In-vivo Therapeutic Efficacy Trial with Artemisinin Derivative, Buparvaquone and Imidocarb Dipropionate against *Babesia equi* Infection in Donkeys

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ABSTRACT. The therapeutic efficacy of imidocarb, artesunate, arteether, buparvaquone and arteether+buparvaquone combination was evaluated against *Babesia equi* of Indian origin in splenectomised donkeys with experimentally induced acute infection. Efficacies of these drugs were tested by administering each drug or drug combination to groups of donkeys (having three donkeys each group). One group of donkey was kept as untreated control for comparing the results. Parasitaemia, haematology (WBC, RBC, PCV, granulocytes and haemoglobin), biochemical parameters (SAST, SALT, alkaline phosphatase, albumin/globulin ratio) were monitored at regular intervals. Individually, arteether and buparvaquone were found to have no parasite clearing efficacy and the treated animals died within 5–6 days after showing high parasitaemia and clinical symptoms of the disease. However, artesunate treated animals were able to restrict the parasite multiplication but only during the treatment period. Animals treated with imidocarb and arteether+buparvaquone combination were able to clear the parasite from the blood circulation after 2–5 days post-treatment (PT). After 55–58 days PT, recrudescence of *B. equi* parasite was observed in both these groups and a mean survival period of 66 days and 69 days, respectively, was recorded in these groups. Results of haemato-biochemical parameters had shown that imidocarb had deleterious effect on the liver function while on the other hand arteether+buparvaquone combination was found to be safe. This limited study indicates that arteether+buparvaquone combination could be a better choice than imidocarb for treating *B. equi* infection, but further trials are required in detail.
KEY WORDS: artemisinin derivative, *Babesia equi*, buparvaquone, imidocarb dipropionate, treatment.

Equine babesiosis is caused by *Babesia equi* and *Babesia caballi* and both protozoa are transmitted by the same tick-vector in an endemic area and hence are closely associated. *B. equi* is more widely distributed than *B. caballi* [19] and is generally more resistant to treatment than *B. caballi*. Efficacy of many babesicidal drugs against *B. caballi* is well documented as compared to *B. equi* infections [17, 18]. So far none of the drug has been found to completely cure the animals from *B. equi*. For treatment of equines infected with *B. equi*, repeated doses of the drug are usually used and this approach controls clinical infection but will not clear the parasite completely from the body. Imidocarb, the only drug of first choice against *B. equi* infection, is also partially successful in eliminating *B. equi* from the infected horses [10, 18]. This drug has also been observed to be harmful to horses [1] and recrudescence of parasitemia in treated animals is common. Parvaquone and buparvaquone, anti-theilerial agents, have also been tried against acute *B. equi* infection but with limited efficacy [18, 31]. Recently, artemisinin (qinghaosu) derivatives viz. artesunate, arteether, artemether have been found very effective in treatment of multiple drug resistant human cases of falciparum malaria [5, 25, 29] and *Babesia* in *in-vitro* culture system (22). The aim of the present study was to test these new drugs against acute *B. equi* infection, experimentally produced in donkeys, and to compare their efficacy against imidocarb treated donkeys.

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MATERIALS AND METHODS

Animals: Eighteen indigenous non-descript, healthy and *B. equi*-free donkeys 1.0 to 2.5 years of age, were used in the present drug trail. The *Babesia equi* free status of these donkeys was ascertained by testing their serum for the absence of specific antibodies by conducting Dot-ELISA [15]. These donkeys were randomly divided into six groups of three donkeys per group. All the donkeys were splenectomised under general anaesthesia [7]. The objective of the splenectomy was to induce acute *B. equi* infection and to relinquish the parasite clearing efficacy of body immune system so that potency of the drug(s) under test can be accurately assessed. After a suitable duration (5–7 days after surgery) of post-operative care, these were infected with *B. equi* parasite (Indian strain) [12] by intravenous inoculation of about 50 m*l* blood, collected in acid citrate dextrose (ACD) solution from a known *B. equi* carrier donkey maintained at National Research Centre on Equines, Hisar, India.

