CLINICO - PATHOLOGICAL CHANGES IN SHEEP EXPERIMENTALLY INFECTED WITH EHRLICHIA RUMINANTIUM (UM BIAGA -ISOLATE)

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المستخلص

تم إحداث عدوى تجريبية في تجربة لمرض الخدر في الضأن باستخدام جرثومة الاريليخيا دومينتشيوم والتي تم عزلها من القراد البالغ لنوع أمبليوما لبيدم أثناء التغذية على الأبقار في منطقة أم بياقة بولاية سنار في يناير عام 2003 وذلك بحقن خليط متجانس من مسحون القراد في الماعز النوبي وبعد حدوث المرض فيه تم سحب دم يحتوي على الطفيل وتم حفظه في النتروجين السائل. تم شراء سبعة حيوانات من الضأن الصحراوي من النوع الدباسي من مدينة شندى الخالية من المرض. وقسمت هذه الحيوانات إلى مجموعتين، المجموعة الأولى تضم أربعة حيوانات تم حقنها بدم ملوث بالجرثومة. أما المجموعة الثانية تتكون ثلاثة حيوانات حقنت بماء معقم كمجموعة تحكم. العلامات السريرية والمرضية أظهرت ارتفاع في درجة الحرارة، أنيميا، موه في التامور والصدر، احتقان وخزب في الرئة والغدد اللمفاوية والدماغ. التغيرات النسيجية المرضية أظهرت زيادة في خلايا الدم البيضاء وانتشارها حول الأوعية الدموية في الرئة، الغدد اللمفاوية، عضلات القلب والدماغ. كما لوحظ توسع حافظات بومان، الأنيبيبات الملتفة والقنوات الجامعة للكلية. النتائج المختبرية لتحليل الدم والبلازما أسفرت عن نقص نسبي في عدد الكريات الحمر، محتوى الهموقلوبين ونسبة العدد التراصي لكريات الدم. ولا توجد تغيرات تذكر في كيميائية الدم ماعدا ارتفاع طفيف في تركيز البليروبين.

Key words: clinico-pathological, Ehrlichia, ruminantium, Sheep.
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Abstract

An experimental infection of sheep with Um Biaga isolate of Ehrlichia ruminantium was made to determine the pathogenisity of the isolate, using in-vitro isolate from Amblyomma. lepidum adults ticks collected from cattle at Um Biaga village (Sennar, State) by inoculating homogenate in adult Nubian goats in January 2003. Seven Desert sheep of Dubassi eco-type purchased from Shendi town known to be a heartwater free area were assigned into two groups. Group I composed of three animals served as uninfected control. Group II composed of four animals that were inoculated intravenously with 5 ml of infected blood from the Nubian goats. The clinical signs and postmortem findings of the infected group were typical of

heartwater. The main pathological findings observed were hydropericardium, hydrothorax, lung congestion and oedema of lymph nodes and brain. The histopathological findings showed intensive leukocyte stasis and severe perivascular infiltration in the lung, lymph nodes, myocardium and brain. The convoluted tubules and collecting ducts of kidneys were dilated and Bowman's spaces were widened. The mean haematological values from the infected sheep indicated that a decrease in PCV, RBCs count and Hb content coincided with fever while a slight increase in WBCs count was observed. Alterations in plasma biochemical parameters were not significant in most of the infected group.

Introduction

Heartwater is a fatal disease of domestic and wild ruminants transmitted by the vector Amblyomma tick and occurs in Sub-Saharan Africa and the Caribbean region (Walker and Olwage, 1987; Peter, et al. 2002). It is caused by infection with the intracellular rickettsia Ehrlichia ruminantium (Dumler, et al. 2001), formerly known as Cowdria ruminantium. The disease is considered to be the most important tick-borne disease of ruminants in South Africa, and second in importance only to East Coast fever in eastern Africa. Its economic importance in cattle in the Sub-Saharan Africa is recognized but not well documented (Norval, et al. 1991).

