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Case report

Expiry of a completely splenectomised calf in post-operative period due to mixed piroplasm infection: a case report

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ABSTRACT. Bovine babesiosis is an infectious protozoan disease and causes significant economic losses in terms of production loss and mortality. The genus *Babesia* belongs to the family Babesiidae order piroplasmida and is transmitted by ticks globally. The signs of disease are particularly prominent in old or immuno-compromised animals. The spleen plays a vital role in defence against hemoparasites like *Babesia*. A young cross-bred cow calf of about 3 months of age was splenectomised to propagate *Babesia in vivo* experimentally. Prior to splenectomy, the calf was examined through microscopy and PCR analysis and was found negative for any kind of piroplasms including *Babesia*. The calf was completely splenectomised, but the calf was naturally infected during its postoperative period. The calf expired after naturally acquiring *Babesia bigemina* and *Theileria annulata* during the 11 th day of postoperative period owing to increased parasitaemia, exhibiting typical mixed parasitic infection stigmata e.g. reddish urine, elevated temperature up to 41.38°C. This study concluded that complete splenectomy along with dexamethasone administration in the postop period caused exceptional increase in parasitaemia. This parasitaemia couldn't be countered by any symptomatic treatment because of the absence of spleen and greatly reduced immunity of the animal.

Keywords: splenectomy, *Babesia bigemina*, *Theileria annulata*, piroplasm, dexamethasone

Introduction

Bovine babesiosis is an important protozoan disease transmitted by ticks [1]. The signs of disease are particularly prominent in advanced aged, immuno-compromised and splenectomised animals [2]. The disease is responsible for high morbidity and mortality in susceptible animals. It is characterized by fever, anaemia, jaundice, weight lost, reduced milk production and, in severe cases, death [3]. Bovine babesiosis is distributed globally and occurs mostly in tropical and subtropical regions of the world. It also has a large impact in South America, Australia and other regions with important cattle industries. Various methods for improved control have been developed by studying key phases in the life-cycle of *Babesia* parasite as well as role of the spleen in clearance of infected RBCs (IRBCs), and age-related disease resistance in cattle [4]. The pathogen attacks and infects the bovine erythrocytes when *Rhipicephalus microplus or Rhiphicephalus annulatus* feeds on bovine blood [5,6]. Despite transstadial survival that occurs in most of the *Babesia* species, not all developmental stages of ticks can transmit the parasite. For example, *B. bigemina* cannot be transmitted until the nymphal and adult stages of the ticks take the blood meal [7]. The incubation period is 1–4 weeks

following the tick bite [8] and in Pakistan the high risk months for tick bites are May to September [9]. Spleen is of vital importance in defence against haemoparasites like *Babesia*. Many *in vitro* studies have elaborated the working of splenic cells in order to activate key mechanisms required for successful resolution of infection [10]. The objectives of the current study were firstly, to develop and sustain an *in vivo* culture of *B. bigemina* to maintain parasite bank. Secondly, we also aimed to report the naturally acquired *B. bigemina* infection after splenectomy as well as the post-mortem findings of a *Babesia*-infected splenectomised calf.

Case presentation

The study was approved from the animal welfare and ethic society of University of Veterinary and Animal Sciences Lahore, Pakistan with No. DR 1112, Dated: 13-10-2017. All the animals were treated after experiments.