Experimental design: Drug treatments in different groups were initiated when parasitaemia was between 6.4–12.5% (refer Table 1) (defined as day '0'). Donkeys in group I were treated by inoculating imidocarb dipropionate (Imizol®, M/s Schering-Plough Animal Health Corporation, Germany) at the dose rate of 5 mg/kg body weight with 4 doses at 72 hr intervals. Group II donkeys were treated with artesunate (Falcigo®, M/s Zydus Cadila Health Care Limited, India) at the level of 2.5 mg/kg body weight daily for 4 days. Group III donkeys were treated by injecting arteether (E-Mal®, M/s Themis Laboratories Limited, India) at a dose rate of 5 mg/kg body weight daily for 3 days. Group IV

donkeys were treated with buparvaquone (Butalex®, M/s Zydus Aqrovet, India) at a dose rate of 5 mg/kg body weight daily for 4 days. Group V donkeys were treated with arteether and buparvaquone combination at a dose rate and duration as mentioned above. Imidocarb dipropionate, arte-sunate and arteether were administered by intramuscular route while buparvaquone was given intravenous route. Group VI donkeys were kept as untreated control. Drug efficacy was defined as the mean percent suppression of parasitaemia as compared with non-treated control donkeys group (VI). It is to certify that the experiment was con-ducted as per the guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Ani-mals (CPCSEA), Animal Welfare Division, Government of India. During the experiment, animals were treated humanely as per the procedure approved by Institutional Animal Ethic Committee (IAEC).

Blood samples and clinical observations: Initially, blood and serum samples, both for haematology and biochemical analysis were collected respectively in heparinised vac-cutainers (Vacuette® Greiner GmbH, Germany) and serum collection tubes from all the donkeys daily for a week, then twice a week up to 35 days post-treatment (PT). Parasi-taemia in peripheral circulation was monitored throughout the observation period by making a thin blood smear from a drop of blood from the tip of ear and its percentage was determined by counting at least about 2000 erythrocytes per stained smear. After recrudescence of parasitaemia, samples were collected twice a week until recovery or death.

Haematological parameters: Haemoglobin (Hb, g/d*l*), packed cell volume (PCV, %), red blood cell count (RBC, 10^6/cmm), white blood cell count (WBC, 10^3/cmm), granu-locytes (%), lymphocytes (%), monocyte (%) were esti-mated in the blood of donkeys (Group I to VI) using MS 9VET Blood Cell Counter (M/s Melet Schloesting Labora-tories, France).

Biochemical Parameters: Serum aspartate aminotrans-ferase (SAST, IU/*l*), serum alanine aminotransferase (SALT, IU/*l*), serum alkaline phosphatase (SAP, IU/*l*), albu-min (g/d*l*), total serum protein (TSP, g/d*l*) were estimated using single step reagent kits (M/s Transasia Biomed Pvt Ltd, India) with the help of auto analyzer (M/s Biosystem BST-370, Germany). Globulin (TSP - albumin = globulin) and albumin/globulin (A/G) ratio was determined mathe-matically.

Statistical analysis: Data was subjected to statistical anal-ysis by Student's *t* test as described by Snedecor and Cochran [28].

RESULTS

Efficacy of different drugs with respect to clinical and haematological observations: Tables 1 and 2 present the information on drugs used and haematological observations. Imidocarb treated animals (group I) recovered from initial parasitaemia two days post-treatment (PT), but after 54 days of treatment recovery, recrudescence of parasitaemia was
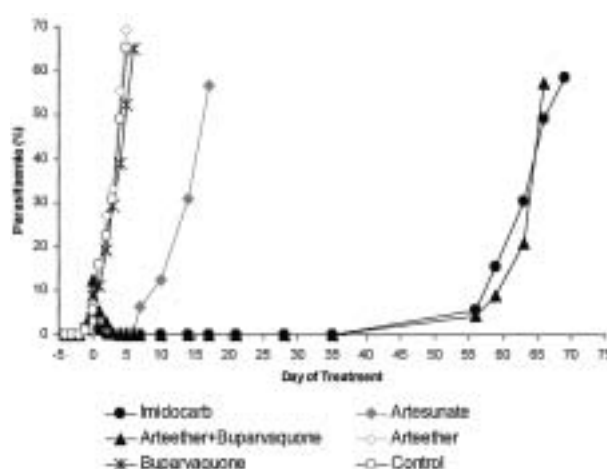

