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Research Article

Buffalo colostrum- A novel substitute of human serum for the cultivation of *Plasmodium falciparum in vitro*

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Abstract
In vitro cultivation of erythrocytic stages of *Plasmodium falciparum* requires supplementing the culture medium with human serum. The present study was carried out to explore an alternative to human serum. Different human serum samples were found to vary considerably in their ability to support the growth of erythrocytic stages of *P. falciparum in vitro*. These results strongly suggested the use of pooled human serum for comparing the growth of parasites in medium augmented with other supplements. Parasites could multiply for a few cycles only in RPMI (Roswell Park Memorial Institute) medium supplemented with serum obtained from pig, goat, sheep or buffalo. Continued cultivation could not be achieved using any one of these animal sera. Ability of bovine colostrum was investigated as an alternative to human serum. Buffalo colostrum, 10%(v/v) 'suitably prepared' supported the continuous growth and multiplication of *P. falciparum* . Morphologically both asexual and sexual stages appeared normal and healthy, but the multiplication rate of parasites grown in colostrum augmented medium was found to be lower than that in serum-supplemented medium. The one month of uninterrupted cultivation of *P. falciparum* registered 10^6 fold increase in parasite density compared to 10^{10} fold multiplication recorded in control culture with 10% serum supplement. Cow colostrum failed to support the growth and multiplication of parasites beyond 6 days in culture. The initial positive results with buffalo colostrum hold promise and should be explored further as a potential substitute for human serum for continuous *in vitro* propagation of erythrocytic stages of the malignant malaria parasite.

Keywords: Bovine colostrum, Human serum substitutes *In vitro* culture, *Plasmodium falciparum*, Pooled human serum

INTRODUCTION

Long-term cultivation of the malarial parasite, *Plasmodium falciparum* is one of the important requirement for research in understanding the biology of the parasite, drug development and immunology. The first successful continuous cultivation of erythrocytic stages of *P. falciparum* was reported nearly five decades ago (Trager and Jensen,1976). Since then commercially available RPMI-1640 has been found to be the medium of choice, but alone it is not sufficient to support the growth of parasites, so it has to be supplemented with human serum. The requirement of human serum poses certain constraints; it is a scarce commodity, varies from batch to batch, and is expensive and moreover, in endemic areas, serum samples may contain inhibitory substances like residues/metabolites of antimalarial drugs and/or antibodies. These constraints and variability factors demand the development of alternatives to human serum for cultivation. Several attempts have

been made earlier to make use of animal sera for cultivation (Ifediba & Vanderberg, 1980; Sax and Rickman, 1980 ; Divo *and* Jensen, 1982; Oduola *et al*., 1985). We have also tried to replace human serum with animal serum. Besides serum, other alternatives like commercial preparations such as bovine serum albumin and C-18 fatty acids (Wilet and Canfield 1984), Nutridoma (Lingnau *et al.*,1994; Flores *et al.,* 1997), Albumax I&II (Binh *et al.,* 1997; Crammer *et al.,* 1997; Srivastava *et al.,* 2007; Dohutia *et al.,* 2017) have also been reported but with varying success and are also less cost-effective. For long-term and large scale production of parasites, relatively inexpensive and readily available alternatives to human serum are required.

The requirement of serum is not only essential for the cultivation of *P. falciparum* but is also a must for *in vitro* culture of human and animal cell lines, where fetal bovine serum (FBS) is almost universally used as a medium supplement. To reduce or completely eliminate the use of FBS, other less expensive biological fluids such as milk, colostrum (Steimer *and* Klagsbrun, 1981), colostrum ultrafiltrate (Pakkanen, 1994) and whey fraction (Paradkar *et al*., 2019) have been successfully used as alternatives for supplementing cell culture media. . Other alternatives to FBS like bovine ocular fluid, sericin protein from silkmoth cocoon, platelet lysate and coelomic fluid of earthworm have been reviewed by Chelladurai *et al*. (2021). Such serum alternatives can also be tried for growing *P. falciparum*

The present study explored using easily available animal sera and bovine (buffalo and cow) colostrum as alternatives to human serum for the long term cultivation of *P. falciparum*. Bovine milk/colostrum has never been reported as a medium supplement for *P. falciparum* culture.