This study presents the procedure to develop an *in vivo* parasite bank of *Babesia*. For this purpose, a crossbred cattle calf was purchased and kept under favourable conditions prior to splenectomy. The age of the calf at the time of purchase was 3 months. No ticks were observed on the body of calf at the time of purchasing. The animal was examined through microscopy and PCR analysis and was found negative for any kind of haemoparasites including *Babesia*. The calf was fed twice on daily basis with fresh fodder and silage. Rectal temperature and urine colour were recorded daily prior to splenectomy. No signs of babesiosis were observed prior to splenectomy. Splenectomy was performed in the standing calf after local anaesthesia. Surgical protocol adopted as reported by Nuss et al. [11] with a few modifications. The animal was given the proximal paravertebral anaesthesia of the 12th and 13th thoracic nerves and the 1st lumbar nerve. Laparotomy was performed in the 12th intercostal space for optimal access to spleen. The laparotomy incision was 30 cm to provide enough visibility and approach for ligation of splenic vessels and removal of the spleen. To provide analgesia when breaking Adhesions between the spleen and peritoneum by hand, Lidocaine (2%, 40 ml) was administered into the adhesions. Splenic artery was ligated at 3 locations before manual breaking the adhesions. The branch of the splenic artery that was entering into the proximal part of the hilum was identified and was ligated at 3 different locations. After freeing the spleen from its attachments to the rumen and diaphragm, the splenic vein was ligated twice, and all vessels were then transected between the ligatures for spleen removal. The abdomen was inspected for haemorrhages and the abdominal incision was closed in layers in a simple continuous suture pattern using absorbable suture material (Vicryl). Skin was closed with silk [11]. The animal was intramuscularly administered with 10 ml of meloxicam (Melonac-ICI Pakistan).

The rectal temperature of the animal was monitored twice daily. The wound was dressed twice a day with Pyodine scrub, and a mixture of mustard oil and phenyl was used as fly repellent. During post-operative care (POC), the animal was found to be infested with ticks naturally. The ticks were collected and blood samples were obtained from jugular vein of the calf for microscopy as well as PCR examination because of its higher sensitivity for parasitic infections even in carrier state [12]. For each PCR amplification 50 ng of DNA was extracted by using the method described elsewhere [13]. The extracted DNA was used as the template in a 20 µl reaction mixture containing 10 µl 2x PCR master mix (Catalog NO.W1401-2 Wiz bio solutions, Korea), 20 p-mole of each primer (Table 1) and 4 µl Diethyl pyro carbonate (DEPC) treated water (Catalog NO. 750023 INVITROGENTM, USA). The PCR reactions were conducted in a SimpliAmpTM Thermal Cycler (Catalog no. A24811; ThermoFisher scientific).

On 4th day post-surgery, the animal underwent a sudden shock and the temperature was reported to be 39.55°C and reddish urine was collected. Upon microscopic examination and PCR analysis the animal was declared positive for *B. bigemina* and *T. annulata* which showed the animal was carrying a mixed infection. Collected ticks were identified as

Table 1. List of primers used for amplification of extracted DNA from sample

Serial no	Primer sequences	Targeted species	Product size
1	TannUNF = GGGAGCTACAGTCATAGGTGGT TannUNR = TCCTGCCATTGCCAAAAGTC	*Theileria annulata*	460 bp
2	*B. bigemnia* F = AGAGGGACTCCTGTGCTTCA *B. bigemnia* R = GACGAATCGGAAAAGCCACG	*Babesia bigemina*	321 bp

Table 2. Post-surgical variation of rectal temperature (RT) with respect to No. of days

No. of days	Rectal temperature [°C]	
	morning	evening
1	38.6	38.6
2	38.8	38.6
3	38.8	39.1
4	39.2	39.7
5	39.7	39.7
6	40.2	40.1
7	39.9	40.0
8	39.5	39.7
9	40.0	40.8
10	41.1	41.3
11	41.3	41.3

