J. Protozool. Res. 27. 13-22 (2017)

Serum-free GIT medium for short-term *in vitro* cultures of *Babesia bigemina*, *Babesia divergens*, and *Theileria equi*

Mahmoud R. AbouLaila[1,2], Bumduuren Tuvshintugla[1,3], Mohamed A. Rizk[1,4], Shimaa A. El-Sayed[1,5], Naoaki Yokoyama[1], Ikuo Igarashi[1,*]

[1] National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-Cho, Obihiro, Hokkaido 080-8555, Japan
[2] Department of Parasitology, Faculty of Veterinary Medicine, University of Sadat City, Sadat City 32511, Minoufiya, Egypt
[3] Laboratory of Molecular Genetics, Institute of Veterinary Medicine, Ulaanbaatar, Mongolia
[4] Department of Internal Medicine and Infectious Diseases, Faculty of Veterinary Medicine, Mansoura University, Mansura35516, Dakahlia, Egypt
[5] Department of Biochemistry and Chemistry of Nutrition, Faculty of Veterinary Medicine, Mansoura University, Mansura35516, Dakahlia, Egypt
*Corresponding author: Ikuo Igarashi; E-mail: igarcpmi@obihiro.ac.jp

ABSTRACT

The present study evaluated serum-free GIT medium for the *in vitro* culture of *Babesia bigemina*, *Babesia divergens*, and *Theileria equi*. Parasite growth was compared with parasites grown in cultures containing M199 or RPMI1640 media with serum. The serum-free GIT medium supported the continuous *in vitro* culture of *B. bigemina* for 40 days. *B. divergens* growth was continuously cultured for 32 days in the serum-free GIT medium, and the parasite growth was similar to that in RPMI1640 with 10 % fetal calf serum and higher than the growth in M199 medium with 10 % fetal calf serum culture systems. Serum-free GIT medium supported the growth of *T. equi* for only 24 days, while the growth was sustained for 32 days in serum-free GIT medium supplemented with hypoxanthine. Parasite growth was similar to that in M199 medium supplemented with 40 % horse serum and hypoxanthine. The morphology of the three kinds of parasites was similar in serum-free GIT and the standard media with serum. Therefore, the present study indicated serum-free GIT medium alone for short-term *in vitro* cultivation of *B. bigemina* and *B. divergens* and supplemented with hypoxanthine for the *in vitro* culture of *T. equi*.

Keywords: GIT; *In vitro*; culture; *Babesia bigemina*; *Babesia divergens*; *Theileria equi*

INTRODUCTION

Babesia bigemina and *Babesia divergens* are causative agents of bovine babesiosis worldwide (Homer *et al*., 2000; Zintl *et al*., 2003). *Theileria equi* is a major pathogen of equine piroplasmosis (Rothschild and Knowles, 2007). Signs of the disease include fever, hemolytic anemia, jaundice, and hemoglobinuria. These piroplasms affect animal production and trade in many areas of the world. Therefore, there is a critical need to develop either an effective vaccine or a safe drug without side effects to control these parasites.

A continuous *in vitro* culture system keeps these parasites in a continuous living state for assessment of chemotherapeutic drugs or antibodies to recombinant vaccine candidates. The

standard culture medium needs either bovine or equine sera as an important component such as medium 199 for *B. bovis* (Levy and Ristic, 1980), *B. bigemina* (Vega *et al*., 1985), and *T. equi* (Zweygarth *et al*., 1995) and RPMI1640 medium for *Babesia caballi* (Avarzed *et al*., 1997) and *B. divergens* (Vayrynen and Tuomi, 1982). Instead, serum in parasite cultures interferes with the isolation and purification of parasite proteins from the culture (James *et al*., 1981) and modifies drug effects in *in vitro* drug screening (Bork *et al*., 2005). Therefore, the use of serum-free medium will help to overcome such problems.