MATERIALS AND METHODS

Parasites

P. falciparum infected blood sample from patients reporting to malaria clinic in Delhi was used to initiate the *in vitro* culture by the Petridish candle jar method of Trager and Jensen,1976 (Mehra and Bhasin, 1996). Stock culture of parasites was maintained in continuous culture using human A+ erythrocytes suspended at 8% hematocrit in RPMI-1640 medium containing 25mM HEPES, 25mM sodium bicarbonate, 40mg/ml gentamicin and 10% heat-inactivated AB+ human serum. The petridish was placed in a glass desiccator equipped with stop-cock and holding a lighted paraffin candle. The lid was sealed with silicone grease with an open stop-cock. As soon as the lighted candle extinguished, the stop-cock was closed and the candle jar was placed at 37°C in an incubator. Medium was renewed daily in the culture. Subcultures were made on day 4 or 6 to restore the hematocrit to 8% and the parasitemia below 1%.

Human and animal sera

Human AB+ blood without anticoagulant was procured from different voluntary donors with informed consent. Blood was allowed to coagulate for a few hrs at room temperature, kept at 4°C overnight and centrifuged to collect serum. Each serum sample was stored in small aliquots. Blood samples from buffalo, pig, sheep and goat (obtained from the licensed slaughter house in Delhi) were also processed in the same way as human blood to separate serum. Animal sera were filter sterilized using 0.45 µm filters. All serum samples were stored frozen at -20°C and heat-inactivated at 56°C for 30 min before use. These serum samples were used to supplement HEPES buffered RPMI-1640 medium (Trager and Jensen, 1976) at a concentration of 10%(V/V).

Bovine colostrum

The first milk secreted after giving birth is called colostrum. For the present purpose, colostrum was collected within four hours of calf birth and milk samples within two days at different intervals.The colostrum/milk samples of buffalo and cow were procured from National Dairy Research Institute, Karnal (India), transported to the laboratory in ice and stored at -20°C. The samples were thawed as needed and spun at 12000 rpm in a Sorvall centrifuge at room temperature for 40 min. The top layer consisting of fat, the bottom layer containing cells and other sediments were discarded. Middle layer was first subjected to coarse filtration followed by sterile filtration through 0.45 µm filter and this processed colostrum/milk was stored at 4°C until used. Adsorption of colostrum was done twice by mixing it with an equal volume of outdated human blood for 1hr at 37°C. Heat inactivation of colostrum/adsorbed colostrum was done at 56°C for 30 min to render the agglutinating factor inactive. Whenever colostrum was used without heat inactivation, it had to be adsorbed 3-4 times with erythrocytes and kept at 4°C for at least 24hrs to eliminate residual agglutinating activity.

In vitro multiplication

Multiplication of parasites was determined in plastic 24 well tissue culture plates using minimum of triplicate wells. Each well contained 0.5ml of cell suspension and initial hematocrit was adjusted to 4%. Thin smears were prepared from each well on every alternate day and stained with Giemsa stain to determine the parasitemia by examining at least 5000 erythrocytes from each smear (Moll *et al.,* 2013).

RESULTS

Multiplication of parasites in human serum

Growth and multiplication of parasites were monitored in HEPES buffered RPMI-1640 medium supplemented at 10% (v/v) rate with different human serum samples

obtained from malaria endemic area. Donors of serum were free from malaria at the time of blood collection. Effect of each serum sample on the parasite multiplication rate (PMR) was compared at the end of day 2 and day 4 of continuous cultivation. Table 1 shows the effect of six different serum samples on PMR at the end of the first and second schizogonic cycle. Statistical analysis of PMR at 48 hrs showed that growth of parasites in the medium containing HS-III serum sample was significantly lower than those containing other serum samples. The rest of the five serum samples were similar in their growth promoting activity as revealed by Newman Keul's test (at p=0.01 level). All these serum samples gave significantly different multiplication rates when analyzed at the end of two schizogonic cycles (at p=0.01 level, Newman Keul's test).