Rhipicephalus and *Hyalomma* under stereo-microscope. The ticks were found suspected for the transmission of both the above-mentioned parasites, respectively. Later on post-surgery day 10, the animal went off feed and showed a mild respiratory disease and progressive anaemia. On 5th day of POC, the calf was symptomatically treated with intramuscular injections of oxytetracycline@ 15mg/kg body weight (OXY-LA, Selmore® Pakistan), buparvaquone@ 2.5mg/kg body weight (Butalex®, Imperial Chemical Industries Pakistan) and imidocarb dipropionate@ 120mg/100kg body weight (Imizol®, Merck Sharp & Dohme Corporation,USA) repeated after 24 and 48 hours, respectively for *T. annulata* and *B. bigemina*. Imidocarb dipropionate is the only chemotherapeutic drug available in the market, which provides protection from clinical diseases from 3 to 6 weeks [14]. As mentioned above, the primary objective of the study was to cultivate and sustain an *in vivo* parasite bank of *B. bigemina.* Therefore, to raise parasitemia the animal was intramuscularly administered with Dexamethasone according to the evidence provided by Ravindran et al. [15] with modifications. On 8th day of POC, Dexamethasone @ 0.1mg/kg bodyweight (Dexavet Star laboratory, Punjab, Pakistan) and Vitamin B-complex @2ml/50kg bodyweight (Lawrence Pharmaceuticals Lahore, Pakistan) were injected intramuscularly and repeated after 24 hours to induce immuno-suppression and to achieve high parasitaemia, after the calf was splenectomised [16]. There was a gradual increase in temperature up to 41.38°C on 10th day of POC. This may be attributed due to raised parasitaemia levels which were quantified on daily basis after the first administration of Dexamethasone at 10th day of POC, as shown in Table 2.

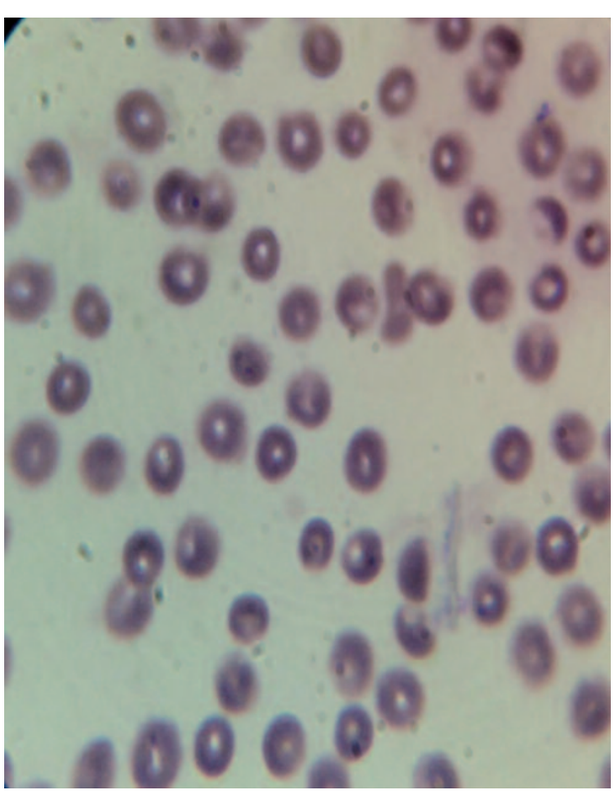

Figure 1. Microscopic image of blood smear of infected splenectomised calf showing intra erythrocytic inclusion bodies-IEBs (thin blood film, magnification 1000×)

Blood smear of infected calf was observed on compound microscope (Microse-MC 30, Austria) under oil immersion lens (1000×). Post-surgical microscopic evaluation of blood smear revealed the presence of haemoprotozoan (Fig. 1). Further confirmatory diagnosis of blood parasites was established using PCR (polymerase Chain Reaction).

For *B. bigemina,* the PCR conditions were used as described in [17] with modifications. The conditions used were; 95°C for 1 min. followed by 37 cycles of 95°C for 30 seconds, 56°C for 30 seconds and 72°C for 30 seconds, followed by an extension step at 72°C for 5 min. Agarose gel electrophoresis showed the clear band on 321 bp length of PCR product. DNA ladder of 100 bp (Genedirex, Catalogue # DM001-R500) was used to compare the amplified product of PCR. Primers targeted 18S Ribosomal Gene of *B. bigemina* as shown in Figure 2. Molecular detection of *T.*