Fig.1.   Per cent parasitaemia in the peripheral blood of *Babesia equi* infected groups during the treatment trial with various drugs.

observed. The mean PT survival period in this group was 69 days (ranged 66 to 71 days PT). In group II, artesunate was observed to restrict the multiplication of the parasite during the treatment regimen only, as initial parasitaemia decreased from 7.1% to 0.5% (Fig.1). But after completion of the reg-imen, the mean maximum parasitaemia increased to 56.7% between 16–19 days PT (mean 17 days PT) and subse-quently all the treated donkeys died. In arteether only and buparvaquone only treated group (III and IV, respectively), no recovery was observed after treatment and they died after showing high parasitaemia as observed in control donkeys also (Table 1). In group V (arteether+buparvaquone combi-nation) recovery after treatment was recorded as parasites were cleared after mean four days PT. However, recrudes-cence of parasitaemia was observed after mean 52 days PT (Table 1) and mean PT survival period was 66 days (ranged 64–68 days PT). All the donkeys in control group (group VI) died within 4 to 6 days of infection.

In group I donkeys, mean minimum haemoglobin value was 11.9 g/d*l* after treatment which decreased to 11.2 g/d*l* after treatment recovery period. Comparatively in group V, a steep fall in haemoglobin mean values (from 11.8 g/d*l* to 5.29g/d*l* on day 10 PT) was recorded, which was subse-quently recovered to mean 10.2 g/d*l* before recrudescence. After recrudescence haemoglobin level decreased drasti-cally in both groups I and V (79.2% and 65.6%, respec-tively). In groups III and IV, acute fall in haemoglobin levels within 4 to 7 days after treatment was observed and mean haemoglobin concentrations before death were 2.5g/d*l* and 2.8g/d*l*, respectively (Table 2). In group II, drop in hae-moglobin was not severe during treatment regimen (from mean 14.7 g/d*l* to 12.7 g/d*l* on day 4 PT), but after treatment regimen a sharp fall was noticed.

Regarding packed cell volume (PCV), group V treated donkeys manifested a rapid fall after treatment and the mean minimum value on the tenth day PT was 16.0% as compared

Table 1.   Efficacy of different drugs against experimentally produced *Babesia equi* infection in splenectomised donkeys

Groups (n=3)	Drugs (trade name)	Drug dosage (mg/kg b.wt) & route	No. of injection	% parasitaemia on the day of treatment	Treatment interval (hr)	Days PT[a] when animals recovered*[b]	Recrudescence	Mean Survival duration after treatment (days)
I	Imidocarb dipropionate (Imizol®)	7.0 (i.m.)	4	6.4	72	Yes (2 PT)	Yes, 52–56 days after treatment recovery	69
II	Artesunate, artemisinin derivative (Falcigo®)	2.5 (i.m.)	4	7.1	24	No	No, died between 16–19 days PT	17
III	Arteether, artemisinin synthetic derivative (E Mal®)	5.0 (i.m.)	3	6.8	24	No	No, died between 4–6 days PT	5
IV	Buparvaquone (Butalex®)	5.0 (i.v.)	4	8.9	24	No	No, died between 5–7 days PT	6
V	Arteether + Buparvaquone (E Mal® + Butalex®, as above)	5 (i.m.) + 5.0 (i..v.)	3+4	12.5	24 (each)	Yes (4 PT)	Yes, 50–54 days after treatment recovery	66
VI	Untreated Control	–	–	–	–	No	No, died between 4–6 days of infection	5

a) PT: Post-treatment.
b) *: Recovery day was considered when no parasite could be observed in the blood smears after treatment.