Multiplication of parasites in animal sera

Multiplication of erythrocytic stages in RPMI-1640 medium containing 10% pooled human serum was compared with that of medium supplemented with the same amount of animal sera obtained either from buffalo, sheep, goat or pig. Fig. 1 shows the parasitemia obtained in different media on various days of continuous culture. Healthy parasites could be observed in all media but growth was less than that observed in control (medium with human serum) wells. Higher parasitemia was attained in a medium supplemented with buffalo and sheep serum than that of pig and goat.

Bovine colostrum as serum substitute in cultivation

Buffalo/ cow colostrum was used as a serum substitute in the cultivation of *P. falciparum*. Buffalo colostrum caused extensive agglutination of erythrocytes and could not be used as such. It had to be adsorbed with human erythrocytes. Heat inactivation alone could not destroy the agglutinating factor(s) in the colostrum. Cow colostrum, on the other hand, never caused agglutination of RBCs. Results of one of the experiments using bovine colostrum as a serum substitute are presented in Fig. 2. Cow colostrum did not support the growth of parasites, whereas buffalo colostrum collected within 4 hrs of delivery supported the growth and multiplication of parasites, although the rate of multiplication was less than that achieved by human serum. Cow colostrum /milk samples collected within four hours of delivery and then at 12 hr intervals for 2 days

Table 1. Multiplication of parasites in RPMI-1640 medium containing different human serum samples. Initial parasitemia in each culture was 0.36% and hematocrit was 4%. Parasite multiplication rate (PMR) was calculated by the following formula : PMR =Log N (day 2 or 4) - Log N (day 0); where N is the number of parasites per 5000 erythrocytes on the day indicated

RPMI-1640 medium + 10% either of the following serum sample	Parasite Multiplication Rate (PMR)*	
	Day 2	Day 4***
HS-I	0.502 ± 0.0208	0.985 ± 0.0043
HS- II	0.467 ± 0.0118	1.078 ± 0.0144
HS-III	0.366 ± 0.01866**	0.357 ± 0.0307
HS-IV	0.482 ± 0.0240	1.015 ± 0.0102
HS-V	0.474 ± 0.0337	0.803 ± 0.0236
HS-VI	0.474 ± 0.0272	0.872 ± 0.0316

* Mean ± SD (n=4);** Mean is significantly different from all other sera as analyzed by Newman Keul's test (p=0.01);*** Mean PMR in each serum is significantly different from others (n=p=0.01 Newman Keul's test).

Table 2. Continuous In vitro cultivation of parasites in RPMI- 1640 medium supplemented with 10% either of buffalo colostrum, obtained within four hours after birth or pooled human serum. The cultures were diluted every 4th or 6th day. Fold multiplication (FM) refers to the times increase in parasitemia during the period of one dilution to the next.

Days in Culture	Buffalo colostrum		Human Serum	
	FM *	Total times dilution	FM *	Total times dilution
6	4.7	1.0	13.3	1.0
12	10.9	8.9	20.8	19.6
16	2.6	64.1	16.9	256.8
22	2.1	429.5	17.8	4288.6
26	3.5	2577.0	8.5	42886
Net increase in parasite density on day 26	2.52×10^6 times		3.03×10^{10} times	

*Mean of triplicate cultures

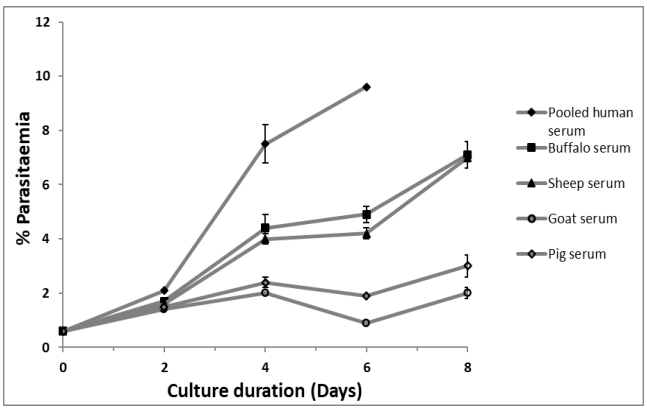

Fig. 1. *Continuous cultivation of P. falciparum in RPMI-1640 medium supplemented with 10 % of either pooled human , goat, sheep, buffalo or pig serum. Each point is an average of triplicate cultures. Standard deviation less than 0.2 is not presented.*