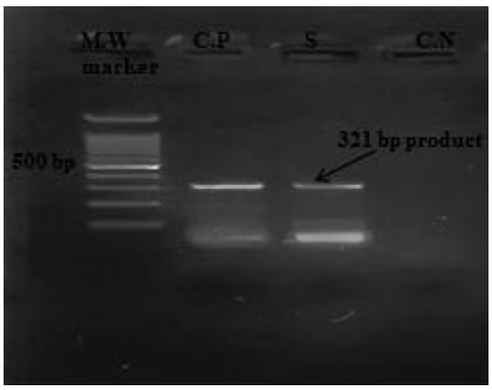

Figure 2. Gel electrophoresis showing 1.5% agarose gel stained with Ethidium bromide showing clear band on 321 bp (base-pairs) using species specific primers for *Babesia* bigemina in calf's erythrocytes
Lane 1: ladder (500 bp DNA); Line 2: positive control (CP); Line 3: positive sample having specific amplicon of 321 bp (S); Line 4: negative control (CN)

annulata was done by modifying the conditions described in [18]. The conditions were also different than that of *B. bigemina* as the thermocycler was operated at 95°C for 5 min. followed by 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 min., followed by an extension step at 72°C for 10 min. The ticks collected from the calf were identified as *Hyalomma* and *Rhipicephalus* under stereomicroscope and were proved to be the cause of mixed infection which ultimately made the prognosis grave. The *Hyalomma* ticks were identified as the ones with longer palps (mouthparts) and medium angular capitulum on the dorsal side as shown in Figure 3a. The legs were spotted and on the ventral side, genital as well as anal pores were present. Striations were seen on the integument texture. The basis capituli was found to have medium angular lateral margins. Eyes were always very convex. The scutum/conscutum was coloured brown. Enamel was absent as usual. The slender legs had pale rings and pulvilli were present as usual. Coxae 4 were of normal size and coxae 1 was noted to have large and equal paired spurs. An anal groove was spotted posteriorly to the anal pore as shown in Figure 3b.

In *Rhiphicephalus* the mouth parts were anterior and the palpi were wider than long and the palp articles were all small. The basis capituli was spotted to have a characteristic hexagonal shape. The legs were slender with pluvilli (pads) and weren't having pale rings.

A scutum was present in the examined ticks. Flat eyes were present and spiracular plates were large and posterior to legs. The anal groove was found posterior to the anus as demonstrated in Figure 4a and 4b.

The calf had an uneventful recovery from surgery but died after naturally acquiring *Babesia* on 11th day post infection presumably due to increased parasitic load, exhibiting typical mixed parasitic infection stigmata e.g. reddish urine, elevated temperature up to 41.3°C, lacrimal discharge and pale mucous membranes of eyes and gums.