Table 2.   Clinical and haematological observations in different splenectomised *Babesia equi* infected donkeys group treated with various drugs

Group (n=3)	Mean parasitaemia (%)			Mean haemoglobin (g/d*l*)				Mean packed cell volume (%)			
	On day of treatment	Max. after treatment	Max. after recrude-scence	On day of treatment	Min. Hb during TR[a]/before death	Hb before recrude-scence	% fall after recrude-scence	On day of treatment	Min. PCV during TR[a]/before death	PCV before recrude-scence	% fall after recrude-scence
I	6.4	–[b] (2)	58.4 (69)	15.0	11.9 (10)	11.2	79.2	30.2	23.7 (10)	24.6	58.9
II	7.1	56.7 (17)	NA[d]	14.7	3.1 (17)	NA	NA	40.9	10.7 (17)	NA	NA
III	6.8	69.2 (5)	NA	9.0	2.5 (5)	NA	NA	24.8	6.9 (5)	NA	NA
IV	8.9	64.9 (6)	NA	10.0	2.8 (6)	NA	NA	28.11	7.47 (6)	NA	NA
V	12.5	– (4)	57.2 (66)	11.8	5.2 (10)	10.2	65.6	40.2	16.0 (10)	36.0	72.9
VI	NT[c]	65.2 (5)	NA	10.0	2.8 (5)	NA	NA	30.0	10.6 (5)	NA	NA

a) TR: Treatment recovery.
b) '–' Indicates no parasite could be observed upon examining at least 20 field in stained blood smears under oil immersion.
c) NT: No treatment.
d) NA: Not applicable.
The values in parenthesis indicate the day of particular observation during post-treatment/post-infection.

to 23.7% in group I (Table 2). During the treatment recovery period, an improvement in PCV values (rose to 36.0%) was observed in group V donkeys. After recrudescence PCV value decreased in both donkeys group I (from 24.6% to 10.1%) and V (36.0% to 9.75%) showing a per cent fall of 58.9% and 72.9%, respectively.

An increase in mean white blood cell (WBC) count was observed in groups I and V donkeys after treatment, but the increase was more pronounced in group V (98% increase PT) as compared to a 56.0% increase in group I donkeys (Fig. 2A). In group II donkeys, WBC decrease was noticed during the treatment schedule and thereafter a sharp increase was observed as in group III, IV and VI (Fig. 2A). Typically the lymphocyte count decreased in all treated groups after treatment (Fig. 2C). In group I and V, the per cent lymphocytes improved during the treatment recovery period but it
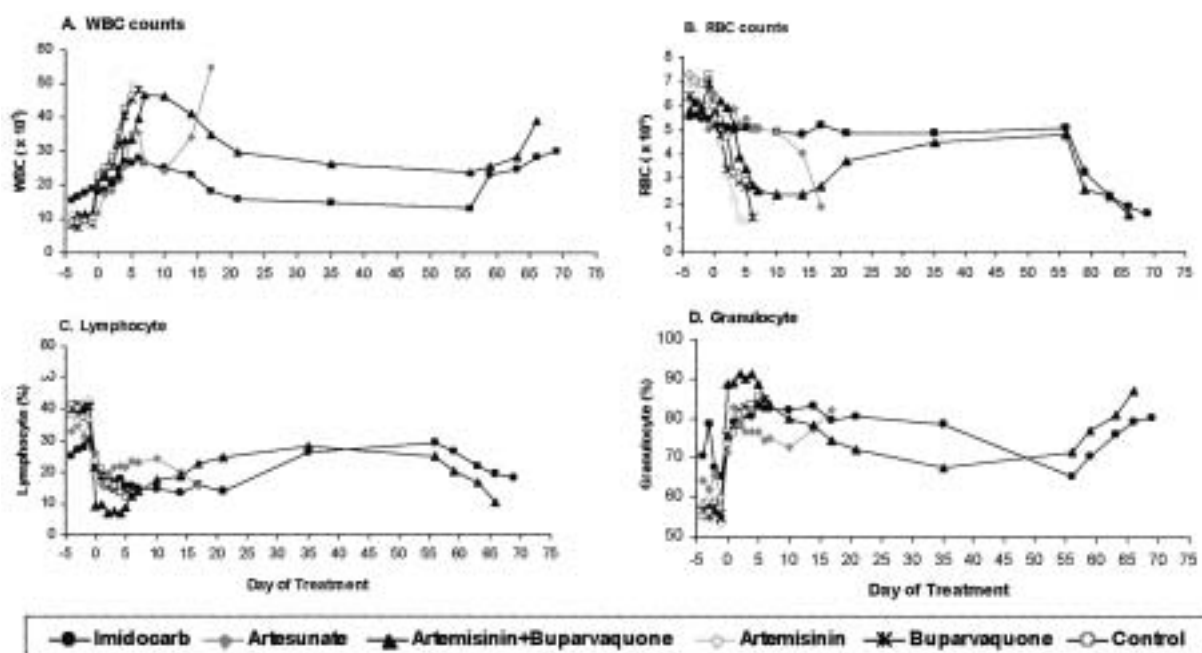
Fig. 2.   WBC counts (A), RBC counts (B), lymphocyte % (C) and granulocyte % (D) estimation in the blood of *Babesia equi* infected
          groups during the treatment trial with various drugs.

