Optimization of Conditions for Cultivation of *Babesia bigemina*-infected RBCs

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ABSTRACT

Background: *Babesia bigemina* is the cause of bovine babesiosis. *B. bigemina*-infected red blood cells (iRBCs) were passaged *in vitro* for the attenuation.

Methods: We cultured Pakistani isolate of the parasite in three different culture media. Whole blood was collected from splenectomized and intact crossbred young calves. The parameters included were hematological profile, catalase activity, osmotic fragility and lipid profile. Cell culture media (M-199, RPMI 1640 and DMEM) were used to find out the longevity of parasites iRBCs.

Result: Highest PPE level was found up to 6.0% on 72 h post-culture in M-199 medium. Furthermore, no significant difference in catalase activity while significant difference in osmotic fragility were observed. However, lipid profile was significantly less in infected animals except in *Babesia*-infected. M-199 was the most appropriate medium for the *in vitro* cultivation of *B. bigemina*. Our findings would help us for *in vitro* cultivation of babesia-infected RBCs for its attenuation.

Key words: *Babesia bigemina*, Culture medium, *In vitro*, Optimization, Percentage parasitized erythrocytes.

INTRODUCTION

Babesiosis is an economically important protozoan tick-borne disease of ruminants, which affects millions of animals around the globe (Terkawi *et al.*, 2012) including Pakistan. *Babesia bigemina* (*B. bigemina*) is highly prevalent in Pakistan and poses serious health and management issues to cattle (Jabbar *et al.*, 2015). The most economical and effective method is vaccination for the control of babesiosis which improved the health and production of the animals (Babiuk, 2002). Moreover, implementation of mass vaccination program reduces the use of medicine and prevents the emergence of resistance for pathogens in animals (Lathers, 2001).

In vitro culture of infected cells is one of the most important techniques for the development of attenuated vaccine. The most important component for cell culture is selection of a suitable growth medium for *in vitro* cultivation of *Babesia*-infected cells. A culture medium in the presence of fetal bovine serum provides essential nutrients for proliferation of parasites (Arora, 2013; Morgan *et al.*, 1955).

Erythrocyte's membrane is composed of polyunsaturated fatty acids that is vulnerable to lipid peroxidation (Esmaeilnejad *et al.*, 2012), causing changes in levels of blood metabolites (iron, calcium and copper) and nutrients (proteins, antioxidant vitamins (A, C and E) and lipids) (Khan *et al.*, 2011). Thus, babesia alters the hemato-biochemical profile including complete blood count (CBC), catalase activity and lipid profile and causes anemia during infection. The presence of *B. bigemina* in the erythrocytes of infected animals has hemolytic effect through increasing lipid peroxidation. In severe cases, there are possibilities of organ failure which leads to morbidity and mortality of infected animals (Turunç and Konaş Aşkar, 2012).

The current study was designed to optimize the suitable culture medium for *in vitro* culture of *Babesia*-

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infected red blood cells (RBCs) as well as to evaluate the catalase activity, osmotic fragility and lipid profile of cattle infected with *B. bigemina* and/or *Theileria annulata*.

MATERIALS AND METHODS

Ethical statement

The current 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.

Selection of animals

A total of twelve piroplasm-free calves were undertaken in this study. The male crossbred calves of 4 to 6 months age were purchased from a local farm and were kept in experimental station of University of Veterinary and Animal Sciences Lahore, Pakistan. The animals were infected individually and collectively with ticks carrying *B. bigemina*

and *T. annulata* reared at temperature 28 °C and at humidity >80% in Biological Oxygen Demand (BOD) incubator (Model ICO105 Memmert, Germany). The classification of the animals was done after screening of piroplasm infection in blood through PCR analysis. These calves were divided into four groups with each group carrying 3 animals. Group A consists of 3 crossbred splenectomized calf (Kusminsky *et al.*, 1995) infected with ticks (containing *B. bigemina*) to use as a control positive, group B includes calves infected with ticks (containing *B. bigemina*), group C consists of calves infected with ticks containing *B. bigemina* and *T. annulata* (mix infection), while group D consists of healthy animals free from any infection to use as control negative. All reared calves were in free access to water and corn silage.

Source of infection and parasitemia level in infected calves

Calves of group A, B and C were exposed to tick infestation. After 14 days pre-exposure with ticks, blood samples from experimental animals were collected in EDTA coated tubes. Thin blood smear of these samples were prepared to examine the PPE. To calculate the parasitemia level in infected calves and in cultured RBCs, three smears from each sample were prepared to count the average number of *B. bigemina*/*T. annulata*-infected RBCs according to previously used method (Maharana *et al.*, 2019). The PPE was measured by thin blood smear. Parasitemia level was calculated according to the previously reported formula (Schetters *et al.*, 1994).

