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Current State in the Serological Diagnosis of Babesiosis and Haematological Changes in Splenectomised Buffaloes

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Abstract: The present study included two phases. The first phase aimed to describe the early changes in haematology of buffalo-calves during a period of one month after splenectomy. Two of these animals were followed up in the second phase which extended for further 45 days. After splenectomy, anemia developed quickly, reached peak on day24 and then with gradual improvement, the erythrogam approaches the normal at the end of second phase (day 75 post operation). Also, there was marked leucocytosis which reached its height at the first week post operation and then diminished gradually. In the second phase, an experiment was carried out to determine clinical picture and haematological alterations in splenectomised calves following experimental infection with *B. bovis* (by inoculation of $5x10^6$ cryopreserved infected cells) and to develop a serological methods and materials for early diagnosis. Both calves developed an acute disease characterized by parasitaemia, fever and anemia for several days after inoculation. The maximum values of MCV, reticulocytes and nucleated red cells were achieved from day 18 onwards. Leucocyte levels fell initially, followed by leucocytosis due to lymphocytosis, which fell again. A soluble antigen was obtained from *B. bovis* infected erythrocyte and purified using affinity chromatography. An enzyme-linked immunosorbent assay (ELISA) and immunoblotting were developed for the detection of anti-*B. bovis* antibodies in experimentally and naturally infected sera. Maximum titers were reached between days 9 and 14 (PI). Out of 112 sera from buffalo calves 34 showed higher antibody levels (30.4%). Immunodominant antigen was identified in the crude and purified fractions at MW of 78 kDa. The purified bound fraction is the most diagnostic antigen. It recognized specific band at MW of 43 kDa by *B. bovis* antiserum, not detected with the other antigens. It was suggested that buffalo-calves had begun to recover from anemia due to splenectomy, after 75 days post operation. Also, blood picture of the splenectomised-infected calves had improved after the *B. bovis* piroplasms eliminated from the blood.

Key words: *Babesia bovis,* · Buffalo · splenectomy · Haematology · ELISA · Affinity chromatography · SDS-PAGE, Immunoblotting

INTRODUCTION

Babesia bovis is intra-erythrocytic protozoan parasite transmitted by ticks to cattle in which they induce babesiosis, a disease that resembles human malaria. Anemia, caused by the destruction of non-infected erythrocytes, is a critical feature of the disease [1].

The spleen functions as a filter for the circulating blood, metabolism of blood cells and a coordinator of the immune response [2].

Characterization of babesial antigens based on pathophysiological characteristics led to the identification of fibrinogen associated antigen complexes [3]. The heparin binding babesial fraction revealed a single protein band in the approximate molecular weight of 43 kDa by sodium dodecyl sulphatepolyacrylamide gel electrophoresis [4].

Many immunodiagnostic tests have been developed for the detection of antibodies to *Babesia* spp. [5]. For *B. bovis* sensitive ELISAs were developed on the basis of antigen preparations made from infected blood [6]. The need for an improved capability to diagnose bovine babesiosis has become urgent in many tropical and subtropical countries. ELISA is desirable technique for

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automated testing of large numbers of serum samples. ELISA is more sensitive than Immuno-fluorscent antibody test (IFA) [7]. However, some samples are ELISA positive but Western blot negative. Therefore, recommendation of a screening by ELISA and confirmation of specificity by Western blot analysis is fair. Potentially, most of the problems associated with crude antigens can be overcome by the production of recombinant antigens. *Babesia orientalis* was detected in buffalo using semi-nested PCR by amplifying a specific 257 bp fragment of *B. orientalis* 18S rRNA gene [8].

Experimental *B. bovis* infections in buffalo, so far mostly focused either on the diagnosis or on the prevention of the disease. In Egypt, there is no clear control program for facing this blood parasite, therefore a scheme of diagnosis and immunization is needed. The present study was undertaken in an effort to furnish information and materials for investigating babesiosis and studing the clinical picture and hematoligical alterations in splenectomised buffaloes before and after infection. *Babesia bovis* soluble antigen and the corresponding antibodies were isolated, purified and evaluated as diagnostic reagents.

MATERIALS AND METHODS

Experimental animals: Four clinically healthy 2 month old male buffalo-calves were used in the current study. The calves were obtained from an area known to be free from Babesia and raised in hygienic barn at Faculty of Veterinary Medicine, Cairo University. The animals were protected against parasitic infections by drenching of albendazole (Pharma- Seed) at dose level of 2ml/20kg body weight and injection of ivermectin (Ivomec Super, Merial) subcutaneously at dose level of 1ml/50kg BWt before beginning of experiments. All calves were splenectomised one month prior to the inoculation of *B.bovis* piroplasm to minimize the immunity against infection and kept under healthy control conditions. The animals were routinely examined clinically during the experimental periods.

