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HEMATOLOGICAL, SEROLOGICAL AND VIROLOGICAL FINDINGS IN SHEEP AND GOATS EXPERIMENTALLY INFECTED WITH LINEAGE III PESTE DES PETITS RUMINANTS VIRUS ISOLATES IN KENYA

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ABSTRACT

The aim of this study was to determine the hematological and serological changes in experimentally infected sheep and goats and to validate use of Real time reverse transcriptase PCR analysis in diagnosing PPRV in Kenya. In this study, five sheep and five goats aged 3-6 months and of different sexes were experimentally infected with lineage III PPR virus. Further, two goats and two sheep that were confirmed using c-ELISA kit to be free from PPR were used as controls. Hematological examination of infected animals revealed a modest decrease in lymphocyte counts (L) and a slight increase in Neutrophils (N) and White Blood Cell Count (WBC) in goats. Parameters of the erythron apart from an increase in HB were unremarkable in goats. An increase in White Blood Cells (WBC), Neutrophilia, lymphopaenia and relative change in red blood cells (RBCs) parameters were consistently observed in infected sheep. Serology revealed the presence of antibodies against PPRV by 10th day post infection with both goats and sheep showing mean competition percentage of 41.10 ±10 and 40.768 ±5.26, respectively. Antibodies against PPRV continued to rise by day 14 where both goats and sheep showed a mean competition percentage of 29.77 ±4.98 and 22.51 ±6.69, respectively. Real-time -PCR revealed positive amplification in ocular swabs, mesenteric lymph nodes, intestines and in lungs. Results of this study indicated that infection with PPR in sheep and goats provide valuable data about hematological and serological findings that can be used for diagnosis of PPR. Samples of choice for real-time PCR diagnosis for PPR are ocular swabs, mesenteric lymph nodes, intestines and lungs.

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1 Introduction

Peste des petits ruminants (PPR) is an acute or sub-acute and highly contagious viral disease of goats, sheep and small wildlife ruminants. The common clinical findings of the disease are high fever, depression, salivation, pneumonia, anorexia, erosive or necrotic stomatitis, purulent ocular and nasal discharges, and ulceration of the mucous membranes and inflammation of the digestive system associated with severe diarrhea (Aruni et al., 1998; Cam et al., 2005; Aytekin, 2008). PPR virus infection has been determined in many countries including Middle East, the Arabian Peninsula, Southern Asia and West, Central and East African countries. It is more commonly seen in North Africa, the Middle East and Turkey (Kwiatek et al., 2007; Saeed et al., 2010) and its mortality is considerably high in these countries. PPR virus has affinity for lymphoid organs contributing to marked immune-suppression as indicated by leucopenia, monocytes depletion and lymphopenia. These observations are predominant particularly during acute phase of disease (Rajak et al., 2005). The number of eosinophils may remain unaltered because these cells are primarily associated with parasitic infections (Sahinduran et al., 2012). A gradual and moderate decrease in lymphocyte count has been reported in animals from days 6 to 8 post infection. Lymphopenia is attributed to lymphocyte trapping in the lymphoid tissues that may occur early during induction of immune response following local replication of the virus (Abraham et al., 2005)