Blood profile

Complete blood count (CBC) of samples from experimental animals was performed by using hematological analyzer (Abbott Cell Dyn 4000). The Hb concentration (gm/dl), PCV (%), RBCs count, WBC's count and other erythrocytic indices (MCV, MCH, MCHC) were compared among the four experimental animal groups.

Catalase activity

Whole blood was collected from venipuncture of jugular vein of individual experimental animals (A, B, C and D groups) in EDTA-coated tubes. These samples were processed for identification of antioxidant status by measuring catalase activity by spectrophotometer at 374 nm absorbance. Linear differential equation was used to determine the catalase activity (Hadwan and Abed, 2016).

Preparation of Medium 199, RPMI 1640 and DMEM

Medium 199 (Cat. No. 11150059), RPMI 1640 (Cat. No. 11875093) and DMEM (Cat. No. 11965092) were purchased from Thermo Fisher Scientific (Vanta, Finland). In this study, we cultivated the infected and non-infected RBCs in three different complete culture media following incubation in 5% CO₂ at 37°C as previously conducted (Adaszek and Winiarczyk, 2011; Erp *et al.*, 1980; Vega *et al.*, 1985). The complete culture media were prepared comprising of 60% (Medium 199, RPMI 1640 and DMEM) with supplementation of FBS 40%, amphotericin B 50 µg ml⁻¹, penicillin 100 IU, streptomycin 100 µg ml⁻¹ and HEPES 15 mM.

In vitro cultivation

Upon confirmation of infection and infection-free calves, blood samples were collected for *in vitro* propagation of *B. bigemina* in infected RBCs. A total of 10 ml whole blood was collected from the calves of all 4 groups in 15 ml EDTA coated tubes and thoroughly mixed. Live and dead cells were calculated by cell counting through hemocytometer. For that, trypan blue was used as a marker for the differentiation of live and dead cells (Rojas-Martinez *et al.*, 2017). Centrifugation of collected blood samples was performed at 3000 rpm for 6 minutes at 4°C to obtain the pellet of cells. The cells were washed 3 times with 0.15 M phosphate buffer saline. The washed RBCs (80 µl) were then cultured within each type of complete culture medium (720 µl well⁻¹) in a 24-well culture plate and incubated in 5% CO₂ at 37°C. After every 24h, 700 µl of culture media was replaced with fresh complete culture media. The infected RBCs were sub-cultured *in vitro* for 9 generations in duration of 36 days.

Osmotic fragility of infected RBCs

To perform erythrocytic osmotic fragility test, the most traditional protocol was followed (Parpart *et al.*, 1947). Briefly, NaCl solution at percentage of 0.9, 0.6, 0.5, 0.4, 0.3 and 0.0 in distilled water (dH₂O) was used to optimize its concentration.

Lipid profile activity

The tests were performed for the profiling of three important parameters of lipids including cholesterol, triglycerides and high-density lipoprotein (HDL). Cholesterol analysis was done using Cholesterol LiquiColor® kit (Quiclet *et al.*, 2019). Triglycerides analysis was done by using Kit (ab65336) (Zhang *et al.*, 2015). HDL cholesterol level was measured by CHOLESTEROL liquicolor test kit (catalog no. INF 1001801 GB) (Zhu *et al.*, 2005).

Statistical analysis

RBCs of *B. bigemina*, *T. annulata* and in mix-infected calf were cultivated *in vitro* in three different media to find out the propagation of parasitemia. Their results were expressed in the form of percentage with respect to their media. Graphical analysis was done by using the GraphPad Prism 7 software (La Jolla, CA 92037, USA). One-way ANOVA was performed to analyze the catalase activity, osmotic fragility and lipid profile of the infected RBCs. The significance among the groups was evaluated by using Post Hoc test (Tukey's test). Significant values obtained were presented as *p<0.05, **p<0.01 and ***p<0.001 and "ns" represented non-significance (p>0.05).

RESULTS AND DISCUSSION

Parasitemia and Blood profile

The results of parasitemia and blood profile are shown in Fig 1 and Fig 2, respectively.

We found decreased PCV (%), Hb and RBC count in babesia-infected as compared to control negative group

animals. All other hematological parameters were statistically non-significant among the groups. Our findings suggest that decrease in these parameters were due to destruction/lysis of infected RBCs. These results are concurrent by the previously reported changes in

Fig 1: Parasitemia level (%) in classified calves on 14 days post-tick infestation.

Fig 2: Complete blood profile (%) of 4 groups calves at 14 day post-tick infection.

hematology of *B. bovis* infected animals (Mahmoud and Abou-Zeina, 2008). In infected animals, WBC count was significantly (<0.05) low as compared to control negative group while only neutrophil count was non-significant. High MCV and decreased MCHC were observed in control positive and babesia-infected animals respectively, but these values were non-significantly different. Decreased hematological values (MCHC, RBCs, PCV and Hb) suggested that anemia was the constant factor in babesia-infected animals. Our findings correlate with the previous study (Rahbari *et al.*, 2008).