Heartwater is of considerable economic importance to the livestock industry and is expected that the greater part of the research

efforts centres on the occurrence of the disease in domestic ruminants particularly sheep and goats, and it can cause mortality in several wild ruminant species (Uilenberg, 1997). The disease can be controlled by a combination of acaricides, chemotherapy and immunophylaxis using an infection and treatment method (Camus, et al., 1996). Several attempts which have been made include the development of inactivated vaccines, attenuated vaccines and also DNA vaccines (Collins et al., 2003; Faburay et al., 2007). Although progress has been made, the antigenic diversity identified amongst different stocks of E. ruminantium resulting in a lack of protection between heterologous stocks, has been identified as a major obstacle in vaccine development. Furthermore, comparative genomic analysis of E. ruminantium isolates showed the presence of active mechanisms of genome plasticity possibly involved in the limited field-efficacy of vaccines (Frutos et al., 2006). Strain-specific diagnosis is thus essential (Vachiery et al., 2008). However, serodiagnosis of heartwater has long been limited by a lack of specificity, sensitivity and cross reactions with other Ehrlichia species occur (Mondry et al., 1998). PCR-based method also proved efficient for detecting E. ruminantiumin hosts and ticks and to characterize isolates of E. ruminantium from related species (Bekker et al., 2002). However, routine strain-specific diagnosis is not yet achieved and additional diagnostic targets are still to be identified (Vachiery et al., 2008).

In the Sudan, little research has been conducted on heartwater apart from the work of Karrar (1960) on isolation and epizootiology of the disease. Shommein and Abdel Rahim (1977), Abdel Rahim and Shommein (1978) and Jongejan et al. (1984) studied isolation, pathology and preservation of the organism. However, no systematic research has been conducted in this filed. Camus, et al. (1996) reported complete cross protection between Um Banein strain and the Gardel strain, which is very pathogenic. The studies presented here attempted to determine the clinico-pathological changes in sheep experimentally infected by Um Biaga isolate of E. ruminantium isolated from Sennar State, Sudan.

Materials and Methods

Experimental animals

Seven naïve sheep (6-9 months old) of Desert sheep Dubassi eco-type were purchased from Shandi town, Northern Sudan (Free zone of heartwater) and transported to the premises of the Department of Parasitology, Faculty of Veterinary Medicine, University of Khartoum

during August 2003. The sheep were assigned into two groups. Group I composed of three animals (Nos. 5, 6 and 7) represented the control. Group II composed of four animals (Nos. 1,2,3 and 4) represented the infected group. Pre-experimental examinations and animal monitoring were carried out.

Ehrlichia ruminantium isolate

In-vitro isolation of Um Biaga isolate was carried out according to Birnie, et al. (1985) method. Adults of A. lepidum partially engorged were collected from cattle at Um Biaga village (5 Km west of Um Banein). The ticks were kept alive and identified according to Hoogstraal (1956), cleaned and washed thoroughly by cool Phosphate Buffer Saline (PBS). One hundred of these ticks (50 males and 50 females) were thoroughly ground using pestle and mortar after adding 50 ml of cool PBS (pH 7.0) as diluent to a volume of 0.5 ml per tick. The tick homogenate was transferred to a clean sterile glass cylinder and allowed to stand at room temperature for 10 minutes. Using a sterile Pasteur pipette, the supernatant was removed into a glass flask and the remainder of the supernantant was cryopreserved after addition of dimethylsulphoxide (DMSO) slowly to give a final concentration of 10% in petri dish with continuous stirring. A volume of 4.5 ml of the supernatant was taken and inoculated intravenously immeditaly into three naïve goats. The inoculated animals were kept in tick proof sheds and rectal temperature was taken daily. At the rise of goats' temperatures to 41ºC, blood in EDTA was taken from the jugular veins and DMSO (1: 10) was added very slowly in petri-dish on ice under continuous stirring. The blood was divided into 2 ml aliquots and placed in cryo-vials. They were then immediately cryopreserved in liquid nitrogen (-197º C) till used. A volume of 5 ml of the infected blood was thawed, inoculated intravenously into other 3 naïve goats. The primary infected goats were then killed and brain-crushed smears were prepared and stained by Giemsa's stain for detection of E. ruminantium colonies.