Sampling: Random blood and serum samples were collected from 112 buffaloes at Basateen slaughter house, Cairo.

Design of experiment

Phase 1: This phase aimed to document the early changes in haematology of buffalo-calves post splenectomy. On day 0, before splenectomy- which is considered as control- and every 3 days thereafter, for a period of one month, 2.5 ml of blood was taken from the Jugular vein into EDTA containing vacutainer tubes for the haematological evaluation. Red blood cell counts (RBCs), packed cell volume (PCV), haemoglobin (Hb), white blood cell counts (WBCs) and differential leucocytic counts were determined [9]. Red blood cell indices; mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated.

Phase 2: This phase describes the haematology of splenectomised calves following experimental infection with *B. bovis* in parallel with those splenectomised- non infected calves. One month later post splenectomy, the splenectomised calves divided randomly into two equal groups. Group 1 (calf no. 1 and calf no. 2) was kept as control while group 2 (calf no.3 and calf no.4) were inoculated at the same time with $5x10^6$ cryopreserved infected cells [10]. Observations were made on the calves before inoculation and daily afterwards for 45 days. 2.5 ml of blood was taken, twice weekly, from the Jugular vein into EDTA containing vacutainer tubes for haematology and daily into plain vacutainer tubes for separation of serum used for the serological tests. The reactivity was evaluated by ELISA. The animal's rectal temperatures were recorded and a temperature of 39.4°C or above was taken as being indicative of a febrile response. Giemsa-stained thin blood smears were examined for the presence of parasites for 21 days. Parasiteamia estimations were made [11].

Prepartion of soluble *B. bovis* antigens: The antigen was prepared at the peak of parasiteamia according to [12].

Afinity chromatography

a- Preparation of immunoglobulins: Immunoglobulins were prepared by precipitation with an equal volume of saturated ammonium sulphate solution and the anti-*Babesia bovis* antiserum obtained from experimentally infected calves. The protein content of the immunoglobulin solution was assessed by A280 nm estimation.

b- Purification of IgG using Protein G column: The immunoglobuline solution obtained from the pervious step was loaded in 2 ml of immobilized protein G column (Immunopure). Antibody titer of the elute was determined at an optical density 280 nm [13].

c- Affinity chromatography of soluble antigen: The obtained IgG was coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the manufacturers' recommendations. Crude babesial antigen in 0.05 M Tris-HCI pH 8.2, were applied to affinity columns containing the homologous anti-*Babesia bovis* IgG. The run-through fractions (2 ml) were monitored at A280 nm. The unbound antigen was collected and designated unbound fraction. Antigen specifically bound to the affinity column was eluted into a small volume of PBS using 0.05 M glycine-HCl pH 2.8. This antigen was then designated bound fraction [14].

Enzyme linked immunosorbent assay (ELISA): ELISA was carried out [6] and utilizes soluble and purified antigens. Sera from calves naturally and experimentally infected with *B. bovis* were screened by this technique. The positive threshold value was determined to be two-fold the mean cut-off value of negative sera.

SDS-PAGE analysis of *B. bovis* proteins: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was undertaken to study the polypeptide profile of crude and affinity purified babesial antigens [15].

Immunoblottig: Protein blotting was performed according to a modification of the method of [16]. The crude as well as the purified fractions were separated by SDS-PAGE and then transferred from the gel to nitrocellulose paper. This paper was incubated with diluted anti-sera raised in splenectomised buffaloes. After washing, papers were incubated with peroxidase-conjugated anti-bovine IgG and then exposed to substrate solution for 30 min. Analysis of the separated bands was performed by software analysis (Gel analyzer).

RESULTS

Data for individual animals within each group have been pooled and consequently only average trends are discussed. No advantage was apparent from undertaking statistical analysis on individuals or groups.

Phase 1: The mean early changes in haematology of four splenectomised buffalo-calves, during a period of one month are summarized in Fig 1. After splenectomy, the animals showed decline in RBCs, compared with 0-time to reach the lowest mean count as 6.04 x10 ^{6}/mm^{3} on day 21.The Hb contents decreased reaching their lowest levels (6.67 g%) coincidentally with lowest mean RBCs, while PCV levels slightly elevated. The MCH and MCHC showed marked decline while MCV increased reaching its peak on day 24 post splenectomy. The mean numbers of WBCs greatly increased to 24.25 x10^{3}/mm ^{3}on the third day of splenectomy, remained high on the 6th day and then decline gradually. Differential leucocytic counts revealed marked elevation in absolute number of neutrophils from approximately 2.83 to 9.97 x10^{3}/mm^{3} on days 3 and 6 and then slowly decline. Eosinophils increased post operation from 0.34 x10^{3} /mm^{3}to reach their peak 1.17 on day12 then decline gradually.