In the last decades, serum-free culture systems were established for *in vitro* culture of *Babesia* and *Theileria* parasites. RPMI1640 medium was supplemented either with high density lipoprotein (HDL) (Schrevel *et al*., 1992) or with Albumax I (Grande *et al*., 1997) for *B. divergens*. The bovine serum was successfully replaced with lipid-rich bovine serum albumin for several strains of *B. bovis* (Jackson *et al*., 2001). Serum-free HL1 medium, buffered with HEPES and supplemented with glutamine and hypoxanthine, was used for the *in vitro* culture of *T. equi* (Zweygarth *et al*., 1997; Schuster, 2002). Serum-free HL1 medium, supplemented with either lipid-rich bovine serum albumin, alone or with chemically defined lipids, was used for *B. caballi* culture (Zweygarth et al., 1999). HL1 medium is a modified DMEM/F12 base contained HEPES buffer, known amounts of ethanolamine, transferrin, sodium selenite, testosterone, insulin, proprietary stabilizing proteins, and a range of unsaturated and saturated fatty acids. GIT medium is a serum-free medium for cell culture that contains basal medium (a mixture consisting of an equal volume of Ham's F-12 medium and Iscove's medium). Ham's F-12 medium contains inorganic salts, vitamins, amino acids (1 mM $_{L}$-glutamine), and other constituents such as putrescine 2HCl. Iscove's medium is a modified Dulbecco's medium formulation that contains potassium nitrate, selenium, vitamins, sodium pyruvate, $_{L}$-glutamine, and HEPES buffer. *B. caballi* (Ikadai *et al*., 2001) and *B. bovis* (Bork *et al*., 2005) were cultured in serum-free GIT medium. GIT medium was not used for the continuous culture of *B. bigemina*, *B. divergens* and *T. equi*. Therefore, the aim of the present study was to assess serum-free GIT medium for the *in vitro* culture systems of *B. bigemina*, *B. divergens* and *T. equi*.

MATERIALS AND METHODS

Parasites

B. bigemina (Argentina strain) (Igarashi *et al*., 1998), *T. equi* (US Department of Agriculture) (Kappmeyer *et al*. 2012), and *B. divergens* (German strain) (Mackenstedt *et al*., 1990) were used in this study.

Culture conditions

The bovine *Babesia* parasites and *T. equi* used in this study were maintained in bovine or equine red blood cells (RBCs), using a microaerophilic stationary-phase culture system (Salama *et al*., 2014; Vega *et al*., 1985). Medium M199 (Sigma-Aldrich, Tokyo, Japan) was used for *B. bigemina*, *B. divergens*, and *T. equi* cultures. RPMI1640 (Sigma-Aldrich) without HEPES buffer was used for *B. divergens* culture. Normal bovine serum was used at 40% for *B. bigemina* culture. Fetal calf serum (Gibco, Thermo Fisher Scientific Inc., CA, USA) was used at 10% of the cultures of *B. divergens*. Normal equine serum was use at 40% of the culture of *T. equi*. Antibiotics and antifungals were added to the culture media at 60 U/ml penicillin G, 60 μg/ml streptomycin, and 0.15 μg/ml amphotericin B (all three drugs from Sigma-Aldrich). Additionally,

T. equi culture was supplemented with 13.6 μg/ml of hypoxanthine (ICN Biomedicals Inc., Aurora, Ohio, USA) (Zweygarth *et al*., 1995). Serum-free GIT medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used for culturing *B. bigemina*, *B. divergens*, and *T. equi*. For *T. equi* cultures, GIT medium was tested alone or supplemented with either hypoxanthine or hypoxanthine and horse serum. Parasite-infected RBCs were diluted with uninfected RBCs to obtain an RBC stock supply with 1% parasitemia. One hundred microliters of RBCs (10%) with 1% parasitemia were dispensed into a 24-well microtiter plate (Nunc, Roskilde, Denmark) with 900 μl of the culture medium and then incubated at 37°C in a humidified multi-gas water jacket incubator with 5% CO_2, 5% O_2, and 90% N_2. Three separate trials were performed, consisting of triplicate experiments, over a period of four days. Subculture was performed on the fourth day to obtain 1% parasitemia. Continuous serum-free GIT medium cultures were performed for 40 days for *B. bigemina*, 32 days for *B. divergens*, 24 days for *T. equi* without hypoxanthine, and 32 days for *T. equi* with hypoxanthine culture systems. The experiments were not continued after indicated time periods due to time limitation. During the incubation period, the overlay culture medium was replaced daily with 900 μl of fresh medium. Parasitemia was monitored daily by counting the parasitized RBCs to approximately 1,000 RBCs in Giemsia-stained thin blood smears. Subculture was performed every four days.