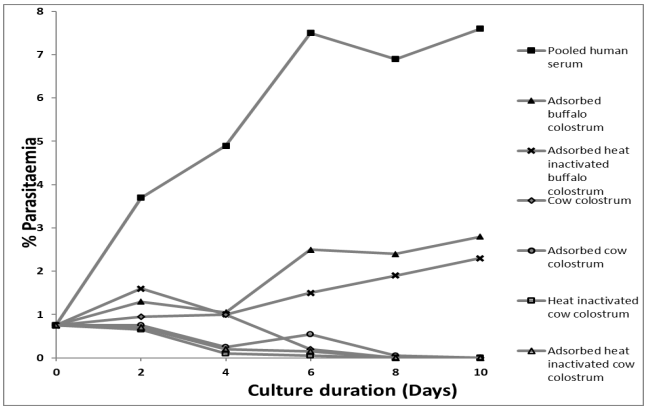

Fig. 2. *Growth of parasites in RPMI-1640 medium supplemented with 10 % of either pooled human serum or bovine colostrum. Buffalo / cow colostrum was collected within 4 hours of calf birth. Each point is an average of triplicate culture*

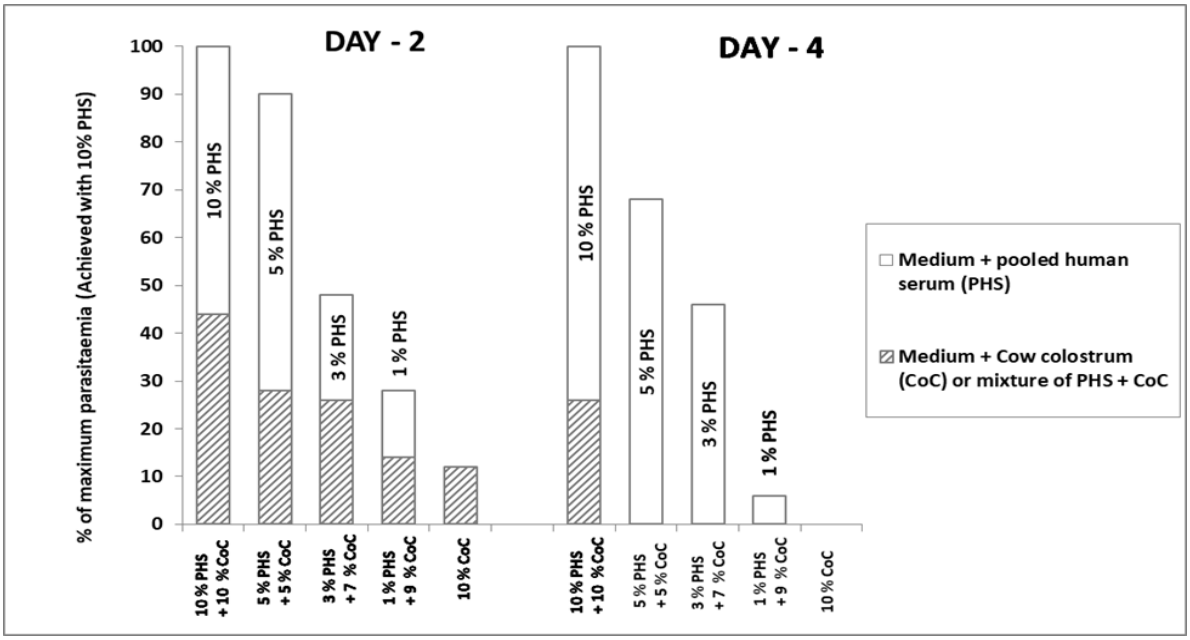

Fig. 3. *Inhibitory effect of cow colostrum supplement on growth of the parasites in relation to parasite growth in RPMI-1640 medium supplemented with 10 % of human serum on day 2 and day 4 of culture. Each bar on the graph represents an average of triplicate cultures*