Phase 2: In Fig 2, the percentages of parasite are plotted against time after inoculation of the two splenectomised calves (calf no.3 and calf no.4). Calf no.3 was parasitaemic from day 6 to day 17, with a maximum parasitaemia of 10% erythrocyte infected on day12.While calf no.4 demonstrated parasite from day 7 to day16, with a maximum parasitaemia of 9% on day13.

-In Fig 3, the individual daily rectal temperature for each of splenectomised- infected calves is plotted against time after inoculation. Calf no.3 had a febrile response from day 8 to day 16, whilst calf no.4 had a response from day 9 to day16.

-Fig. 4, describes the haematology of the two splenectomised-infected calves compared to those splenectomised non-infected. Both infected calves (calf no.3 and calf no.4), showed a marked decline in RBCs, PCV and Hb levels after day 9, remained depressed until the 21th day of infection and then rose steadily until the 45th day. The MCV rose, while the MCHC decline on the 12th day 9 Post-infection and returned to pre-infection levels by day 45.The rise in the MCV corresponded with the appearance of reticulocytes, nucleated erythrocytes and anisocytosis in blood smears. Leucocyte numbers fell during the onset of parasitaemia in both calves reflecting the trends of lymphocyte and neutrophil numbers, but rose continuously after day15- 18 due to lymphocytosis and then fell again after day 27. The neutrophils and lymphocytes declined from approximately 3.66 - 3.9 to 1.7 - 1.9 and 7.07 - 7.42 to 3.66 - 3.92x10^{3}/ mm^{3}, respectively and then rose slowly again. Although the numbers of lymphocytes and neutrophils decline during the early reaction period, the numbers of monocytes rose from approximately 0.54 - 0.61 to a peak of 2.63 - 2.9x 10^{3} / mm^{3} on day 12 and declined gradually to normal levels by day 21. During leucocytosis, lymphocytes reach to a peak of

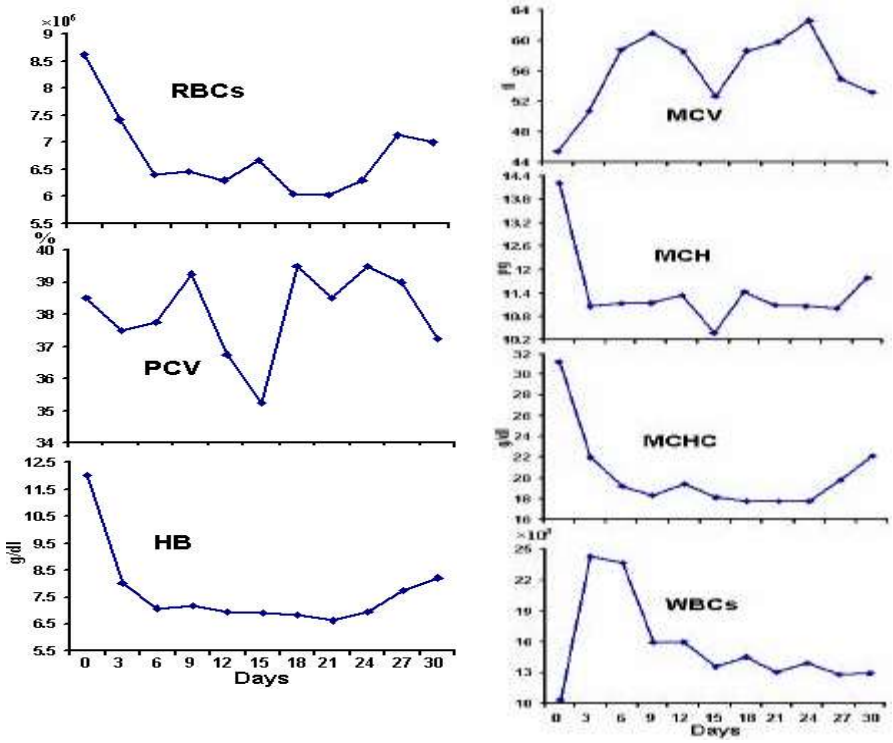

Fig. 1: Mean haematological data of four buffalo- calves after splenectomy

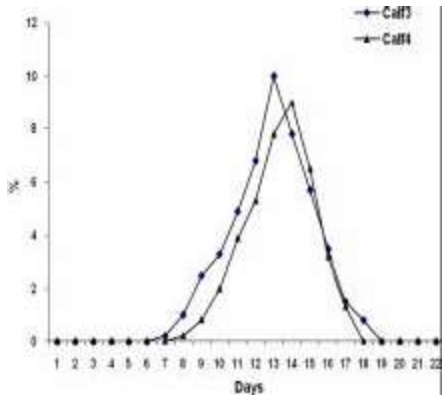

Fig. 2: Daily parasitaemia of the two splenectomized buffalo-calves inoculated with *B. bovis*.