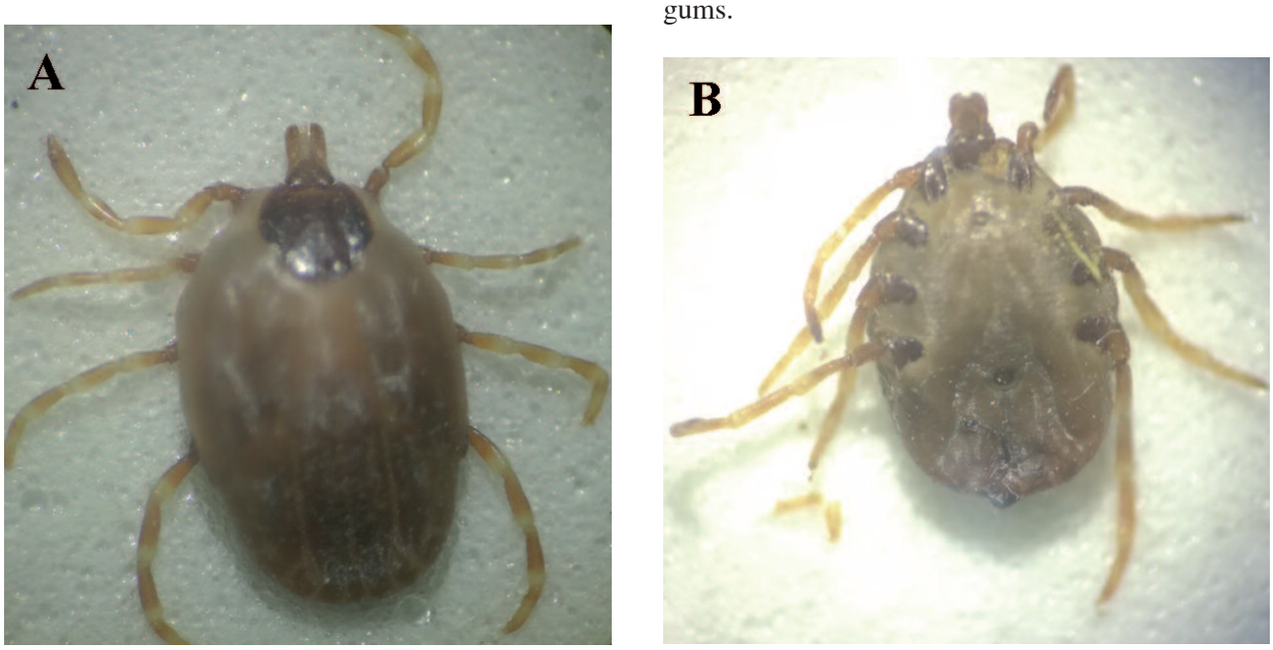

Figure 3. Dorsal and ventral representation of *Hyalomma* tick showing: long palps, scutum, medium angular capitulum, spotted legs, anal groove, anal and genital pores under stereomicroscope at 4× magnification

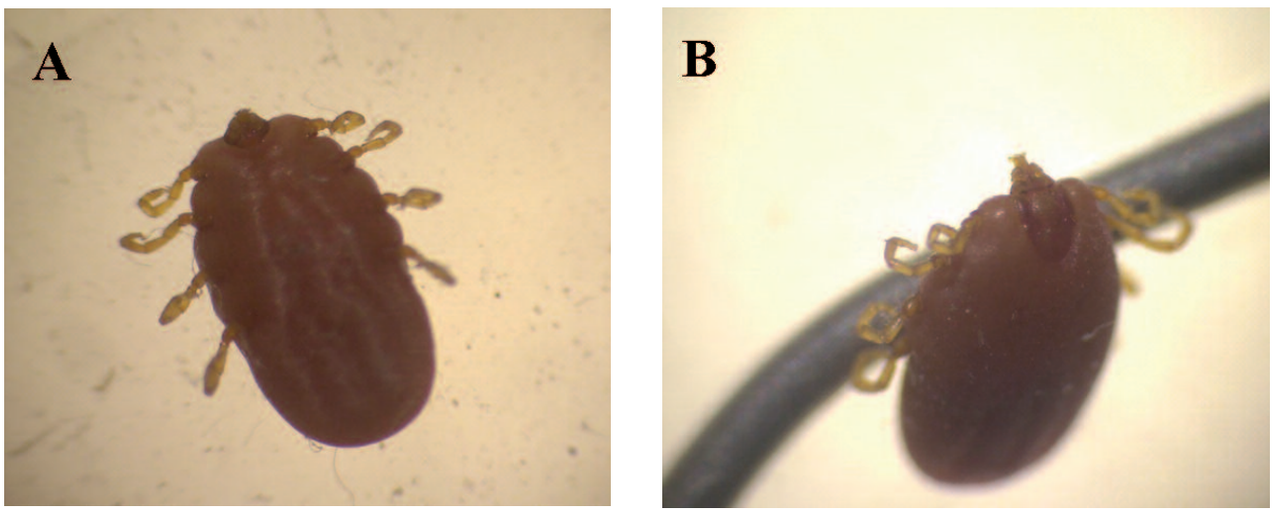

Figure 4. Dorsal and ventral representation of *Rhiphicephalus* tick showing: jointed legs, palps, hexagonal shaped basic capitulum, genital and anal pores under stereomicroscope