failed to support the growth of parasites. Cow colostrum sample collected soon after birth when combined with varying concentrations of human serum was found to inhibit parasite multiplication (Fig. 3). Medium supplemented with 10% buffalo colostrum on day 1 and 2 after birth showed the presence of healthy parasites till day 6 but parasitemia never increased to heights requiring sub culturing. Continuous cultivation of *P.falciparum* was thus achieved only in a medium supplemented with buffalo colostrum collected within 3-4 hour of delivery (Table 2). Although maximum parasitemia reached in the medium supplemented with buffalo colostrum was never more than 5%, one month of continuous cultivation represented a $2x10^6$ fold increase in parasite density. The effect of various concentrations of buffalo colostrum on parasite multiplication was evaluated in an experiment (Fig. 4). It was found that parasitemia increased with increasing concentration of colostrum in the medium and morphologically both asexual and sexual parasites looked similar to that of parasites cultivated in the medium with human serum (control).

DISCUSSION

Human serum provides all the essential metabolic precursors needed for the growth and multiplication of erythrocytic stages of *P. falciparum* (Sherman, 1977; Vial *et al.* 1982; Trigg, 1985). However, the amount of various nutritional components or inhibitory factors pre-

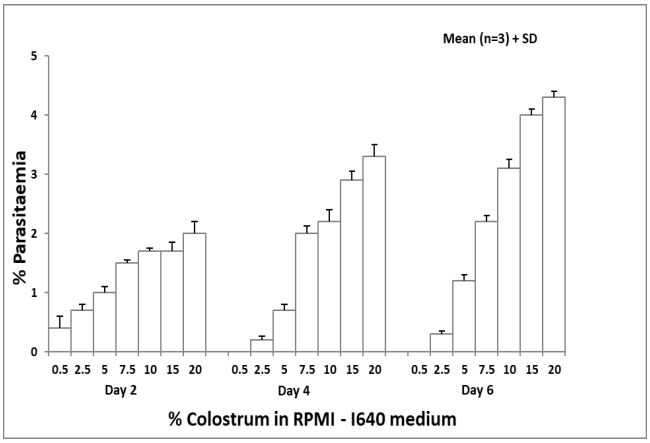

Fig. 4. *Percent parasitemia on day 2, 4 and 6 in RPMI-1640 medium supplemented with different concentrations of buffalo colostrum*

sent in different serum samples might vary from donor to donor (Agueusop *et al.*, 2020). To determine the impact of these variations, if any, on parasite multiplication, individual effect of six different serum samples collected from donors of an endemic area was evaluated. The present results substantiate the earlier findings (Jensen, 1979; Jensen *et al*., 1982) that human sera vary considerably in their ability to support the multiplication of *P. falciparum* in cultures. The impact of serum samples is most pronounced in cultures with high parasitemia. These results, therefore, made us strictly adhere to using pooled human serum for the continuous cultivation of parasites and to use this as the control for comparing the results of parasite growth in the presence of other supplements.

Due to several constraints regarding the easy availability and variability of human serum samples many attempts have been made to replace them with various animal sera for cultivation of erythrocytic stages of *P.falciparum* (Ifediba and Vanderberg,1980; Divo and Jensen, 1982). Most of these trials have met with limited success and some also require either long parasite adaptation period or the inclusion of additional substances along with animal serum for continuous cultivation of parasites. Serum from horse, rabbit and goat have been reported to support the growth of parasites without preadaptation or supplementation (Butcher, 1979; Sax and Rieckman, 1980; Oduola *et al*., 1985; Srivastava *et al.,* 2007). Horse serum supported the continuous cultivation of *P.falciparum* upto 100 days (Srivastava *et al.,* 2007). Considering cost considerations and technical limitations in procuring horse and rabbit serum, we examined the possibility of supplementing RPMI medium with other readily available animal sera like that of buffalo, pig, sheep and goat for continuous cultivation. The results indicated that goat and pig serum are less suitable for parasite propagation than buffalo and sheep serum. These findings differ from the observations reported by Oduola *et al.* (1985), who reported that media supplemented with sheep and bovine serum do not support parasite multiplication. They had used serum from Holstein cows, whereas we employed buffalo serum for supplementation. Animal sera used in the present study do not require any absorption with RBCs as they neither caused agglutination nor hemolysis of human erythrocytes in cultures. Also, parasites can be cultivated directly in these sera-supplemented media without any preadaptation. These advantages together with the easy availability of buffalo and sheep serum throughout most parts of India at low cost, make them suitable substitute material for human serum in cultivation. However, more efforts are needed to develop these sera as true substitutes having capability equivalent to that of human serum in supporting parasite growth *in vitro*.