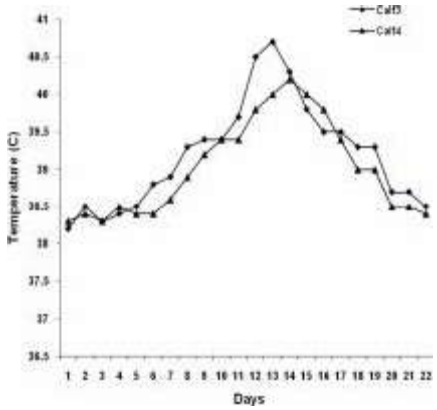

Fig. 3: Daily rectal temperaters of the two splenctomized buffalo-calves inoculated with *B. bovis.*

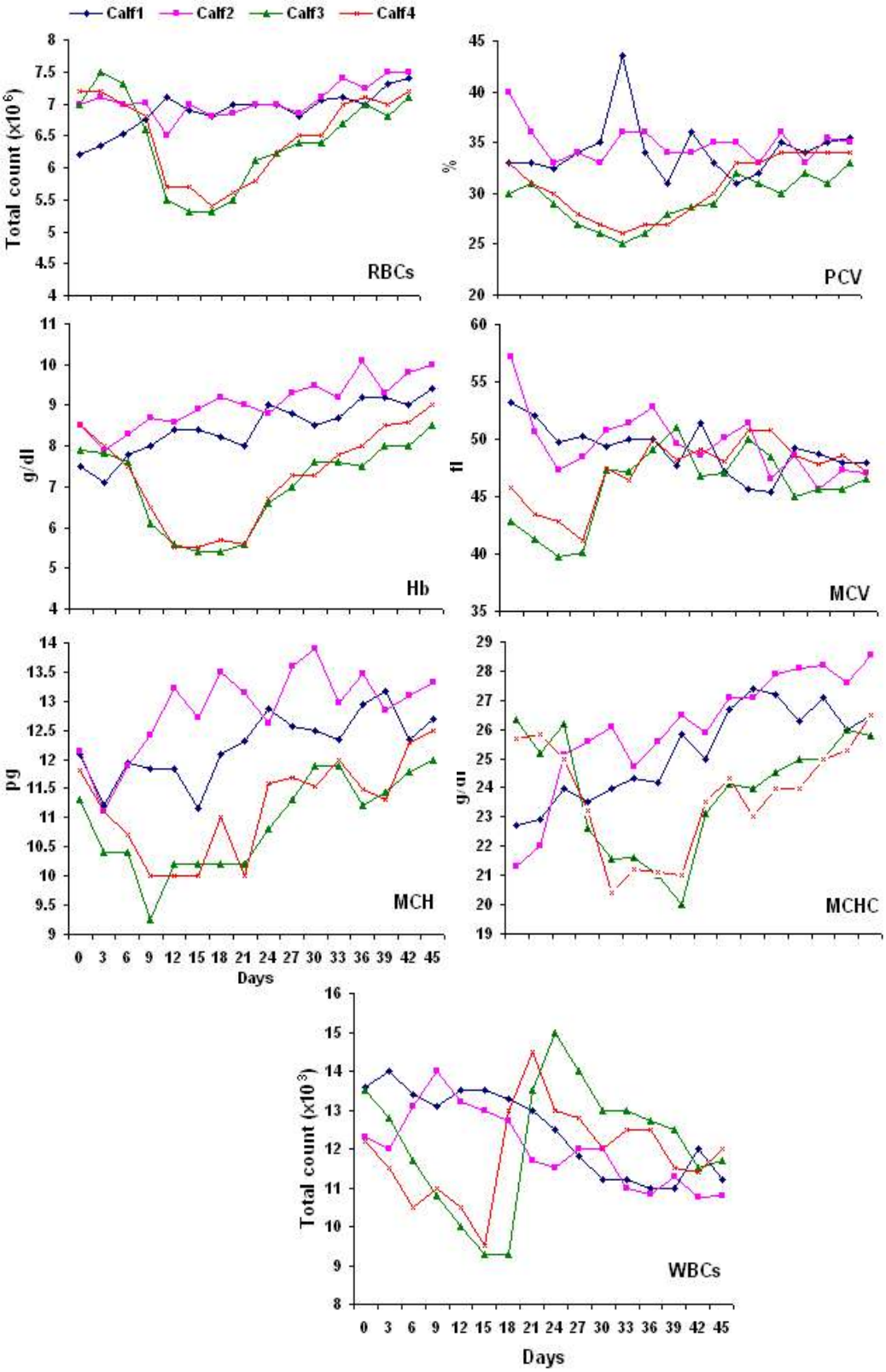

Fig. 4: Haematological parameters of two splenectomised buffalo – calves inoculated with *B.bovis* (calf no.3 and calf no.4) compared with two splenectomised non infected buffalo-calves (calf no. 1and calf no. 2)