Experimental infection

Each sheep in group II was inoculated intravenously by 5 ml of infected blood which was collected from the primary infection goals during the febrile stage. The animals were daily observed for clinical changes, body temperature and respiratory rates. Blood samples for

haematological and biochemical analysis were collected in EDTA. Packed cell volume (PCV) and haemoglobin (Hb) values, erythrocyte and leukocyte counts were determined. Total plasma protein, plasma albumin, urea and bilirubin were also determined as described by Evans (1968). The sheep were killed and conducted macroscopic **lesions** were noted, and tissue samples for histopathology were taken using Bancroft et al. (1996) methods.

Statistical analysis

Data of haematology and biochemistry were subjected to appropriate general linear model (GLM) procedure of statistical analysis system (SAS) package. The SAS was used to perform analysis of variance (ANOVA) while mean separations were performed using Ryan- Einot-Gabriel- Welsch (REGW) multiple range test (Day and Quinn, 1989).

Results

Clinical manifestations of infected sheep with E. ruminantium

Ehrlichia ruminantium infected sheep developed mild clinical changes during a week after inoculation. These were manifested by fluctuating fever, followed by inappetence, depression, mild incoordination and respiratory distress. The course of symptoms and duration of clinical signs of the disease were considerably variable within individual animals. Three animals in group II developed subacute infection, which was characterized by prolonged fever and coughing. The rise of body temperature over 40^{o}C was observed on day 21. These animals were killed on day 23 after infection. A mild or subclinical form of the disease was observed in one animal that showed a transient febrile response. The body temperature reached 40ºC on day 18 and then dropped to normal until the animal was killed on day 30 after infection.

Macroscopic appearance

Most of E. ruminantium infected sheep showed moderate effusion of body cavities, hydrothorax and hydropericardium (Plate 1). The lungs were congested, oedematous (Plate 2) and serous froth and bronchitis were observed. Other lesions included oedema of the mediastinum and associated lymph nodes. The brain was congested and oedematous in all cases. Subendocardial petechial haemorrhages and flabbiness of heart muscles were observed. A mild to moderate splenomegaly, hepatomegaly

and slight swelling of kidneys were evident. Crushed smears made from brain cortex showed presence of E. ruminantium colonies (Plate 3).

Histopathological findings

The histopathological alterations in various organs of E. ruminantium infected sheep, were similar in all animals. The heart showed congestion of blood vessels. Muscle cells appeared thin with a focal proliferation of lymphocytes (Plate 4). The lungs showed areas of collapse, focal oedema, haemorrhage, massive congestion of blood vessels and emphysema (Plate 5). A mild to moderate brain oedema, capillary proliferation and perivascular spaces were seen. Moderate dilatations of proximal, distal and collecting tubules were observed. Glomerullar appeared tuft lobulated with widened Bowman's space (Plate 6). The spleen showed prominent white pulp with germinal centres and marked congestion. The lymph nodes were widened with medullary sinuses containing large macrophages, lymphocytes and erythrocytes. Hepatocytes appeared swollen with disintegrated cytoplasm and slight sinusoidal congestion.

Haematological findings

The mean haematological values of E. ruminantium infected and control sheep are illustrated in Fig. 1. A gradual decline in Hb content in infected sheep was observed during the first three days post infection. Then, a slight increase in Hb concentration was observed up to day 9. Thereafter, Hb content began to decrease significantly ($P< 0.05$) on day 12 and remained low until the end of the experiment. Little or no changes in Hb content were observed in only one infected animal and control group. The mean values of PCV gradually declined for 9 days post infection. Thereafter, the values fluctuated with small level of changes and a significant reduction ($P< 0.05$) was observed on day 21 post infection. The mean erythrocytes count in infected sheep gradually declined during the first week. A significant reduction ($P<0.05$) was recorded on day 6 and slight increase was observed on day 9. Thereafter, there was a gradual reduction that remained lower until the end of experiment. The control animals showed a slight increase up to day 6 and then fluctuated at the same levels up to the end of the experiment. A gradual decrease in WBCs count observed up to day 6 post infection. But, there was slight increase which reached a peak on day 12. Thereafter, the

count fluctuated within a slight range up to day 18. The average counts of the two groups then were not significantly different till the end of the experiment, with the exception of one animal that showed a slight decrease below normal count ($P< 0.05$).