The most interesting finding of the present study was that RPMI medium supplemented with buffalo colostrum could support and promote the growth and multiplication of parasites in the absence of serum. Colostrum, like the serum, is an exceptionally rich nutrient medium containing casein instead of albumin as the major protein. Also, colostrum is fortified with growth factors, hormones and immunoglobulins (Uruakpa *et al*., 2002). These factors are very important for the growth and development of cells and tissues of newborn during the first few days. As the newborn matures, the need for these factors declines. Correspondingly there is a change in the composition of milk during the lactation period in terms of growth factors and hormones (Klagsbrun, 1978; Playford and Weiser, 2021). It has been found that colostrum obtained within a few hours of delivery is a better substitute for human serum in supporting parasite growth than older milk. Although a lower parasite multiplication rate is achieved in colostrum supplemented medium, continuous cultivation is possible. Unaltered morphology of parasites over a period of one month of cultivation indicates that colostrum could be successfully used in place of human serum in *P.falciparum* cultivation. Lower multiplication of parasites in colostrum supplemented medium may be due to insufficient amount of some factors required to support maximal parasite growth. Lee and Hossner (2002) reported that low molecular weight ultrafiltrate fraction of colostrum supported better growth and differentiation of a preadipocyte cell line than the whole colostrum. So instead of whole colostrum, different fractions of colostrum may be investigated as serum substitutes to assess their impact on the growth of *P.falciparum*.

Buffalo and cow are two closely related bovine species but their colostrum vary tremendously in promoting parasite propagation. Cow colostrum has been found to be inhibitory to parasite growth. This may not be due to a lack of nutrients or growth factors but might be due to

certain substances in cow colostrum inhibiting parasite multiplication. Bovine colostrum has never been attempted previously for cultivating any of the parasitic protozoan. Only certain cell lines like fibroblasts, epithelial cells, a mouse hybridoma, preadipocytes have been cultivated in media containing bovine colostrum (Klagsbrun, 1980; Steimer *et al.,* 1981; Steimer and Klagsbrun, 1981; Ramirez *et al.,* 1990; Pakkanen, 1994; Belford *et al.*, 1995; Lee and Hossner, 2002; Chelladurai *et al*., 2021). Cow colostrum has also been reported to be selective in supporting growth of cell lines (Steimer *et al*., 1981; Barman and Rajput,1994). These initial positive results indicate the potential of buffalo colostrum-supplemented media for the cultivation of erythrocytic stages of *P. falciparum in vitro*.

Conclusion

The present study concluded that buffalo colostrum can be used as a substitute for human serum for *in vitro* cultivation of erythrocytic stages of *P. falciparum.* Bovine colostrum has never been reported as a supplement for cultivating blood parasites. Being relatively inexpensive and readily available, its use can reduce the maintenance cost of the culture of parasites.

Ethical approval

No animal was used in conducting the experiments. Only animal products such as milk / colostrum and animal blood were used. Milk and colostrum are natural secretions of cows and buffalo which were procured from dairy. Animal blood which is a waste product in slaughter house was obtained from licenced slaughter house. Human blood samples were procured from voluntary blood donors after taking their consent. Since no human /animal was directly involved in conducting the experiments, the ethics committee approval was not required.

Conflict of interest

The authors declare that they have no conflict of interest.

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