Animal experiment approval

Animal experiments were conducted under approval number 27-65 from animal welfare Committee at Obihiro University of Agriculture and Veterinary Medicine.

Statistical analysis

The differences in the percentages of parasitemia for the *in vitro* cultures were analyzed with GraphPad Prism 5 (GraphPad Software, Inc., CA, USA) using the two-way ANOVA followed by Bonferroni's *post-hoc* tests. A P value of < 0.05 was considered statistically significant.

RESULTS

The GIT medium supported the culture of *B. bigemina* for four days. The percentages of parasitemia were significantly different between the parasites grown in M199 medium supplemented with 40% bovine serum and in GIT medium on days 3 ($P < 0.01$) and 4 ($P < 0.001$) (Fig. 1A). Parasites grown in serum-free GIT achieved parasitemias of approximately 12% on the fourth day, as compared with approximately 8% for those grown in M199 medium supplemented with 40% serum. Moreover, the *in vitro* culture of *B. bigemina* in serum-free GIT medium continued for 40 days, as compared with the M199 culture medium supplemented with 40% serum (Fig. 1B). Parasite growth was higher in serum-free GIT medium, achieving approximately 20% parasitemia; however, it was only significantly different between the parasitemia in M199 medium cultures and GIT medium cultures on days 8 ($P < 0.01$), 20 ($P < 0.05$), 24 ($P < 0.001$), and 36 ($P < 0.001$) (Fig. 1B). There were no observed changes in the parasite morphology from serum-free GIT medium (Fig. 2A) as compared with the parasites from M199 medium supplemented with serum (Fig. 2B).

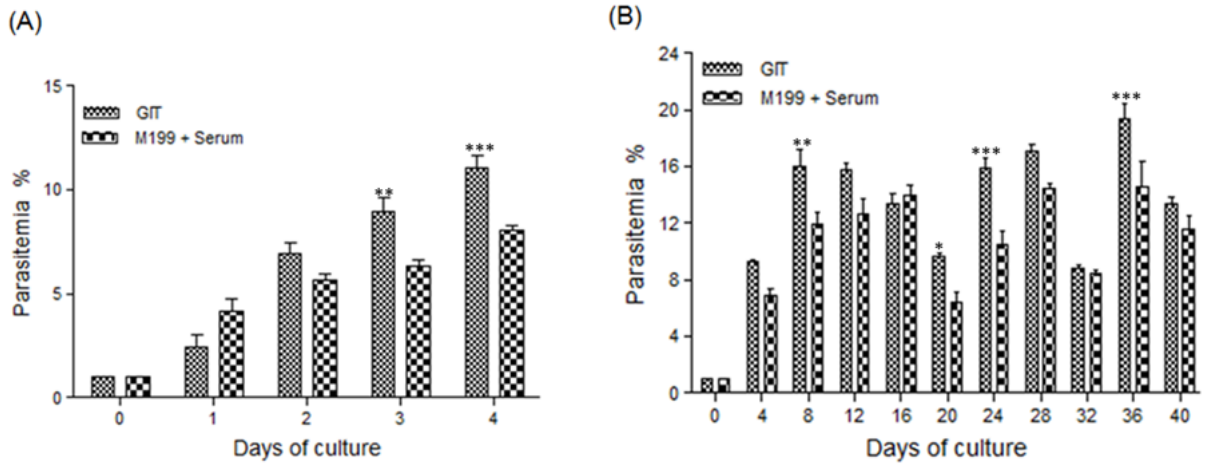

Fig. 1. *In vitro* growth of *Babesia bigemina* in serum-free GIT and M199 with 40% serum culture systems. (A) Cultures were maintained for four days. The curves represent the results of three experiments. (B) Cultures were maintained for 40 days of continuous culture. Each value represents the mean ± standard deviation in triplicate (two-way ANOVA followed by Bonferroni's *post-hoc* tests). Asterisks indicate significant difference between serum-free GIT and M199 with 40% serum culture systems on same days (*$P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