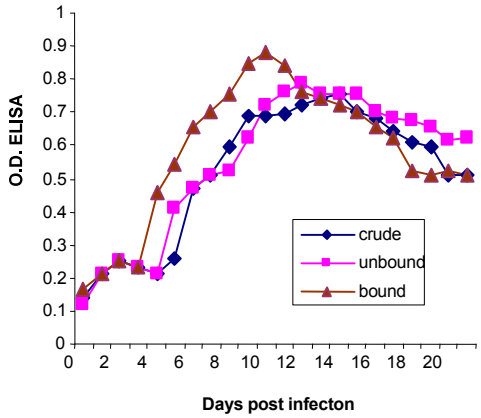

Fig. 5: The antibody level (meam optical density) of serum from splenectomized buffaloe No. 1 experimentally infected with B. bovis using ELISA

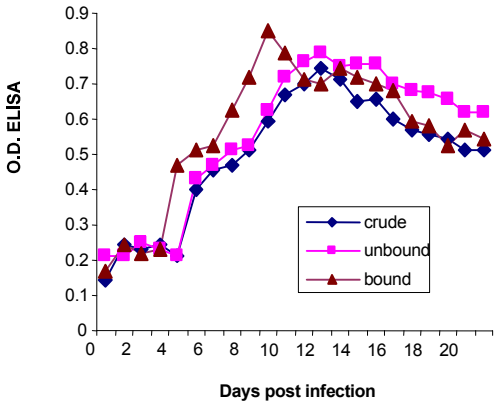

Fig. 6: The antibody level (meam optical density) of serum from splenectomized buffaloe No. 2 experimentally infected with B. bovis using ELISA

9.6 – 11.7x10^3/ mm^3 on day 24. The blood pictures of both splenectomised-non infected calves (no.1 and no.2) gradually improved, but still lower than the pre splenectomy levels.

ELISA results: The crude and affinity purified fractions were evaluated by ELISA for diagnosing of buffalo babesiasis. The antibody levels of experimentally infected calf No.1 appeared at 6, 5 and 4 day (PI). The antibodies were gradually increased and reached the highest concentration at 14, 12 and 9 days (PI) by using crude, unbound and bound antigens, respectively (Fig 5). While in the calf No.2, the antibody levels appeared at 5, 5 and 4 day (PI) and the antibodies were gradually increased and reached the highest concentration 12, 12 and 9 days (PI) by using crude, unbound and bound antigens,

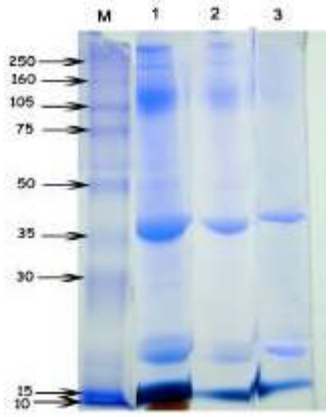

Fig. 7: SDS-PAGE pattern of Babesial antigenic fraction obtained from affinity chromatography. M: molecular weight standard; 1: soluble crude antigen 2: unbound fraction; 3: bound fraction

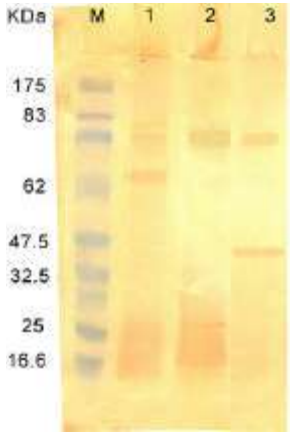

Fig. 8: Different Babesia bovis antigenic fractions recognized by homologous antiserum M: molecular weight standard; 1: soluble crude antigen 2: unbound fraction; 3: bound fraction

respectively (Fig 6). It was clear that the purified bound fraction is the most diagnostic antigen.

Out of 112 buffaloes, 30.4% were seropositive using ELISA with mean O.D. values at 405 nm ranged from 0.688 to 0.596.

Analysis of different antigenic fractions obtained from affinity chromatography: The crude antigens as well as the purified antigenic fractions (unbound and bound) were analyzed by SDS-PAGE. Crude and unbound antigens were fractionated into six polypeptide bands at molecular weight ranged from 340–13.8 kDa (Fig 7, Lane 1 and2). Bound antigen was separated into 3 polypeptide

bands 37.59, 19.29 and 15.7 kDa (Fig 7, Lane 3). The shared polypeptides bands between the three antigens were at MW of 37 and 19 kDa.