Biochemical analysis of plasma

Plasma constituents of total protein, albumin, total bilirubin and urea of infected and control sheep fluctuated within the normal range during the experiment period.

Discussion

Sheep experimentally infected with Um Biaga isolate developed subacute and subclinical forms of heartwater. The results were in contrast to previously reported field and experimental studies carried out by Jongejan, et al. (1984) who reported an acute form of the disease in sheep, goats and exotic cattle. However, the incubation period (16-21 days) of infected sheep was similar to their studies in goats (16-20 days) and cattle (12-23 days).

The observation that the disease underwent subacute may be attributed to the low level of the infective materials of tick homogenate. Infection rate of ticks is believed to play an important role in the epidemiology of heartwater. Tick infection rates vary according to Amblyomma species and from a place to another (Peter, et al. 1999). In this study, small sample sizes were used and in most cases, the ticks used had been feeding on animals for unknown periods of time. It should be proposed that the disease might be valid only for the combination of the Dubassi sheep and E. ruminantium concentration in the blood. This host may not be the most susceptible for heartwater and the infective materials may be insufficient to infect them. It was noticed that the susceptibility to heartwater varied from an animal to another within a herd and from a herd to another within the same population (Norval, et al. 1991).

However, when considering the resistance observed in indigenous breeds, a distinction should be made between acquired immunity and hereditary factors. A higher resistance on the part of local breeds were observed, in so- called “black headed” Persian sheep, the disease is mild and without complications, and for many years it has been postulated that the infections might be endemic in Iran. However, this breed of sheep originates from the Horn of Africa not from Iran. In Guadeloupe,

resistance in Creole goats appears to be genetically controlled. This manifests as a resistance to severe disease but not to mild reactions, such as rise in temperature (Camus, et al. 1996). Uilenberg (1983) pointed out that it might not be a question of breed but of population. Therefore, he argues that the difference in susceptibility does not appear to be linked to any particular breed or species but probably depends mainly or exclusively on inherited resistance acquired by local livestock through long, natural selection. Similarly, Karrar (1968) stated that it seems more likely that local cattle population have acquired inherited resistance to heartwater through long natural selection.

The gross pathological changes in sheep infected with Um Biaga isolate of E. ruminantium, corresponded to a large extent with those previously described in sheep, goats and cattle naturally or experimentally infected with E. ruminantium (Prozesky, 1987; Prozesky and Du Plessis, 1985a). Although the disease underwent subacute or subclinical form, the pathological gross changes confirmed the findings of Jongejan, et al. (1984) in natural and experimental infections of Watish sheep (local type), goats and an exotic breed of cattle. These animals were infected with Um Banein strain and underwent an acute form of heartwater with slight hydropericardium, pulmonary oedema and enlargement of mesenteric lymph nodes. Other changes reported were brain oedema, moderate hepatomegaly, moderate splenomegaly and slightly swollen kidneys. These findings closely resembled the findings of Prozesky and Du Plessis (1985a) in heartwater-infected Angora goats, which were treated with oxytetracycline during the febrile reactions.