PPR virus infections and vaccination induce immunity that is protective for the rest of the host's life. This protection is independent of the PPRV lineage. Suckling lambs acquire passive immunity via the colostrums from previously exposed or vaccinated dams. This immunity last for 3-4 months and antibodies are detectable until four months of age in virus neutralization test but only 3rd month in competitive ELISA (Libeau et al., 1992). Reports from other authors indicate that serum samples collected from experimentally infected goats and tested for PPRV antibodies by c-ELISA revealed traces of antibodies from 7th day post infection, that continued to rise till day 28 post infection and dropped on 30th day post infection (Osman et al., 2009). The titer of antibodies induced by PPRV started from weak positive (PI 51-70%) at 7th day post infection to moderate (PI 73-82%) at day 21 post infection to strong positive (PI 85-90%) at following days till day 28 post infection. At day 30 the antibody titer recorded was less than in day 28 post infection (Osman et al., 2009). Rapid and specific taqman based one step real time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) has been described for detection of PPRV. Primers and probes are labeled on the nucleocapsid protein gene sequence. The real time PCR has been used to detect PPR isolates from very distinct geographical areas including Africa, Middle East and Asia (Bao et al., 2007). Real time-PCR generates complete results within 3 hours hence can be used as a rapid diagnostic tool. The sensitivity of RT-PCR assay increases one log unit over that of conventional RT-PCR (Shaw et al., 2007) The aim of this study was to determine hematological and serological responses in sheep and goats infected with lineage III PPR virus isolates in Kenya and to validate use of Real time PCR analysis in diagnosis of PPR.

2 Materials and Methods

2.1 Experimental animals

Five goats and five sheep aged between 3-6 months that were experimentally infected with PPR virus isolates from natural field cases in Kenya were used for this study. In a previous study by the same author, it was demonstrated that the positive tissue samples had viral RNA from a lineage III PPRV and the first available complete genome sequence was determined (Dundon et al., 2014). The animals were intra-nasally infected with 2 ml of mixed tissue suspension (mesenteric lymphnodes, spleen, lungs and intestines) and observed for development of PPR specific signs. Two goats and two sheep that were examined to be clinically healthy and free of PPR antibodies were used as control group. Animal experimentation was conducted under the approval of Board of Postgraduate Studies, University of Nairobi, following the recommendations of Faculty of Veterinary Medicine which ensured that all bio-safety, animal use and ethical issues had been addressed.

2.2 Hematological analysis

Blood for hematology was collected from all animals in the study before infection and on day 10 post infection. Two ml of blood was collected from jugular vein and placed in vials containing EDTA. Blood samples were used to determine white blood cell count (WBCs), hemoglobin concentration (HB), red blood cell count (RBCs), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and platelets (PLT) using a blood cell counter (Beckman Coulter Gen-S Hematology Analyzer, USA). Changes in hematological values were analyzed by calculating the differences of the parameters before and after infection. The mean of these differences and its standard error were used to calculate the significant difference. These means were considered statistically significant where the P value was lower than 0.05 ($P<0.05$).

2.3 Serology

Blood for serum was collected from all animals before they were infected and on days 6, 10 and 14 post infections. About 5ml of blood was collected from each animal and emptied into a sterile universal bottle. The bottle was placed in a slanting position for the blood to clot at room temperature for 1 hour. The expressed serum was centrifuged at 2000 rpm for 15 minutes to sediment the erythrocytes and clear straw colored serum was harvested. PPR c-ELISA kit designed to detect antibodies directed against the nucleoprotein of peste des petits ruminants (PPR) virus, developed by FAO reference laboratory (CIRAD-EMVT, Montpellier, France) was used. The test was

performed according to the instructions in the manufacturer's manual.

2.4 Collection of ocular swabs and tissues for PCR

The ocular swabs for virus RNA detection by reverse transcription polymerase chain reaction were collected on days 13 post infection when all infected animals exhibited ocular discharges. All animals were humanely euthanized by the end of the study (20^{th} dpi) and tissue samples for PPR antigen detection by PCR aseptically collected. From sheep, mesenteric lymph nodes, intestines and lungs were collected while from goats, mesenteric lymph nodes and intestines were harvested. These tissues were placed in a sterile universal bottle and stored in a cool box containing ice packs and transported to the laboratory.

2.5 Extraction and Purification of PPRV RNA from tissues

Extraction and purification of PPRV RNA from fresh frozen tissue was performed according to manufacturer's description in QIAGEN RNeasy Mini kit, Ref: 74104 ou 74106.

2.6 Extraction and Purification of PPRV RNA from Ocular swabs

Purification of PPRV RNA extracted from nasal and ocular swabs was performed as described for the frozen tissues above.