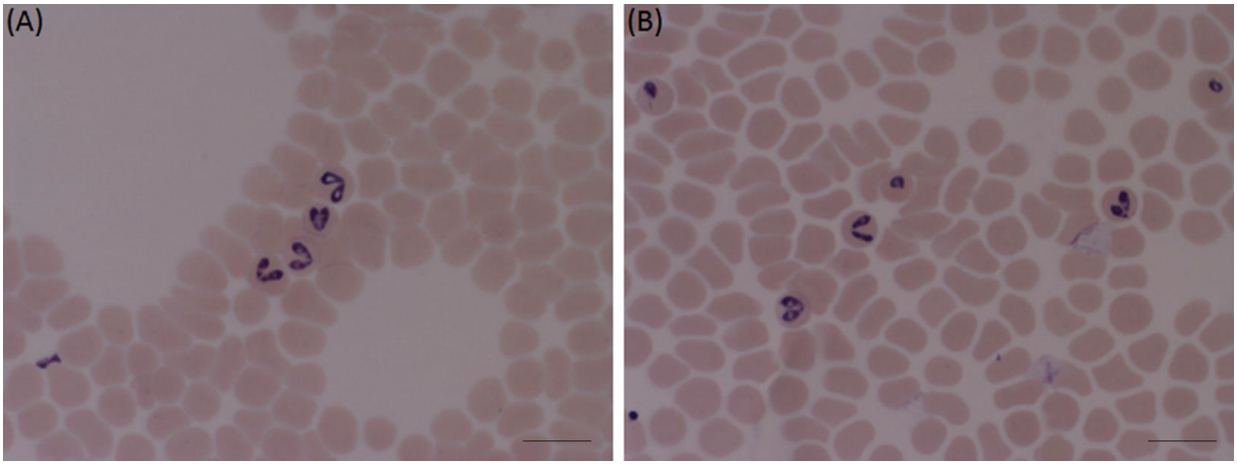

Fig. 2. Light micrographs of *Babesia bigemina* from M199 with 40% serum (A) and serum-free GIT (B) culture systems. Micrographs were taken on day forty of *in vitro* culture. Bars, 10 μm.

B. divergens parasitemia in RPMI1640 supplemented with 10% fetal calf serum was significantly higher than the parasitemia in M199 medium supplemented with 10% fetal calf serum on the third ($P < 0.01$) and fourth ($P < 0.001$) days of culture achieving parasitemia of about 16% (Fig. 3A). While parasitemia of *B. divergens* in RPMI1640 showed only a significant difference ($P < 0.001$) from the serum-free GIT medium culture systems on the third day (Fig. 3B). The serum-free GIT medium culture system supported the growth of *B. divergens* for 32 days. The growth was significantly different between medium RPMI1640 supplemented with 10% fetal calf serum and serum-free GIT medium at days 12, 16, 24, and 28 ($P < 0.05$, Fig. 3C). Parasitemias were between 12-16%. The parasite morphologies were identical in RPMI1640 (Fig.

4A) and serum-free GIT (Fig. 4B), while morphological changes were observed in parasites from M199 medium culture systems (Fig. 4C).

