fermentas Revert Aid First Strand cDNA kit. The RNA (5µL) from suspected clinical specimens was heated at 65°C for 5 min with 1µL of a random hexanucleotide mix (0.2µg/µL conc.), chilled on ice and reverse transcribed at 42°C for 1 hr in a 20 µL reaction mixture containing 5 µL of 5X RT buffer, 1µL of Moloney-murine leukemia virus (M-MuLV), reverse transcriptase (200 U), 1µL RNase inhibitor (20U) and 2µL of 10mM dNTP mix (Tiwari, 2004; Niamat *et al.*, 2019).

Using primer pair NP3: (TCTCGGAAATCGCCT CACAGACTG); NP4: (CCTCCTCTGGTCTCCAG AATCT) and Taq polymerase (Thermo Scientific, Cat # EP0402), a conventional PCR was carried out with a pre-denaturation at 95°C for 4 min and 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, followed by 72°C for 10 min. PCR product was electrophoresed on 1% agarose gel and stained with ethidium bromide. The correct amplification product was shown as a DNA band of about 353bp with NP primer pair (Couacy-Hymann *et al.*, 2002; Khan *et al.*, 2018). The resulting PCR products were purified using the gel extraction kit (Biobasic, USA) and sent for sequencing to Macrogen Inc. (Korea) for PCR confirmation and sequence fidelity. For the determination of virus lineage, the nucleic acid sequence of a 353bp segment of the N gene was aligned with reference PPR virus sequences available in the NCBI database. The neighbor-joining method was used to determine phylogenetic relationships with 1000 bootstrap replications. The student's t-test was used for comparison of hemato-biochemical parameters among infected and healthy ones (Sullivan *et al.*, 2009).

RESULTS

Clinical signs and necropsy findings: Morbidity 100% and mortality 24% (25/105) were recorded. Affected animals showed high body temperature (102.4-105.4°F) which remained for 3-5 days. Affected animals showed watery diarrhea that may or may not be blood-stained. Affected animals were emaciated, anorexic, depressed with a dull coat, and had a dry muzzle and sunken eyeballs (Fig. 1a). There were nasal discharge and erosive

stomatitis, involving the dorsum of the tongue and oral mucosa. Grossly, ulcers were observed in the oral cavity, with sloughing of the mucosal layer as pseudo-membrane (Fig. 1b and 1c). PPR affected goats showed watery discharge from the eyes, typically reflecting catarrhal conjunctivitis (Fig. 1b). Sick goats were suffering from respiratory distress in the form of rales and abdominal breathing. The animals were in sternal recumbency in later stages with respiratory grunting sounds.

At necropsy, carcasses were emaciated. Abomasum was congested with multiple mucosal hemorrhages (Fig. 1d). The duodenum mucosa was reddish to dark in color; jejunum and ileum exhibited small streaks of hemorrhages. Large intestines were more severely affected with zebra stripes on the mucosal folds of the posterior part of the colon. The size of the mesenteric lymph nodes was increased with hyperemia and hemorrhages seen from the outer surface (Fig. 1e). The liver and kidneys were enlarged and congested. The cardiac lobes of lungs were congested (Fig. 1f) while the trachea was full of froth and petechial hemorrhages. The heart was found normal.

Histopathological findings: Microscopically, PPR lesions were most characteristics in the intestines, lungs, and lymphoid organs. The intestinal villi exhibited blunting/stunting and fusion (Fig. 2). Tips of the villi were mostly necrosed, sloughed off and present in the intestinal lumen (Fig. 2a-2c). Infiltrating in the lamina propria were mostly mononuclear cells along with very few neutrophils (Fig. 2a). Degenerative and necrotic changes were seen in glandular epithelial cells.

Destruction of lymphoid follicles was partial to complete in the Peyer's patches. The white pulp of the spleen exhibited extensive depletion of lymphocytes. Similar lesions were present in mesenteric and retropharyngeal lymph nodes and tonsils. Coagulative necrosis and vacuolation of hepatocytes and congestion were seen in the liver at so many places.

Histopathological lungs were severely involved showing interstitial, broncho-interstitial, or bronchopneumonia. The bronchiolar epithelium was sloughed off and lumen of bronchioles was full of cell debris that was suggestive of bronchopneumonia (Fig. 3a). The alveoli were filled with edema fluid that was mixed with fibrin, alveolar macrophages, and neutrophils (Fig.