2.7 Real-time Reverse Transcriptase PCR

TaqVet^{TM}Peste des petits ruminants virus real time RT-PCR kit (Ref:PPRP/50) developed by Laboratoire Service International in collaboration with Institute of Animal Health, UK was used. The test was performed according to the instructions in the manufacturer's manual.

3 Results

All experimentally infected animals in this study developed clinical signs similar to those of naturally infected animals while the control animals remained apparently healthy. Clinical signs observed included, nasal and ocular discharges, oral lesions and watery diarrhea that resulted into severe dehydration and death**.**

3.1 Hematological findings

There was a significant increase in hemoglobin concentration (HB), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) in goats after infection with PPRV. Goats also developed a modest decrease in lymphocyte counts (L) and a slight increase in Neutrophils (N) and White Blood Cell Count (WBC) but no significant change occurred in Neutrophil: lymphocyte (N/L) ratio ($p > 0.05$); platelet count also increased notably but not significantly after the goats were infected with PPRV. There was no significant change in Red Blood Cell counts (RBC), Packed Cell Volume (PCV) and Mean Corpuscular Volume (MCV) in goats infected with PPRV (Table I).

Table 1 Mean hematological parameters and their standard deviation for infected goats before and after infection.

Parameters	Before infection (n=5)	After infection (n=5)	Mean of the difference	P-values	T
	X±Sd	X±Sd	đ±Sd		
WBC (X 10^3/ml)	19.51±4.16	22.63±5.29	3.112±4.39	0.2232	1.58
HB (g/dl)	8±1.39	10.12±0.84	2.12±1.69	0.0488	2.8*
RBC (X10^6/ml)	12.65±1.89	12.41±1.29	0.23±2.67	0.8539	0.20
PCV (%)	20.52±2.27	18.24±1.44	2.28±2.21	0.0819	2.3069
MCV (fl)	16.42±1.57	15.3±0.91	1.12±1.68	0.2261	1.4907
MCH (pg)	6.46±0.23	7.8±0.51	1.34±0.55	0.0055	5.4478*
MCHC (g/dl)	39.96±4.87	51.64±5.66	11.68±6.99	0.0202	3.7364*
Platelets (10^3/ml)	382.6±213.4	597.8±278.72	215.4±227	0.1016	2.12
Neutrophils (%)	31.2±12.56	44.4±12.01	13.2±16.87	0.1551	1.75
Lymphocytes (%)	68±13.62	55.8±11.65	12.2±17.41	0.1419	1.5669
N/L	0.536±0.307	0.719±0.100	0,255±0.23	0.1123	2.42

Subscript * in the same row indicate there was a significant difference ($P<0.05$)
Key: X – Mean, Sd - Standard deviation of mean, **đ -** Mean of the difference

Table 2 Mean hematological parameters and their standard deviation for infected sheep before and after infection.

Parameters	Before infection (n=5)	After infection (n=5)	Mean of the Difference	P-value	T
	X±Sd	X±Sd	đ±Sd		
WBC (X 10^3/ml)	19.57±7.70	61.68±25.42	42.11±28.04	0.0305	3.3581*
HB (g/dl)	9.46±2.15	10.34±1.57	0.88±1.12	0.1535	1.7569
RBC (X10^6/ml)	8.69±2.29	12.56±1.54	3.88±1.97	0.0117	4.404*
PCV (%)	21.72±7.23	26.42±3.64	4.68±4.83	0.0949	2.17
MCV (fl)	16.42±1.57	21.6±2.49	5.18±2.77	0.0139	4.18*
MCH (pg)	10.94±0.58	8.14±0.55	2.8±1.08	0.0044	5.7972*
MCHC (g/dl)	45.04±6.56	38.64±2.04	6.4±5.97	0.0746	2.40
Platelets (10^3/ml)	838±542.18	209.6±32.62	628.4±515.94	0.0528	2.7235
Neutrophils (%)	46.2±15.51	58.4±15.53	12.2±3.90	0.0022	6.9949*
Lymphocytes (%)	53.2±16.39	36.4±11.55	16.8±5.81	0.0029	6.47*
N/L	1.098±0.951	1.942±1.378	0.8436±0.461	0.0012	4.09*