Upon post-mortem examination, pale carcass, icteric viscera, abnormal discoloration of eyes and mucous membranes were observed as shown in Figure 5a. Ruminal as well as intestinal bloating was also observed. Haemorrhage was noted around the jugular vein. Figure 5b is showing a flask containing 200 ml pericardial fluid obtained during post-mortem. The prescapular and prefemoral lymph nodes were normal in size.

In conclusions, the spleen and red pulp macrophages (RPMs) not only clear older red blood cells (RBCs) but also keep RBCs "healthy." RBCs on the other hand, contain enzymes that are capable to protect the cells from oxidative damage [19]. According to various studies parasitized RBCs are found in the red pulp of spleen [20]. Splenectomy in calf resembles the situation of functional asplenia which makes the patients to become more prone to blood-borne infections. The role spleen is critical to the survival of host in case of hemoparasitic infections of bovines. Spleen is considered to be the site of removal of infected erythrocytes and therefore is the central site of activity in response to hemoparasitic infections. Removal of spleen prior to infection makes the adult more susceptible and compromises the innate resistance of young calves. The initial splenic response is antibody-independent and involves the phagocytosis of both the infected erythrocytes and free parasites [21]. The surgical approach was used for splenectomy in standing calf under local anaesthesia was used instead of transthoracic approach using general anaesthesia with positive pressure ventilation. This technique doesn't need 360° rotation of spleen to bundle all splenic vessels for easier ligation [11]. Irrespective of the difference in sizes the larval, nymphal and adult ticks, mouthparts penetrate to a similar depth towards the base of the Malpighian layer. The penetration within host may occur within 5 min. of the arrival of the tick. Attachment is accomplished by the secretion of a cement substance in which the mouthparts are embedded and which adheres firmly to the host's skin [22]. The clinical signs in the calf after splenectomy allowed only a tentative diagnosis of blood parasites. Microscopic examination is still considered to be the standard criteria for the detection and diagnosis of tick-borne blood parasites [23]. Microscopy is usually adequate for detecting acute infections and not for detecting the carrier state animals because of low parasitaemia. PCR instead is a sensitive and highly specific method for the diagnosis of piroplasms [24]. Species-specific polymerase chain reactions (PCR) have been developed for the diagnosis and identification of *Theileria* and *Babesia* species [25]. After their development in the bone marrow, RBCs have a life span of approximately 120 days during which they have numerous interactions with macrophages of the spleen and liver. With time the plasma membrane of the RBC undergoes deleterious changes that make the cell susceptible to clearance by macrophages present in the red pulp of spleen and known as RPMs. In case of complete splenectomy these RPMs are not present, and RBCs increase in number along with the blood parasites like *Plasmodium* and *Babesia* species. Macrophages in the spleen play a key role in removing both older RBCs as well as blood-borne pathogens from the