The histopathological changes in Um Biaga isolate of. E ruminantium infected sheep corresponded closely with those previously reported in sheep, goats, cattle and mice artificially or naturally infected with heartwater. Pulmonary lesions observed in this study are in agreement with those observed in sheep and mice, which showed focal oedema (Prozesky and Du Plessis, 1985b). Prozesky (1987) stipulated that an alveolar and interstitial oedema occurs in most animals but is not always discernible histopathologically because the fluid in the alveolar spaces is washed out during the routine processing of the tissues. The myocardium appears thin with focal aggregates of lymphocytes observed between the muscle fibres. These are attributed to pericardial distension due to the excessive fluid pressure (Camus et al. 1996). The observations of prominent lymphoid follicles with germinal cells, widened medullary

sinuses containing large macrophages and lymphocytes in lymph nodes and prominent white pulp with germinal centre of the spleen. These confirm the suggestion of Shommein and Abdel Rahim (1977), that lymph nodes and the spleen may be possible sites for E. ruminantium replications. The proximal and distial convoluted tubules and collecting ducts of the kidneys were dilated. Likewise, Bowman's spaces were dilated and contained variable amounts of eosinophilic fluid. These closely resembled the findings of Prozesky and Du Plessis (1985a) in heartwater-infected Angora goats, especially treated after the first day of the febrile reaction.

The haematological alterations in Um Biaga isolate of E. ruminantium infected sheep showed significant ($P \leq 0.05$) reduction in PCV values, RBCs count and Hb content. These changes coincided with the febrile reactions. The results corresponded to a large extent to previous studies by Abdel Rahim and Sommein (1978) in goats, Van Amestl et al. (1988) in cattle and Martinez et al. (1999) in sheep that were infected subclinically with E. ruminantium. However, in contrast to previous studies a slight level of leucopenia was observed during the first two weeks with no significant changes between the infected and control groups of animals. The plasma biochemical constituents showed some variable alterations in sheep infected with Um Biaga isolate of E. ruminantium throughout the clinical course of the disease. However, these sheep did not show significant changes in the plasma total protein and albumin concentration. Similar findings were reported by Prozesky (1987) who postulated that the plasma protein concentrations remain unchanged. However, Van Amestl, et al. (1988) found reductions in all protein fractions in their study of experimentally- induced heartwater in calves.

Finally, it should be proposed that the disease might be valid only for the combination of the Dubassi sheep and E. ruminantium concentration in the blood. This host may not be the most susceptible for heartwater and the infective materials may be insufficient to infect them. It was noticed that the susceptibility to cowdriosis varied from an animal to another within a herd and from a herd to another within population (Norval et al., 1991).

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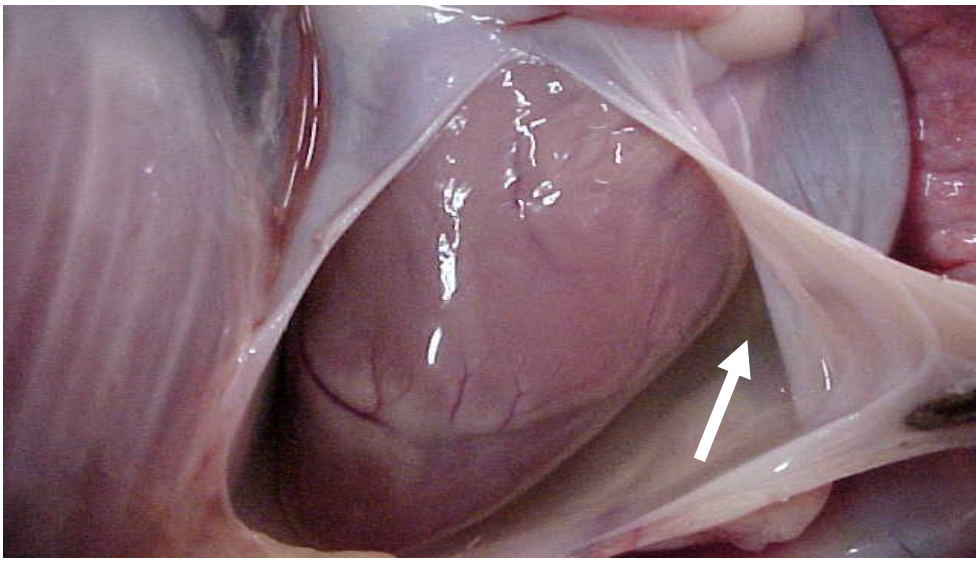

Plate 1. Heart of sheep No.3 infected with *E. ruminantium.* Note hydropericardium (arrow).