Subscript * in the same row indicate there was a significant difference (P<0.05)
Key: X – Mean, Sd - Standard deviation of mean and **đ -** Mean of the difference

The following parameters significantly increased (P<0.05) in sheep that were clinically infected with PPRV: White Blood Cells (WBC), Neutrophils (N) and Neutrophil: Lymphocyte (N:L) ratio and Red Blood Cell Count (RBC). Haemoglobin Concentration (HB) and Packed Cell Volume (PCV) increased proportionately but not significantly after infection with PPRV in sheep. Lymphocyte count (L) decreased significantly in sheep after infection with PPRV. Platelets (P), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) showed a notable but not significant decrease in sheep after infection with PPRV (Table 2).

3.2 Serological findings

No PPR specific antibodies were detected from serum that was collected before experimental infection. On day 6 post infection, Peste des petits specific antibodies were detected in two goats and three sheep while two goats and two sheep were doubtful at serology and had a competition percentage of 50-60%. Only one goat tested negative with a competitive percentage greater than 60%.

All infected animals showed detectable antibodies by 10^{th} day post infection with both goats and sheep showing mean competition percentage of 41.10±10 and 40.768 ±5.26 respectively. Antibodies against PPRV continued to rise by day 14 where both goats and sheep showed a mean competition percentage of 29.77 ±4.98 and 22.51 ±6.69 respectively. No antibodies were detected in control group. The rise in percentage competition has been illustrated in Figures 1.

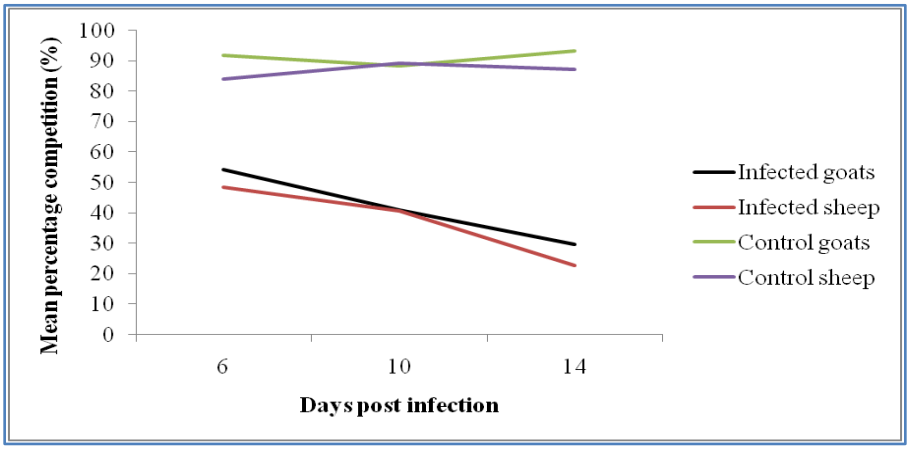

Figure 1 Mean Percentage Competition values for PPRV antibodies in serum collected from experimentally infected goats and sheep and control animals on days 6, 10 and 14 post infection.

Table 3 Real time RT-PCR; threshold cycle values in ocular swabs on 13th day post infection from experimentally infected goats.

Animal No	C_TFAM	C_TVIC
Sheep 1	37.65	29.12
Sheep 2	38.63	31.27
Sheep 3	36.51	29.67
Sheep 4	37.52	38.28
Sheep 5	No C_T	35.29
Goat 1	38.81	28.14
Goat 2	40.63	30.94
Goat 3	No C_T	30.53
Goat 4	41.68	31.68
Goat 5	39.84	30.53

C_TFAM- Indicate threshold cycle for the tissue sample and C_TVIC- indicate threshold cycle for the internal positive control

Table 4 Real time RT-PCR; Ct value, (threshold cycle value) in fresh frozen tissues from experimentally infected sheep with PPR virus.