again declined after recrudescence. Increase in per cent granulocytes (Fig. 2D) occurred in all the treated donkeys groups. Granulocytes percentage was higher in group V (91.1% on day 4PT) as compared to group I (83.0% on day 14PT). Red blood cells (RBC) counts decreased almost in all the treated donkey's groups (Fig. 2B). In group V, it decreased to 3.7×10^6/cmm (on day 21PT) from the initial mean treatment value of 5.9×10^6/cmm, while in group I, it decreased from initial mean value of 5.79×10^6/cmm to 4.82×10^6/cmm (on day 14PT).

Biochemical evaluations: In group I, after treatment a very sharp increase (about 86.7%) in the activity of SAST was observed, as the level of enzyme increased from initial mean 481.0 IU/*l* to mean 898.0 IU/*l* on 14th day PT IU/*l* (Fig 3A). In group V, this increase was from mean 404.4 IU/*l* on '0' day to mean 474.6 IU/*l* on day five PT while in artesunate treated donkeys, a minor decrease in SAST values i.e., from 500 IU/*l* on day '0' to mean 464.6 IU/*l* on third day PT was observed. In rest of the groups, after development of proliferative parasitaemia, a profound increase in SAST values was observed. After recrudescence in group I and V also, an increase in SAST values was observed. SALT values also increased in group I from mean 42.34 IU/*l* to mean 85.5 IU/*l* on 14 day PT, while in group V, this increase was from mean 33.2 IU/*l* to 70.8 IU/*l* on day 4th PT as compared to '0' day value. Increase in SAP activity was also observed during post treatment period. SAP values increased to 33.7% (mean value 191.7 IU/*l* on 14 days PT) from the basal '0' day (mean 143.4 IU/*l*) values in group I as compare to 13.8% (mean 177.5 IU/*l* on 4 days PT) in group

V (on day '0' mean values were 155.9 IU/*l*). Albumin/globulin (A/G) ratio decreased in all the donkeys groups (I to VI) indicating increase in globulin fraction in the serum (Fig. 3D). In group I, it decreased from initial mean value of 0.64 to 0.24 on day 69 PT, while in group V this decreased was from 0.48 to 0.34 (66 day PT).