Comparison of Media

The results for the comparison of different media are shown in Table 1 and Fig 3. Out of culture media used, M-199 was found to be the most suitable for the proliferation of parasite in *in vitro* cultivation in RBCs and remained viable for duration of >72 h.

Catalase activity

The results of catalase activity are shown in Fig 4.

Non-significant difference in catalase activity among groups of calves infected with and without blood protozoan was found. Previous outcomes support our findings (Wang *et al.*, 1999). According to the current findings, high level of catalase activity was observed in mix infection. Our findings are in close agreement to previous outcomes (Rezaei and Dalir-Naghadeh, 2006), where they found decreased catalase activity in healthy and severely anemic animals, while high catalase activity was recorded in mild to moderate *Theileria*-infected calves (Ahmed and Hassan, 2007; El-Deeb and Younis, 2009; Hamid *et al.*, 2014; Razavi *et al.*, 2012).

Table 1: Comparison of *in vitro* PPE in three different culture media.

Media	PPE		Culture darkness period (days)
	Initial	Final	
Medium 199	1.58%	6.0%	7
RPMI 1640	1.58%	5.5%	3
DMEM	1.58%	5.75%	5

Fig 3: Percentage parasitemia in infected erythrocytes relative to culture medium and incubation time period. Parasitemia increased until 72 h while decreased beyond that incubation period due to addition of fresh RBCs.

Fig 4: Graphical representation of catalase activity in 4 groups (A, B, C and D) of calves. X-axis is showing the catalase activity in different groups and Y-axis indicating the values of catalase activity in KU/L.

Fig 5: Graphical representation of erythrocytic osmotic fragility at 0.4 % NaCl. X-axis showed the result of infected RBCs in classified animal groups while on Y-axis represents hemolysis at 0.4 % NaCl. There was no significant ($p>0.05$) difference between the animal groups.

Fig 6: Graphical representation of lipid profile activity in *in vitro* cultured RBCs from classified animal groups.

Osmotic fragility

The results of osmotic fragility test are shown in Fig 5. Our results showed that erythrocytic osmotic fragility of mixed infected RBCs was statistically higher than of single infection. There might be several reasons including infection propagation, reduction of nutrient in culture media and hemolytic anemia (Fazio *et al.*, 2016; Slappendel, 1998). Highest osmotic fragility was observed at the concentration of 0.4% NaCl in all samples. The influx of water happens at 0.4% NaCl because of hypo-tonicity of the culture media and the integrity of their membranes is disturbed. This disturbance permits the release of cell hemoglobin exterior to the cell membrane that dissolves in the external media. In mix-infection, there led to expand oxidative stress as shown by a significant rise in activities of chemicals like lipid peroxidation in erythrocytes. A noteworthy rise within the activities of antioxidant enzymes was observed which may not lower this oxidative stress. This could be the cause of increase in erythrocytic osmotic fragility because of membrane lysis and lower Hb values (Grewal *et al.*, 2005). Noteworthy increase in erythrocytic osmotic fragility was in accordance with the previously reported studies in which the parasitemia expanded the erythrocytic osmotic fragility within *Theileria sergenti*-infected calves (Singh *et al.*, 2001).

Lipid analysis

The results of lipid analysis are shown in Fig 6. Our findings were in contradiction with the results recorded by Goodger *et al.*, (1981) in which the cholesterol and lipoproteins values were significantly decreased (Goodger *et al.*, 1981). Cattle breed, susceptibility to infection and infection propagation could be the reasons for the difference in current and previous studies. Our findings for lipid profiles are supported with previous studies on cattle infected with babesiosis (Alam and Nasr, 2011). These results of triglycerides are similar with already findings that levels of triglycerides increased significantly (Turunç and Kontaş Aşkar, 2012). Similarly, we also found non-significantly increased levels of cholesterol and HDL in *B. bigemina* infected red blood cells (iRBCs) and these finding are

concurrent with previously reported study (Hamoda *et al.*, 2014). Another study on cattle infected with *B. bigemina* showed a significant decrease ($P<0.05$) in HDL level and a non-significant decrease in the levels of other serum lipids (Ganguly *et al.*, 2017). According to some previous studies, changes in lipid profiles were recorded non-significantly but our results noted only significant increase ($P<0.05$) in the concentrations of triglycerides when compared with control group and the standards values (Razavi *et al.*, 2012). The reason behind this deviation could be a change in cattle breed, susceptibility to infection and most importantly the parasitemia rate.

CONCLUSION

Non-significant changes in lipid profile showed that rate of RBCs lysis was not higher in babesia-infected RBCs during *in vitro* cultivation. It is a satisfactory indicator to maintain *B. bigemina* in iRBCs for maximum duration in optimized culture medium to attenuate it for vaccine production against babesiosis in future studies.

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Conflict of interest

The authors declare that there is no conflict of interest.

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