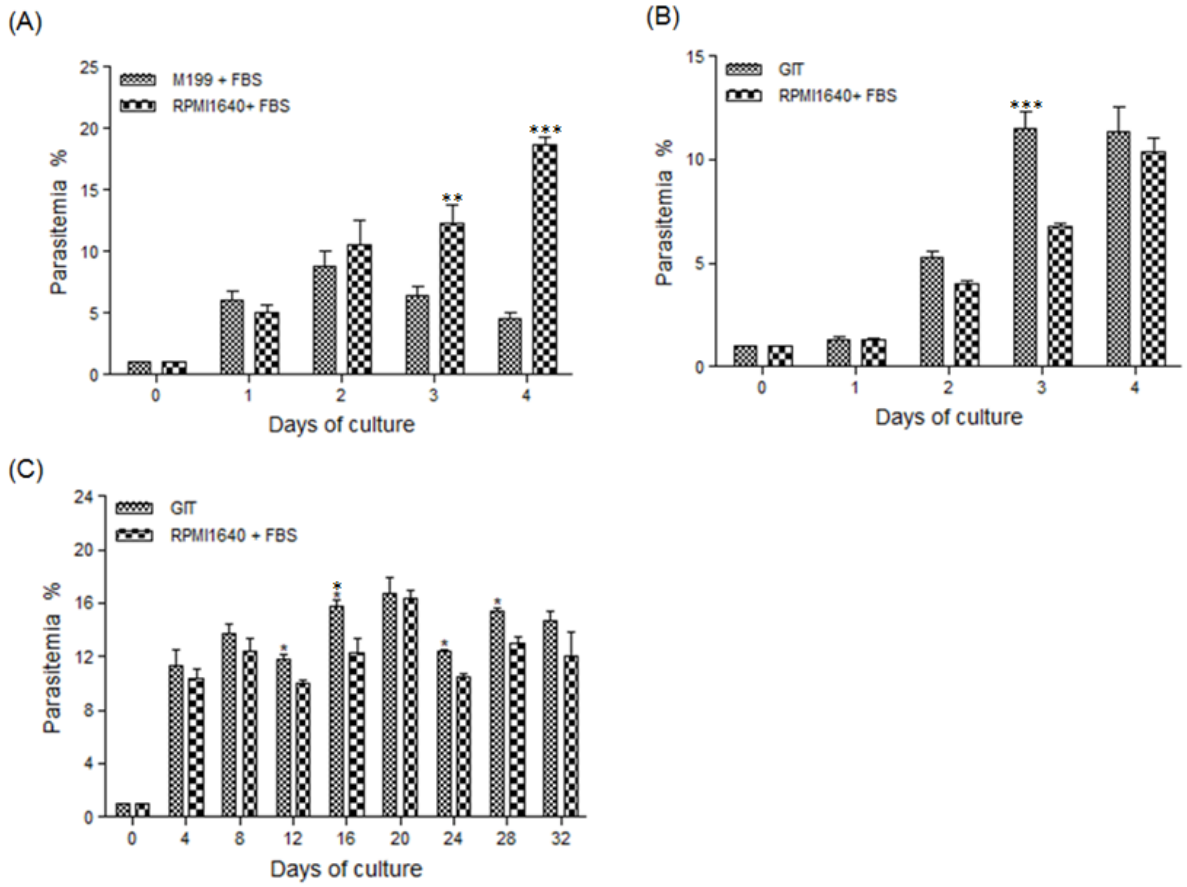

Fig. 3. *In vitro* growth of *Babesia divergens*. Cultures were maintained for four days in M199 supplemented with 40% serum (A) and serum-free GIT (B) media, as compared with RPMI1640 medium supplemented with 10% fetal calf serum. The curves represent the results of three experiments. (C) Continuous culture of *B. divergens* in serum-free GIT, as compared with RPMI1640 supplemented with 10% fetal calf serum. Each value represents the mean ± standard deviation in triplicate (two-way ANOVA followed by Bonferroni's *post-hoc* tests). Asterisks indicate significant difference between two culture systems on same days (*$P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