Analysis of different antigenic fractions obtained from affinity chromatography recognized by Babesia antiserum: The best diagnostic features were presented by immuno-blotting using anti-*B.bovis* experimentally infected serum, whereas, it reacted with all antigens (soluble and purified fractions). Analysis of different antigenic fractions was illustrated in Fig 8. The results revealed that anti-*B. bovis* anti-serum recognized 5, 3 and 2 epitopes in crude soluble, unbound and bound antigens, respectively. These results showed one common reactive epitopes in the three antigens at 78 kDa. The purified bound fraction recognized specific band at MW of 43 kDa by *B. bovis* antiserum not detected with the other antigens.

DISSCUSION

Although there are numerous publications on the experimental *B.bovis* infections in splenectomised cattle, very little literature describe the characteristics of the disease in buffaloes. The first phase of this paper describes the early changes in haematology of buffalo-calves during a period of one month post splenectomy, which follow up for a larger period in the second phase. As shown in Fig 1, splenectomy is followed by marked decrease in RBCs and Hb contents as compared to reference values for this species [9]. The observations indicated that a hypo chromic-macrocytic anemia developed almost immediately after splenectomy as manifested by reduced MCHC and increased MCV, reached its greatest severity at the end of about three weeks and half. After gradual improvement, erythrogram picture returned to approach the normal at the end of phase 2, but still less than the pre splenectomy. These results appear to demand a supplementary study on a larger number of animals and for longer periods. In this respect, buffalo-calves behaved similar to splenectomised small animals [9, 17]. Although other cells in the body can metabolize iron in a similar fashion of spleen, a splenic patients display lower iron for a considerable amount of time after loss of spleen, which is essential for Hb synthesis and RBCs regeneration [18].

Concerning leucogram, the splenectomised calves developed leucocytosis immediately after operation, with rapid fall after one week and then a more gradual decrease, reaching almost to the normal levels at the end of the second phase. The initial leucocytosis was mainly due to an increase in poly morphonuclear neutrophils and eosinophils. In attempting to explain the gradual return toward normal after splenectomy, that hyperplasia of other tissues of the reticulo–endothelial system such as the lymph or haemolymph nodes occurs and take on the activities of the spleen [2, 19]. In phase 2 of the current study, the reactions of the two splenectomised calves to the inoculation of *B. bovis* (calf no. 3 and calf no. 4) correspond well with those reported previously for animals infected with other species of *Babesia* [20-22]. Reactions of both calves to inoculation of *B. bovis* were similar except that there was extended parasite and fever period in calf no.3. The first appearance of *B.bovis* piroplasms in calf no. 4 was delayed compared to calf no. 3. Both calves had febrile responses which tended to reach a peak coinciding with the peak of parasitaemia and declined to normal levels as the parasites disappeared. After patency, both infected calves showed a steady decline in RBCs, PCV and Hb levels until the parasites were eliminated. It was noted the rate of erythrocyte destruction appears to parallel the parasitaemia. The induced hypochromic – macrocytic anemia in both infected calves may result from the haemolytic process [23]. An increased haemopoiesis then occurred, which was evidenced in stained blood smears by presence of nucleated erythrocytes, polychromasia and anisocytosis. There was marked fluctuations in numbers of WBCs. After patency, there was decline in WBCs reflecting a decrease in numbers of neutrophils and lymphocytes, while monocytosis occurs. Terminally there was a leucocytosis accompanied by a lymphocytosis probably as a result of an immune response to babesia infection [20].

The crude and affinity purified antigens for diagnosing buffalo babesiasis was evaluated by ELISA. Maximum titers were reached between days 9 and 14 (PI). Out of 112 sera from buffalo calves 34 showed higher antibody levels (30.4%). It was concluded that the purified bound fraction is the most diagnostic antigen. In this respect, [24] screened sera from calves experimentally infected with *B. bovis* by ELISA from day 9 to day 233 (PI). Maximum titers reached between days 29 and 149 PI. Sera from calves heifers and cows, raised in tick-infested areas showed higher antibody levels in heifers and cows.

In the present study the soluble crude babesial antigen was purified using affinty chromatography. Anti-erythrocyte antibodies were purified from the sera of

infected calves by coupling to a Sepharose-4B column and than used for anti-idiotypic antibodies purification. Results confirmed the presence of anti-erythrocytes antibodies in higher amounts in the serum of infected cattle [1]. Murine monoclonal antibodies (MAbs) were produced against the 'beta' fraction of *Babesia bovis*. The antigen identified by (MAbs) was extracted from *B. bovis* infected erythrocytes by affinity chromatography [13].