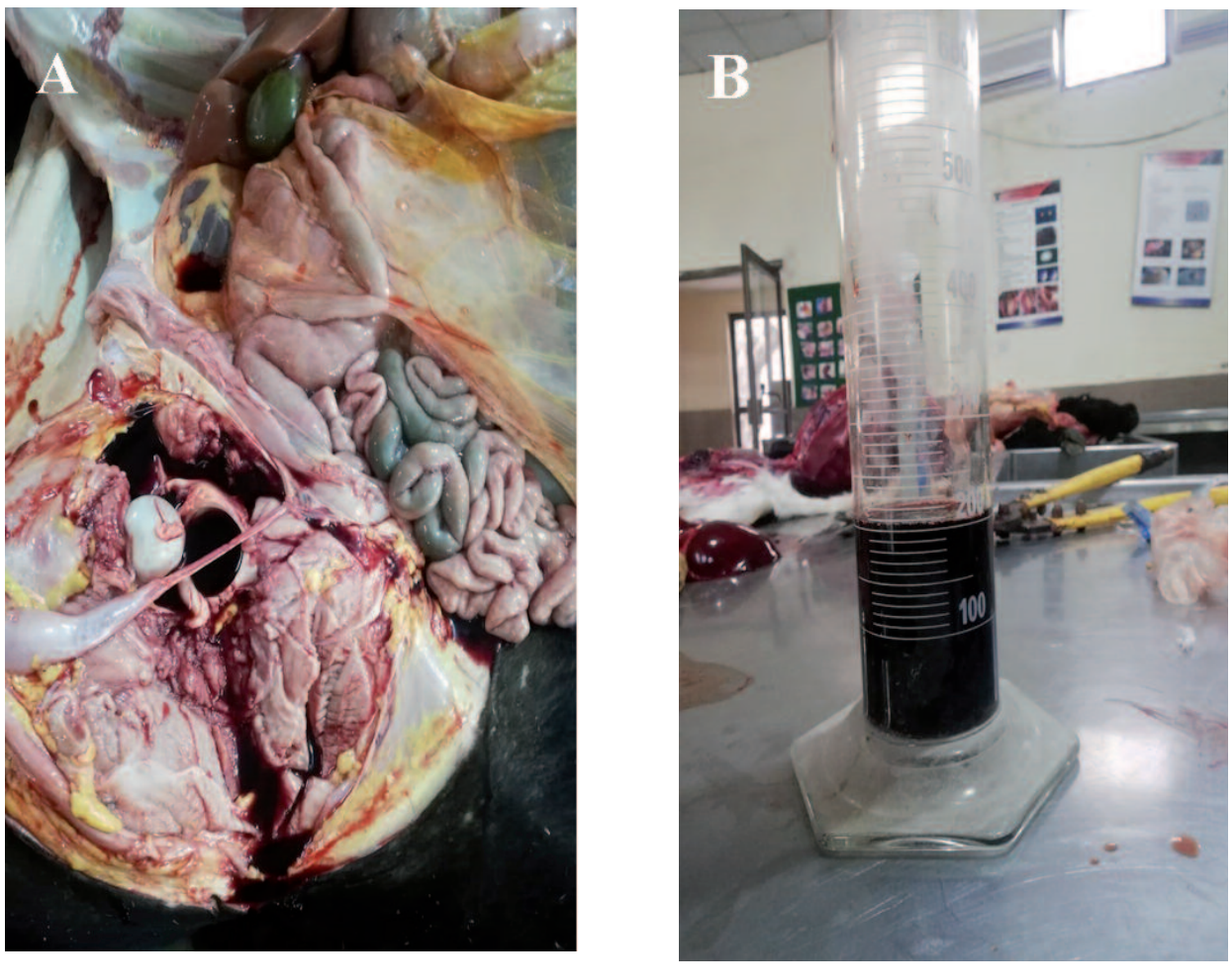

Figure 5. Postmortem image showing icteric visceras and pericardial fluid

circulation [26]. During infections, fever and mild anaemia usually occur between 3 and 8 days after infection. The anaemia is initially mild and non-regenerative but progresses to a more severe, regenerative anaemia by the 15th day following inoculation. Pyrexia occurs during the initial few days, regresses, and then recurs by the 15th day. Splenectomised animals are more severely affected and often die of hyper acute disease [27]. Dexamethasone@ 0.1mg/kg bodyweight (Dexavet Star laboratory, Punjab, Pakistan) was administered to calf to achieve the peak parasitaemia levels as described [15]. The splenectomised calf in this study died of babesiosis was severely jaundiced and haemorrhages were present. The organs were pale and reddish brown urine in bladder was observed as described by Jongejan et al. [28] showing that babesiosis is characterized by pyrexia, haemoglobinuria, anaemia and jaundice with demonstration of *Babesia* parasites in capillary blood.

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