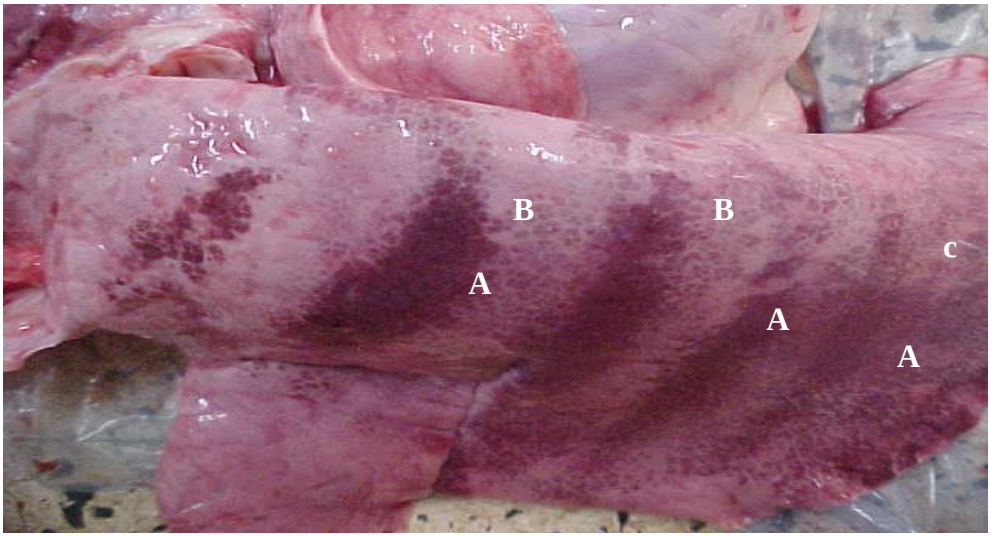

Plate 2. Lung of sheep No 2 infected with *E. ruminantium* shows areas of congestion (A). Note fairmness dark red area due to pulmonary oedema (B), enlargement of the lung and ribs impression due to emphysema.

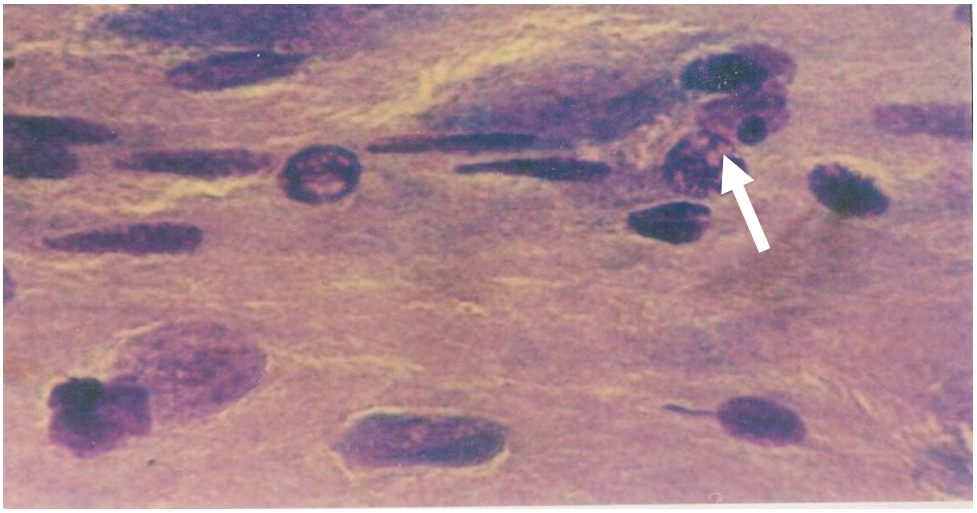

Plate 3.Crushed smear of brain cortex of sheep No.4 infected with *E. ruminantium* showing *E. ruminantium* colonies (arrowhead). (Giemsa's stain) X 1000.