In this study immunoblotting has identified proteins of similar molecular mass in the crude and purified fractions at MW of 78 kDa. The bound fraction is specifically recognized by *B. bovis* antiserum at MW of 43 kDa and therefore could be useful for the production of the recombinant replica and to employ these in further test systems. Babesia bigemina infection of mature bovine erythrocytes results in new proteins specifically exposed on the parasitized cell surface [25]. Also, previous work with both *B. bigemina* and *B. bovis* has demonstrated the existence of isolate as well as species cross-reactivity among merozoite proteins [26]. A *Babesia divergens* merozoite antigen was purified by affinity chromatography using a monoclonal antibody. Silver staining of SDS-PAGE gels revealed 2 bands of MW 50-60 and 24-29 kDa. An esterase was isolated from a crude extract of *Babesia bovis* by affinity chromatography, using soy bean trypsin inhibitor as a ligand [27]. Three (MAbs) were produced against a partially purified lysate antigen. Antigens affinity purified by the respective MAbs had molecular weights of 44, 100 and 130 kDa [28]. Electrophoresis and Coomassie blue staining of heparin eluate revealed only a single band with an apparent mobility of 43 kDa [29]. Ten MAbs were generated against five surface-exposed proteins (16 kDa, 42 kDa, 44 kDa, 60 kDa, 225 kDa) on merozoites of *Babesia bovis* [30].

CONCLUSION

The observations are too few in number and not sufficiently to allow definite conclusions but it was suggested that buffalo-calves had begun to recover from temporary anemia due to splenectomy, after 75 days post operation. Also, haemogram of the splenectomised-infected calves had improved after the *B. bovis* piroplasms eliminated from the blood. The purified bound fraction is the most diagnostic antigen and it is specifically recognized by *B. bovis* antiserum at MW of 43 kDa. Therefore could be useful for the production of the recombinant replica and to employ these in further test systems.

REFERENCES

1. Góes, T.S., V.S. Góes, M.F. Ribeiro and C.M. Gontijo, 2007. Bovine babesiosis: anti-erythrocyte antibodies purification from the sera of naturally infected cattle. Veterinary Immunology and Immunopathology, 116: 215-288.
2. Greer, J.P., J. Foerster, J.N. Lukens, G.M. Rogers, F. Paraskevas and B.E. Glader, 2004. Wintrobs,s Clinical Hematology, 11th Edn. Philadelphia: Lea and Febiger.
3. Goodger, B.V., M.A. Commins, I.G. Wright D.J. Waltisbuhl and G.B. Mirre, 1987. Successful homologous vaccination against *Babesia bovis* using a heparin-binding fraction of infected erythrocytes. International Journal of Parasitology, 17: 935-940.
4. Gangwar, S., J.R. Rao, A.K. Mishra and A.K. Tewari, 2000. Fractionation of heparin binding and fibrinogen associated proteins from *Babesia bigemina* infected erythrocytes and their electrophoretic analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Journal of Veterinary Parasitology, 14: 13-16.
5. Weiland, G. and I. Reiter, 1988. Methods for the measurement of the serological response to *Babesia*. In: M. Ristic (Editor), Babesiosis of Domestic Animals and Man. CRC Press, Boca Raton, FL, pp: 143-162.
6. Waltisbuhl, D.J., B.V. Goodger, I.G. Wright, M.A. Commins and D.F. Mahoney, 1987. An enzyme linked immunosorbent assay to diagnose *Babesia bovis* infection in cattle. Parasitology Research, 73: 126-131.
7. Ravyn, M.D., J.L. Goodman, C.B. Kodner, D.K. Westad, L.A. Coleman, S.M. Engstrom, C.M. Nelson and R.C. Johnson, 1998. Immunodiagnosis of human granulocytic ehrlichiosis by using culture-derived human isolates. Journal of Clinical Microbiology, 36: 1480-1488.
8. Liu, Q., Y.Q. Zhou, D.N. Zhou, E.Y. Liu, K. Du, S.G. Chen, B.A. Yao and J.L. Zhao, 2007. Semi-nested PCR detection of *Babesia orientalis* in its natural hosts *Rhipicephalus haemaphysaloides* and buffalo.Veterinary Parasitology, 143: 260-266.
9. Feldman, B.F., G. Joseph, J.G. Zinkl and N.C. Jain, 2000. Schalm's Veterinary Hematology. 5th Edn., Lippincott Williams and Wilkins Co. Philadelphia.