3b). Inter-alveolar septa (Fig. 3a) was thickened indicative of interstitial pneumonia. Mononuclear cell infiltration in the alveoli and increased population of histiocytes were obvious. Intranuclear eosinophilic inclusion bodies were present in the alveolar lumen as well as walls.

Hematological findings: At hematological levels, all the erythrocytic, leukocytic and platelet indices in clinically affected and healthy goats differed significantly ($P < 0.05$) except MCHC which did not differ significantly. Among erythrocytic indices, red blood cell count and hemoglobin quantity were significantly decreased while hematocrit and MCV were significantly increased in PPR affected goats (Table 1). All the leukocytic indices decreased significantly in PPR affected goats. Platelet counts and platelet distribution width significantly decreased while the mean platelet volume significantly increased in PPR affected goats.

Biochemical findings: Serum total proteins, albumin, and glucose were decreased significantly ($P < 0.05$) while cholesterol, uric acid, creatinine, bilirubin, and alanine aminotransferase increased significantly ($P < 0.05$) in PPR affected goats (Table 2). Non-significant difference was found in the serum concentration of calcium, magnesium, sodium, potassium, triglyceride and aspartate aminotransferase in PPR affected goats. On urine examination, albuminuria was observed in infected goats.

RT-PCR based detection of PPRV in clinically infected animals: RT-PCR was applied to detect the PPRV target genome in a total of 25 samples collected from clinically PPR infected animals with primer pairs NP3. 18 samples were found positive and produced a DNA band of about 353 bp with NP3 and NP4 primer pair (Fig. 4).

Sequence analysis of the partial nucleoprotein gene of PPRV: Nucleotide-nucleotide pairwise comparison of PPRV NP partial genes revealed that the identified gene sequence (Yasmin-Pak-16) was closely related to Pakistani, Saudi, Israeli and Iranian isolates and made a cluster representing Lineage IV viruses (Fig. 5). The homology was 92.6% with Shiraz Isolate, 91.5% with Dhaka isolate, 92.2% with Kurdistan, 93% with each of Pakistani, Saudi and Israeli isolates. Indian isolate was 70% homologous.

Table 1: Erythrocytic, leukocytic and platelets indices (mean \pm SE) of Peste des petits ruminant diseased (n=10) and apparently healthy (n=5) goats

Parameters	Units	PPR Group	Control Group	P value
Erythrocytic indices				
Red Blood Cells	$\times 10^6/\mu\text{L}$	1.80 \pm 0.11	11.21 \pm 1.83	0.006
Hemoglobin	g/dL	7.50 \pm 0.28	11.17 \pm 1.24	0.044
Hematocrit	%	20.2 \pm 0.66	27.58 \pm 1.55	0.004
Mean Corpuscular Volume	fL	114.7 \pm 3.2	19.33 \pm 2.44	0.000
Mean Corpuscular Hemoglobin Concentration	g/dL	39.3 \pm 1.06	34.9 \pm 1.77	0.067
Leukocytic Indices				
White Blood Cells	$\times 10^3/\mu\text{L}$	2.67 \pm 0.46	7.60 \pm 0.31	0.000
Lymphocytes	%	11.8 \pm 1.97	59.6 \pm 2.8	0.000
Neutrophils	%	6.1 \pm 1.54	34.80 \pm 1.63	0.000
Platelets Indices				
Platelets	$\times 10^3/\mu\text{L}$	1.14 \pm 0.07	435.4 \pm 25.7	0.000
Platelet Distribution Width	fL	10.2 \pm 0.24	19.12 \pm 0.63	0.000
Mean Platelet Volume	fL	14.09 \pm 0.18	10.10 \pm 0.49	0.000
Platelet Large Cell Ratio	%	58.10 \pm 2.82	—	—

Data analysis by student's t-test.