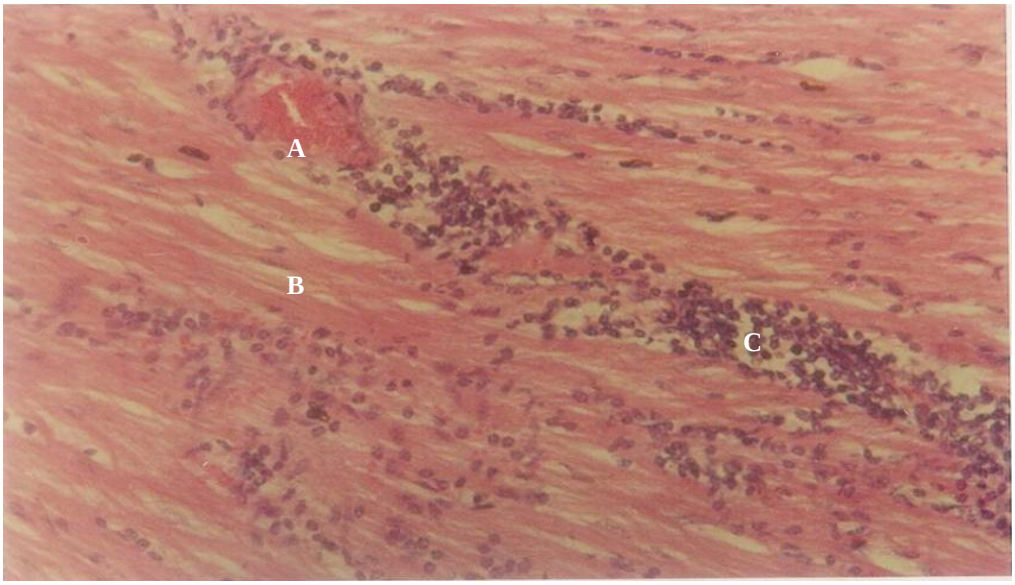

Plate 4.Heart of sheep No.3 infected with *E. ruminantium* showing congestion (A) and myocardial atrophy (B) with focal proliferation of lymphocytes (C). (H&E) X 250.

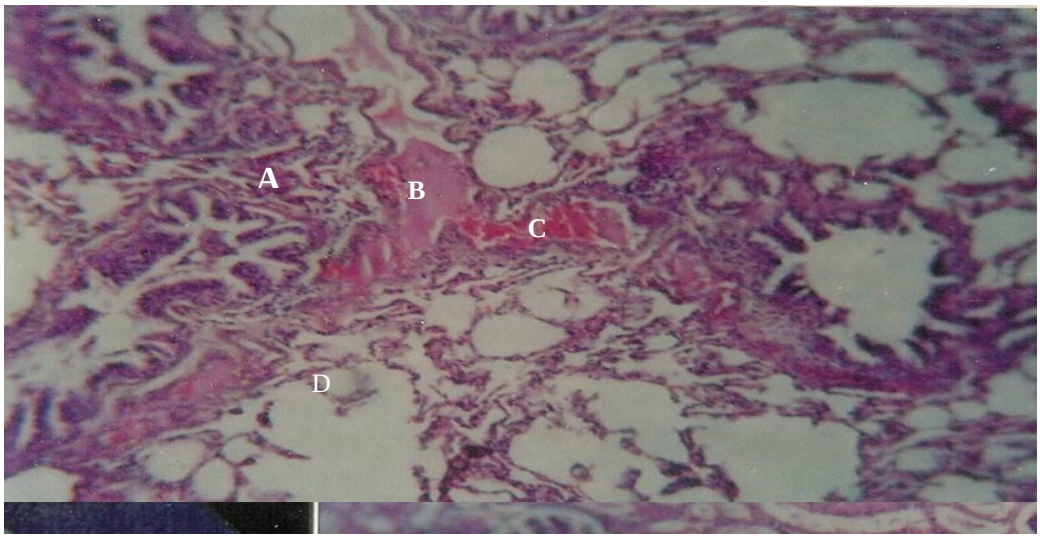

Plate 5. Lung of sheep No.2 infected with *E. ruminantium* showing areas of collapse (A), focal oedema (B), haemorrhage (C) and emphysema (D) . (H&E) X 100.