Animal No	Mesenteric l/n		Intestines		Lungs	
	C_TFAM	C_TVIC	C_TFAM	C_TVIC	C_TFAM	C_TVIC
Sheep 1	No C_T	23.02	No C_T	No C_T	No C_T	No C_T
Sheep 2	41.56	25.57	41.84	25.62	33.32	31.13
Sheep 3	39.39	22.77	34.52	30.28	No C_T	24.49
Sheep 4	43.38	24.97	43.21	26.63	43.71	38.42
Sheep 5	39.67	28.07	No C_T	23.69	No C_T	24.14

C_TFAM- Indicate threshold cycle for the tissue sample and C_TVIC- indicate threshold cycle for the internal positive control

Table 5 Real time-RT-PCR; Ct value, (threshold cycle value) in fresh frozen tissues from experimentally infected goats.

	Mesenteric lymph nodes		Intestines	
Animal No	**C_TFAM**	**C_TVIC**	**C_TFAM**	**C_TVIC**
Goat 1	No C_T	No C_T	42.05	26.66
Goat 2	44.55	33.38	41.7	22.58
Goat 3	No C_T	31.26	40.66	25.62
Goat 4	38.71	24.49	No C_T	27.51
Goat 5	No C_T	24.22	No C_T	28.82

C_TFAM- Indicate threshold cycle for the tissue sample and C_TVIC- indicate threshold cycle for the internal positive control.

3.3 Real time-Reverse transcriptase polymerase chain reaction

Real time reverse transcriptase PCR assay was performed on ocular discharges that were collected from sick animals on day 13 post infection and in frozen body tissues obtained from animals that indicated severe histopathological changes. Ocular swabs revealed positive amplification in 8 out of 10 experimentally infected animals (Table 3) (four sheep and four goats) with sheep and goats having a mean threshold cycle (C_T) value of 37.578 ±0.87 and 40.24 ±1.22 respectively. The highest virus load was in sheep 3 where the PPR viral antigen was detected by cycle 36.51 of amplification. Four sheep tested positive for PPR viral antigen in mesenteric lymph nodes, 3 in the intestines and 2 in the lungs (Table 4). Two goats tested positive for PPR virus in mesenteric lymph nodes while 3 tested positive in the intestines (Table 5). In all samples that were tested, only three were invalidated since no reading either for the test sample or for the internal positive was recorded from the invalidated cases. No PPR virus antigen was detected in formalin fixed tissues.

Discussions

The diagnosis of PPR is based on clinical examination, gross pathology, histopathological findings and laboratory confirmation by virus isolation, serology or genome detection (OIE, 2009). Most laboratory methods require collection of clinical and post mortem materials of high quality and these needs to be transported in a cold chain system from distant places. Sometimes this is hard to achieve especially when PPR outbreaks occur in remote and inaccessible parts of the country. The evaluation of the hematological parameters is a good way of assessing the health status of the animal as it reflects the physiological, nutritional and pathological status of the animal.

The following parameters significantly increased ($P<0.05$) in sheep that were clinically infected with PPRV: A significant increase in White Blood Cells (WBC), Neutrophils (N) and Neutrophil: Lymphocyte (N: L) ratio and a concurrent lymphopaenia that occurred in PPRV infected sheep was expected since the virus causes inflammation in tissues and lymphocytolysis. The proportionate increase in Red Blood Cell Count (RBC), Haemoglobin Concentration (HB) and Packed Cell Volume (PCV) in PPRV infected sheep could be attributed to dehydration that accompanied loss of fluid that was manifested clinically in PPRV infected sheep. The notable decrease in Mean Corpuscular haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) in PPRV infected sheep were derived from an increase in RBC, PCV and HB observed above. PPRV infected goats had less spectacular haematological changes and this was consistent with their clinical disease response in the two species. Coincidentally, Yarim et al. (2006) and Kataria et al. (2007) determined low WBC, high RBCs count, HB and PCV values in infected lambs and sheep.