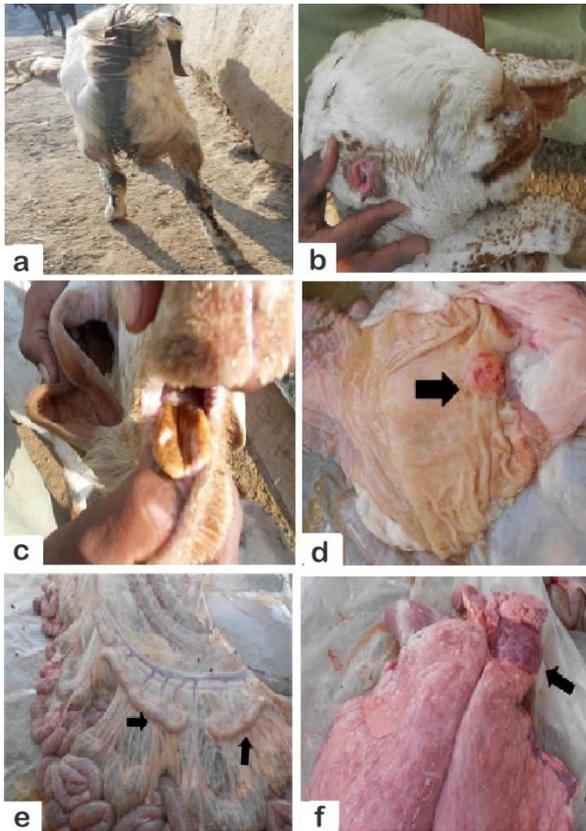


Fig. 1: Gross lesions in various organs of goats suffering from Peste des petits ruminant disease showing a) soiling of hindquarter due to watery diarrhea, b) oculonasal discharge and sunken eyes, c) severe erosive stomatitis, d) congested abomasum mucosal surface, e) hyperemic and hemorrhagic mesenteric lymph nodes and f) congested cardiac lobes of lungs.

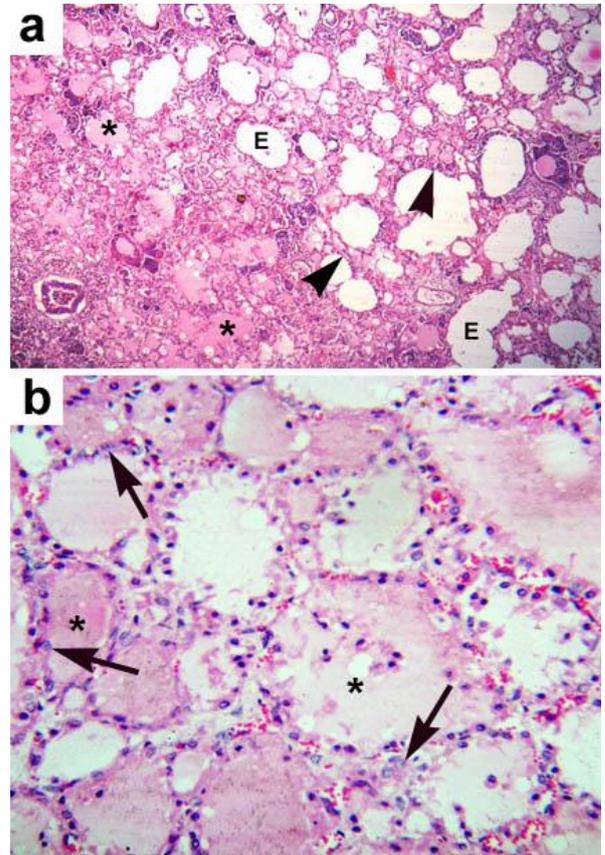


Fig. 3: Photomicrographs of lungs of a goat died of Peste des petits ruminant showing filled alveoli with serofibrinous edema fluid (a & b; asterisks), alveolar macrophages and moderate numbers of neutrophilic leukocytes (a & b); thickened alveolar septa and emphysema (a; arrowheads) and intranuclear eosinophilic inclusion bodies in alveolar macrophages (b; arrows). H and E Stain. a = X 100; b = X 400.

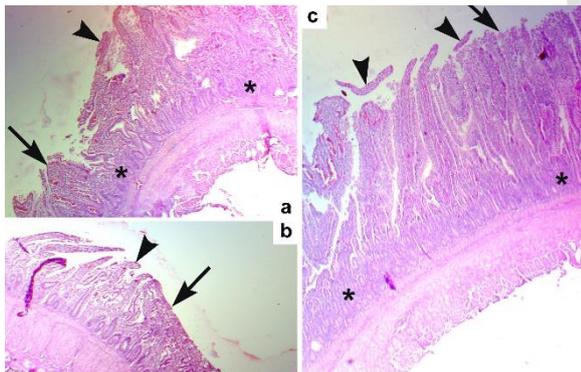


Fig. 2a-2c: Photomicrographs of intestines of a goat died of Peste des petits ruminant showing stunting and fusion of villi (arrow). Tips of the villi are necrosed and sloughed off (arrowheads). Infiltration of mononuclear cells along with a few neutrophilic leukocytes in the lamina propria are evident (asterisk). H and E Stain. X 100.