10. Yao, B., J. Zhao, L. Ma and Z. Liu, 1997. Studies on the pathogenicity of *Babesia bovis* in water buffaloes after cryopreservation and resuscitation. Tropical Animal Health and Prodroduction, 29: 40S-42S.
11. Parker, R., 1973. A direct counting tech nique for estimating high parasiteamia in infections of *Babesia argentina, Babesia bigemina* and *Plasmodium berghei.* Annals of Tropical Medicine and Parasitology, 67: 387-390.
12. Goodger, B.V., I.G. Wright, D.J. and G.B. Mirre, 1985. *Babesia bovis*: Successful vaccination against homologous challenge in splenectomized calves using a fraction of haemagglutinating antigen. International Journal of Parasitology, 15: 175-179.
13. Woollard, D.J., C.G. Gauci and M.W. Lightowlers, 2000. Synthetic peptides induce antibody against a host protective antigen of *echinococcus granulosus*. Vaccine, 18: 785-794.
14. Goodger, B.V., D.J. Waltisbuhl, I.G. Wright and M. White, 1992. *Babesia bovis*: analysis of and preliminary vaccination studies with a defined infected erythrocyte membrane binding antigen. International Journal of Parasitology, 22: 533-535.
15. Laemmli, U.K., 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T_4. Nature, 227: 680-685.
16. Towbin, H., T. Staehelin and J. Gordon, 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proceeding Natural Academic Science. USA, 176: 4350-4354.
17. William, O., D.V.M. Reece and R. Snodgrass, 1972. Effect of splenectomy on postfeeding changes in packed cell volume of dogs. American Journal of Veterinary Research, 33: 635-637.
18. Lee, G.R., J. Foerster, J. Lukens, F. Paraskevas, J.P. Greer and G.M. Rodgers, 1999. Wintobe's Clinical Hematology, 10 th Edn. Philadelphia: Leaand Febiger.
19. Athens, J.W., 1993. the Reticulo endothelial (Mononuclear phagocyte) system and the spleen. In: Lee, G.R. *et al*.,eds,Wentrobe's Clinical Hematology, 9th Edn., Philadelphia:Lea and Febiger, 1704-1722.
20. Wright, I.G., 1973. Observations on the haematology of experimentally induced *Babesia argentina* and *B. bigemina* infections in splenectomised calves. Research Veterinary Science, 14: 29-34.
21. Purnell, R.E., E.R. Young, D.W. Brocklesby and D.J. Hendry, 1977. The haematology of experimentally-induced *B. divergens* and *E. phagocytophila* infections in splenectomised calves. Veterinary Record, 100: 4-6.
22. Marco, I.R., J. Velarde, D. Castella, A. Ferrer and S. Lavin, 2000. Presumptive *Babesia ovis* infection in a Spanish ibex *(Capra pyrenaica)*. Veterinary parasitology, 87: 217-221.
23. Hillman, R.S., 1998. Iron deficiency and other hypo proliferative anemias. In: Faucci, A.S., Braunwald, E., Isselbacher, K.J., Wilson, J.D., Martin, J.B., Kasper, D.L. Hauser, S.L., Longo, G.L. (Eds.), Harrison's Principles of Internal Medicine, 14th Edn., McGraw-Hill, Madrid, pp: 638-645.
24. Machado, R.Z., H.J. Montassier, A.A Pinto, E.G. Lemos, M.R. Machado, I.F. Valadão, L.G. Barci and E.B. Malheiros, 1994. An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against *Babesia bovis* in cattle. Veterinary Parasitology, 15: 17-26.
25. Echaide, I.E., S.A. Hines, T.F. McElwain, C.E. Suarez, T.C. McGuire and G.H. Palmer, 1998. *In Vivo* Binding of Immunoglobulin M to the Surfaces of *Babesia bigemina*-Infected Erythrocytes. Infection and Immunology, 66: 2922-2927.
26. McElwain, T.F., G.H. Palmer, W.L. Goff and Mcguire, 1988. Identification of *Babesia bigemina* and *Babesia bovis* merozoite protein with isolate and species common epitopes recognized by antibodies in bovine immune sera. Infection and Immunology, 56: 1658-1660.
27. Wright, I.G., B.V. Goodger, K.R. Rode-bramanis, M.J.S. Matick, D.F. Mahoney and D.J. Waltisbuhl, 1983. The characterization of an estrase derived from *Babesia bovis* and its use as a vaccine. Ziecsrift Parasitnkend, 69: 703-714.
28. Wright, I.G., G. Mirre, B.K. Rode-bramanis, M. Chamberlain, B.V. Goodger and D.J. Waltisbuhl, 1985. Protective vaccination against virulent *Babesia bovis* with a low molecular weight antigen. Infection and Immunology, 48: 109-113.
29. Mahoney, D.F., I.G. Wright and B.V. Goodger, 1981. Bovine babesiosis: The immunization of cattle with fractions of erythrocytes infected with *Babesia bovis* (syn. *B. argentina*). Veterinary Immunology and Immunopathology, 2: 145-156.
30. Reduker, D.W., D.P. Jasmer, W.L. Goff, L.E. Perryman, W.C. Davis and T.C. McGuire, 1989. A recombinant surface protein of *Babesia bovis* elicits bovine antibodies that react with live merozoites. Molecular Biochemistry and Parasitology; 35: 239-247.

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