The study by Aytekin et al. (2011) partly corroborate with present study findings on the leucon since they also recorded significant increase in WBC, MCV, MCH, MCHC but a low HB, RBCs and PCV in a PPR outbreak affecting lambs. Sahinduran et al. (2012) has also reported significant reduction in number of erythrocytes and hematocrit values. Increase in neutrophil lymphocyte ratio reported in this study is in agreement with the findings of Mohammed et al. (2011), but contrary to findings by Aikhuomobhogbe & Orheruata (2006). Increase in neutrophil: lymphocyte ratio is a good indicator of neutrophilia and lymphopaenia.

The differences observed in hematological parameters may be due to the different phase of disease, presence of secondary infection, nutrition and dehydration degree (Heaney et al., 2002; Kataria et al., 2007). Examination of serum samples revealed detectable antibodies on 6^{th} day, 10^{th} day and 14^{th} day post infection with antibodies titers increasing from 6^{th} to the subsequent days. The presence of detectable PPR antibodies indicated that humoral immune response develops in experimentally infected animals as early as 6 days post infection. In contrast, there were no detectable antibodies in the control group. This observation was in concordance with those reported by Taylor et al., (1979) and Osman et al. (2009). Predominantly, confirmatory diagnosis of PPR in Kenya is done serologically. The findings in this study indicate that acutely infected animals can be serologically confirmed positive for PPR as early as 6^{th} to 10^{th}day post infection and therefore control measures can be instituted early.

In recent years, several attempts have been made to improve diagnosis of PPR and several conventional RT-PCR assays have been developed to allow efficient virus detection in vivo from swabs and biological samples (Balamurugan et al., 2006; Bao et al., 2007). High sensitivity, specificity and rapidity of RT-PCR in comparison with conventional methods such as virus isolation and ELISA, renders RT-PCR assay the first choice of PPR diagnosis. However, conventional RT PCR is technically demanding and requires 4-8 hours for a complete diagnosis.The TaqVet™ Peste des Petits Ruminants Virus kit was used and the amplified product was detected by measuring the fluorescence released by labeled probe hydrolysis. In this study, PPR virus was detected from mesenteric lymph nodes, intestines and lungs of experimentally infected animals. This was in agreement with previous studies where virus isolations were made from mesenteric lymph nodes (Furley et al., 1987), or intestinal epithelial smears (Taylor, 1984). Out of ten experimentally infected animals, eight tested positive for PPRV RNA from ocular swabs. This could be due to the fact that these samples were taken from surviving animals during the clinical phase of the disease when the virus was being secreted. This was in agreement with a study done by Sharawi et al. (2010) who detected PPR antigen from oculo-nasal swabs taken from infected gazzelles. Two animals tested negative for PPRV RNA from ocular swabs. This could be attributed to secondary bacterial infection that result in purulent ocular discharges in two animals at the time of sampling. PPRV is very labile and bacterial contamination could compromise the quality of RNA (OIE, 2009). Detection of PPRV RNA in ocular swabs of 4 sheep and 4 goats; mesenteric lymph nodes of 4 sheep and 2 goats, intestines of 3 sheep and 3 goats and lungs of 2 sheep underscores that these are important target organs for the virus and they should included as first line diagnostic samples for the disease.

Conclusion

From this study, it is clear that antibodies against PPR can be detected as early as 6^{th} day post infection. This finding indicates that early diagnosis can also be achieved through serology. Samples of choice when targeting PPR virus RNA are; ocular swabs, body tissues such as mesenteric lymph nodes, intestines and lungs. In addition, the hematological parameters should be providing supportive evidence in diagnosis of PPR outbreaks.

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