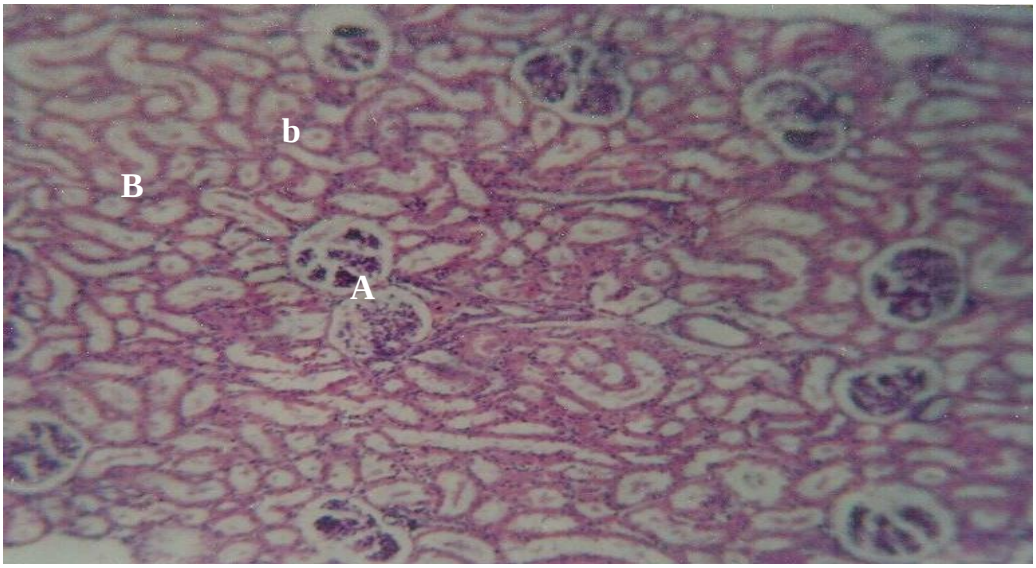

Plate 6. Kidney of sheep No.4 infected with *E. ruminantium*. Note widened Bowman's spaces (A) and lobulated glomerular tuft, moderate dilatation of convoluted tubules and collecting ducts (B) (H&E) X 100.

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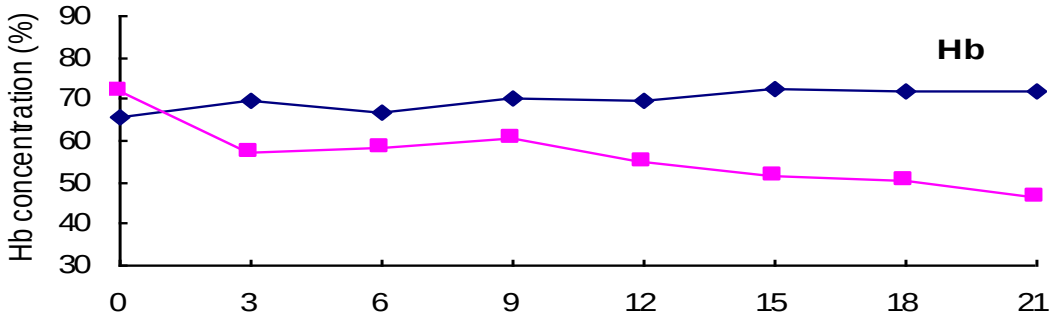

Fig. 1. Mean haematological values in E. ruminantium infected and control sheep.