DISCUSSION

Babesia equi is a known parasitic problem of equines and infection is worldwide. The importance lies in the fact that infected animals become carriers for life and potential intrauterine infection can occur throughout the breeding life of the mare. A high seroprevalence of *B. equi* antibodies have been reported in equids from many tropical countries specially India [15, 16], African continent and Madagaskar [8] and clinical cases are very often. Losses due to intrauterine infection leading to abortion and strong regulation on export of *B. equi* carrier equine species are relatively common. None of the drugs tested so far have been observed to be effective in clearing the parasite. In this paper, an attempt was made to study the efficacy of new anti-theilerial and anti-malarial drugs against experimental *B. equi* infection either individually or in combinations.

No literature is available regarding the *in-vivo* therapeutic efficacy of new anti-malarial artemisinin derivatives (artesunate and arteether) against any intraerythrocytic haemoprotozoan of horse or cattle. Although growth inhibition efficacy of artesunate against equine's *Babesia* in *in-vitro* culture has been demonstrated by Nagai *et al.*, 2003 [22].
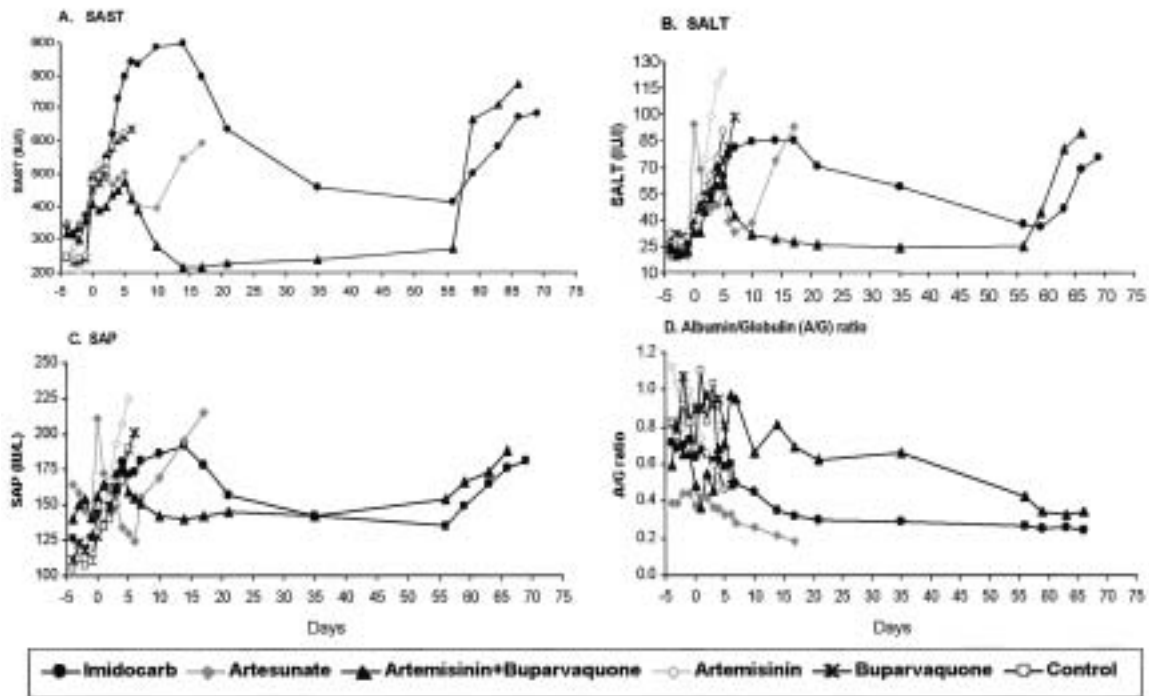
Fig. 3. Activities of SAST (A), SALT (B), SAP (C) and A/G ratio (D) in the serum of *Babesia equi* infected groups during the treatment trial with various drugs.