DISCUSSION

The present study reported the investigation of an outbreak of PPR at Nankana district of Pakistan. The animals are mostly reared for slaughtering at the festival (Eid-ul-Azha) and there is a large scale shifting of the animals from the countryside to big cities, where there is a possibility of direct contact of animals to animals resulting in the spread of disease to healthy animals (Latif *et al.*, 2018). During the outbreak, the disease caused high mortality (24%) with respiratory signs and diarrhea.

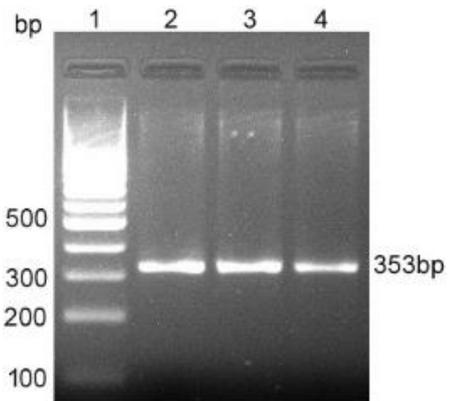


Fig. 4: Agarose gel electrophoresis (1.5%) of RT-PCR product of Peste des petits ruminant virus. Lane 1: DNA ladder; Lanes 2-4: PCR product of 353 bp obtained with primer pair NP3/NP4.

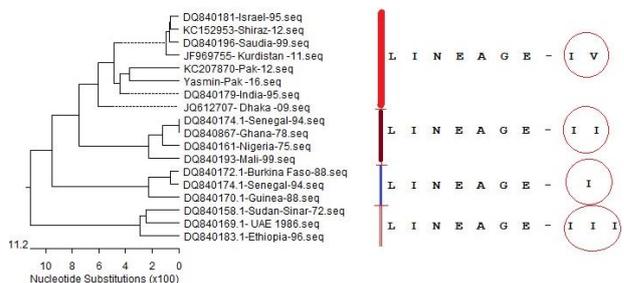


Fig. 5: Clustering of Peste des petits ruminant virus isolates PPRV/NIAB/15 into Lineage-IV viruses.

Table 2: Biochemical parameters of peste des petits ruminant diseased (n=10) and apparently healthy (n=5) goats

Parameters	Units	PPR Group	Control group	P value
Total Proteins	g/dL	8.70±0.56	11.30±0.59	0.009
Albumin	g/dL	3.10±0.33	5.12±0.31	0.001
Globulin	g/dL	5.54±0.77	6.48±0.84	0.063
Calcium	mg/dL	11.7±0.84	10.2±0.40	0.133
Cholesterol	mg/dL	87.2±4.38	71.2±2.74	0.011
Magnesium	mg/dL	2.67±0.26	2.63±0.13	0.893
Glucose	mg/dL	21.6±2.24	68.6±6.13	0.000
Sodium	mEq/L	150.7±13.9	139.3±1.05	0.434
Triglyceride	mg/dL	23.8±1.01	26.8±1.60	0.143
Potassium	mEq/L	5.42±0.63	4.52±0.32	0.231
Uric Acid	mg/dL	1.95±0.35	0.42±0.06	0.001
Creatinine	mg/dL	1.50±0.13	0.82±0.13	0.004
Bilirubin	mg/dL	0.19±0.02	0.13±0.01	0.022
Aspartate aminotransferase	IU/L	107.2±17.8	124.2±15.8	0.491
Alanine aminotransferase	IU/L	80.7±8.17	19.9±9.95	0.000

Data analysis by student's t-test. Mean±SE values along with ranges values are given. P<0.05 was considered as significant.

The necropsy findings showed that carcasses were emaciated, with sunken eyeballs. These lesions might be due to diarrhea and dehydration. Moreover, different lesions in the respiratory tract including lungs, trachea and mucosal surfaces were observed. Similar PPR lesions are already reported and helpful for the diagnosis and characterization of the disease (Khan *et al.*, 2018). Different necropsy lesions observed in this study have also been reported in goats and sheep died due to PPR (Balamurugan *et al.*, 2012). Different microscopic ailments observed in goats such as blunting/stunting and fusion of intestinal villi, destruction of lymphoid follicles in spleen, coagulative necrosis and vacuolation of hepatocytes, interstitial pneumonia and congestion in kidneys have also been observed in PPR infected small ruminants (Kumar *et al.*, 2014; Khan *et al.*, 2018). In published literature (Sahinduran *et al.*, 2012), coagulative necrosis at multiple places and vacuolation of hepatocytes along with abnormal liver function tests were recorded in small ruminants naturally infected with PPRV.