We used 2.5 mg/kg b. wt and 5 mg/kg b. wt dose rate for artesunate and arteether, respectively. It was selected by doubling the effective therapeutic dosage as used in human beings [2, 27, 32]. Imidocarb dosage was based on the previous studies in horses and donkeys [10, 24, 26]. Buparvaquone was used at previously suggested dosage in splenectomised *B. equi* infected horses [31]. A slight swelling at injection site and restlessness were noticed after intramuscular imidocarb inoculation while no such reaction could be appreciated after inoculation of artesunate or arteether or bupavaquone or arteether + buparvaquone, in the present study.

Individually arteether and buparvaquone were found ineffective in suppressing the fulminating parasitaemia. However, a synergistic effect was recorded in terms of parasite clearing efficacy when combination of arteether and buparvaquone were used at the same individual dose rate. It is difficult to explain the mechanism of this synergism (arteether and buparvaquone) in the present study. Buparvaquone acts selectively against the parasite's electron transport system at ubiquinone-cytochrome c reductase of the respiratory chain [14, 11] while artemisinin-type compounds are found to block heme polymerization, causing a buildup of monomeric free heme toxic to the parasite [23]. Hence the combination might have worked well to suppress the increasing parasitaemia. Nagai *et al.* [22] reported complete growth inhibition of *B. equi* in *in-vitro* culture system at 12.44 μg/ml of artesunate concentration, while in the

present study a transient *in-vivo* growth inhibition effect of artesunate was observed. Similar transient *in-vivo* efficacy of the artesunate against *Babesia microti* in the hamster model has been observed by Marley *et al.* [21].

Simultaneously fall in haematocrit and haemoglobin in donkeys treated with arteether+buparvaquone combination was appreciably more than imidocarb treated donkeys. Recent studies suggested that artemisinin induces a decrease in parasitized erythrocytes deformability, hence more likely to be disrupted during their blood capillary passage or can be selectively destroyed by the immune system [6]. A transient decrease in haematocrit values in arteether+buparvaquone combination group may be due to this phenomenon, enabling clearance of parasitized erythrocytes, but body recoup the RBC counts to pre-treatment levels within few days after withdrawal of treatment regimen (Fig. 2). Further, per cent granulocyte (Fig. 2) and *Babesia equi* specific IgG levels (detected by performing Dot-ELISA, data not shown) during the first seven days after treatment in arteether+buparvaquone treated donkeys (Dot-ELISA titre ranged from 1:3,200 to 1:12,800) was more pronounced as compared to the imidocarb treated group (Dot-ELISA titre ranged from 1:3,200 to 1: 6,400).

Elevation of SAST, SALT and SAP levels in imidocarb treated group indicates hepatocellular damage beside damage to skeletal/cardiac muscles, osteoblast and intestine membranes [3, 4]. Frerich *et al.* [10] reported that liver of imidocarb treated horses has highest drug concentration up

to 6 days after first injection. Adams [1] also observed elevated SAST, SALT and SDH levels in horses treated with imidocarb. He reported that imidocarb toxicity mortality is attributed to acute renal cortical tubular necrosis and acute periportal hepatic necrosis. The dose rate selected for arteether and artesunate was lower than the previous reported neurotoxic, anorectic toxic dosage (> 25 mg/kg/day multiple doses of arteether) in rats [20]. Buparvaquone has been reported to be safe when tried in Bo-RBC-SCID mouse model against *Theileria sergenti* [13] and in horses and ponies [31].

Decrease in A/G ratio indicates hyperglobinemia. A decrease in albumin level and increase in globulin fraction in the serum was observed in all the donkeys groups. De Waal *et al.* [9] and Yeruham *et al.* [30] also recorded similar observations during *B. equi* and *Babesia ovis* infection in horses and sheep, respectively.

In conclusions, we demonstrated that giving repeated doses of imidocarb or combination of arteether and buparvaquone at appropriate time interval and dose rate could control the *B. equi* (Indian origin) parasitemia in infected donkeys. However, arteether and buparvaquone combination was observed to be safer than imidocarb alone. Further more *in-vivo* trial would be required in splenectomised and non-splenectomised animal species to examine various dosages of arteether+buparvaquone and their side effects before this drug combination can be applied on infected animals in field.

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