The results on hematological level showed a marked decrease in leukocytes and erythrocytes cell count indicating the involvement of the hemopoietic system. Previously, the PPRV caused marked immunosuppression evident from the decreased leukocytic and platelet counts (Scott, 1990; Khan *et al.*, 2018). The immunosuppressive properties of the PPRV have also been reported in experimentally infected animals (Rojas *et al.*, 2017) and the lymphoid system infection (Yan *et al.*, 2019). A significant decrease in WBC counts and the percentage of lymphocytes and neutrophils recorded in the present study have also been reported in the past (Aikhuomobhogbe and Orheruata, 2006) although there are some studies reporting vice versa (Bisalla *et al.*, 2011). A marked decrease in hemoglobin concentration and increased MCV suggestive of large-sized immature red blood cells in the circulation in goats were recorded (Khan, 2008). The higher MCHC values indicated macrocytic anemia and decreased oxygen-carrying capacity of the red blood cells (Begum *et al.*, 2018). The presence of hemorrhagic areas in the liver and other organs of the digestive system also caused a marked decline in erythrocyte counts and

hematocrit values. The significant reduction in platelet counts might be due to decreased platelet production or accelerated consumption (Parida *et al.*, 2015). Usually, increased platelet consumption takes place during severe or disseminated intravascular coagulation (Sahinduran *et al.*, 2012). Increased hematocrit levels in goats could be due to diarrhea and water loss.

The increase in serum sodium and potassium values was not found statistically significant as already reported in lambs (Aytekin *et al.*, 2011). The differences might result from the severity and stage of infection, presence of secondary infections, age, and species of the affected animal. There was a significant decrease in glucose levels which might be due to low intake of feed since the onset of infection (Balogun *et al.*, 2017). In the present study, significantly increase in the mean serum concentrations of ALT in infected goats was determined. Pathophysiology for elevated ALT might be an injury to hepatocytes (Begum *et al.*, 2018). Moreover, the increased activities of enzymes could also be due to oxidative stress (Khan *et al.*, 2009) which was evident as the infected goats were lethargic and depressed. The significant decrease in the total serum proteins and albumin along with urine albuminuria might be due to poor protein synthesis in the liver and increased protein excretion through the urine leading to hypoproteinemia in PPR infected goats. The lesions might be due to the involvement of GIT and kidneys as reported in PPR infection (Khan *et al.*, 2018).

In the present study, increased serum creatinine and uric acid could be due to impaired kidney function (Balogun *et al.*, 2017) and also suggestive of the presence of albumin secretion in urine. The PPRV causes endothelial damage, which might be the reason for the release of albumin in the urine. Similarly, congestion of glomeruli and cortical blood vessels in natural and experimental PPRV has been reported (Khan *et al.*, 2012; Khan *et al.*, 2018).

The PPRV outbreak in the present study was confirmed by highly sensitive and accurate test, RT-PCR was carried out targeting the N gene which also has been reported as a reliable tool for diagnosis and confirmation of PPR (Mahapatra *et al.*, 2015). In the present study, 18 samples from clinically infected animals were found positive with an amplicon size of 353 bp of N gene. This is similar to the previously reported studies where 66.7% (12/18) samples yielded positive results using N gene-based primers (Tiwari, 2004). The detection of lineage IV in the current outbreak indicated that PPRVs circulating in Pakistan are so far genetically stable and homologous.

The present investigation confirmed the outbreak of PPR in Nankana district of Pakistan. RT-PCR was found to precisely identify and differentiate the PPR virus from rinderpest virus. PPRV has a great impact on small animal rearing communities and pushes them to poverty. The risk of the outbreak becomes more with the gathering of animals at livestock markets on different occasions. PPR infection impairs the functioning of many organs and systems in goats which is evident by alterations in hematological and biochemical values. The endemic situation of PPR in Pakistan reminds us to stay alert and reinforce the proper immunosurveillance strategy to eradicate this disease.

Acknowledgments: The authors acknowledge the travel grant provided by the Higher Education Commission of Pakistan for presenting this research work in the conference "Emerging Viruses of Zoonotic and Veterinary Importance" held at Churchill College, Cambridge on 24-26th July 2017.

Authors contribution: MH and MM conceived the concept. SAK, MM, RH, MA, and AAN collected and analyzed the samples. Data were analyzed by SAK, MM and AMD. Manuscript was written by MM, RH, and AK. The language was corrected by AK, XXD, and JBY. All authors approved the final version of the manuscript.

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