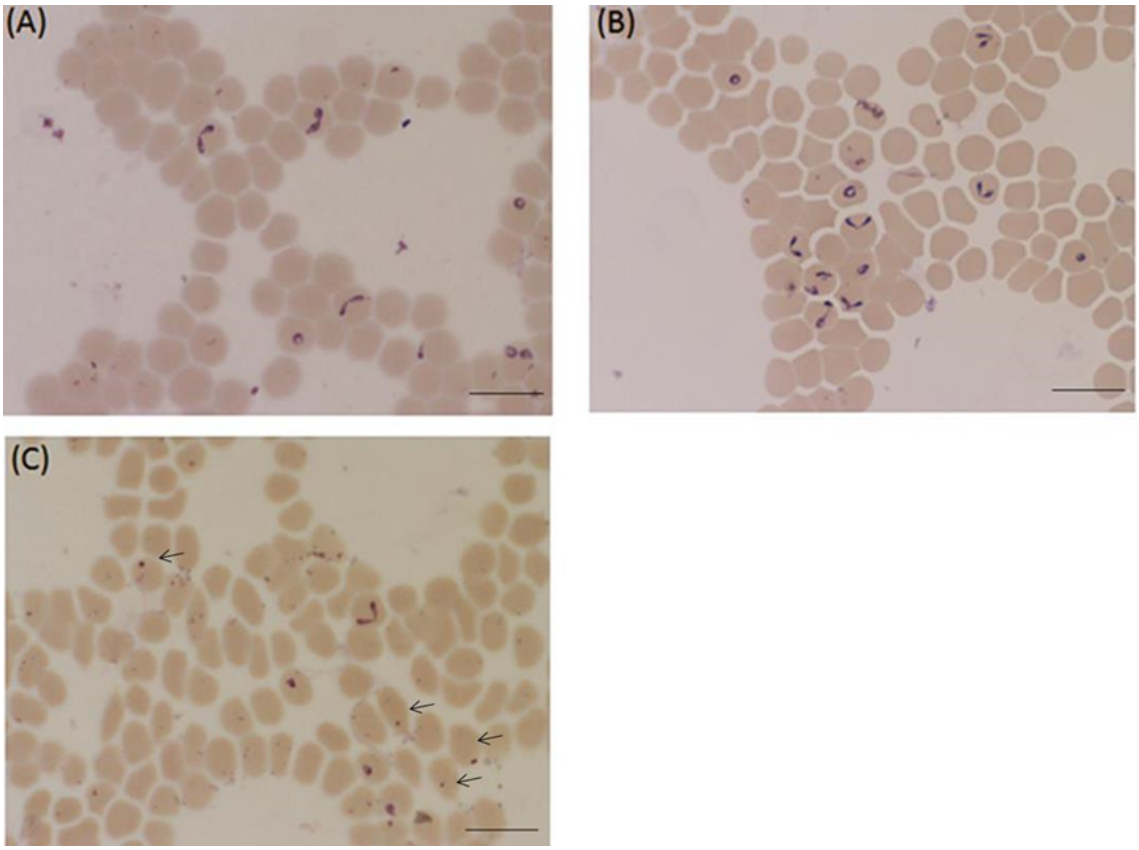

Fig. 4. Light micrographs of *Babesia divergens* from RPMI1640 supplemented with 10% fetal calf serum (A), serum-free GIT (B), and M199 supplemented with 10% serum (C) medium culture systems. Micrographs were taken on day thirty two of *in vitro* culture in A and B and fourth day in B. Note the pycnotic appearance of the parasite in C, indicated by arrow. Bars, 10 μm.

The parasitemias for *T. equi* were not significantly different in cultures systems of M199 and GIT media supplemented with 40% horse serum and hypoxanthine (Fig. 5A). Parasitemias levels achieved around 15% in GIT culture supplemented with serum and hypoxanthine. *T. equi* parasitemias in M199 standard medium were significantly different from either serum-free GIT or GIT supplemented with hypoxanthine culture systems on days 2 ($P < 0.001$) and 4 ($P < 0.01$) (Fig. 5B). Parasitemias levels around 17% were achieved in either serum-free GIT or GIT supplemented with hypoxanthine culture systems. The serum-free GIT culture system supported the *T. equi* growth for only 20 days with parasitemias around 10% (Fig. 5C), while parasite growth was sustained in serum-free GIT medium supplemented with hypoxanthine for 32 days (Fig. 5D). The parasitemia percentages were only significantly different in serum-free GIT supplemented with hypoxanthine compared with M199 culture systems on day 24. Parasitemia in GIT supplemented with hypoxanthine attained about 12% (Fig. 5D). The morphologies of the parasites were similar in M199 supplemented with 40% horse serum and hypoxanthine (Fig. 6A), GIT supplemented with hypoxanthine (Fig. 6B), and serum-free GIT (Fig. 6C) medium culture systems.

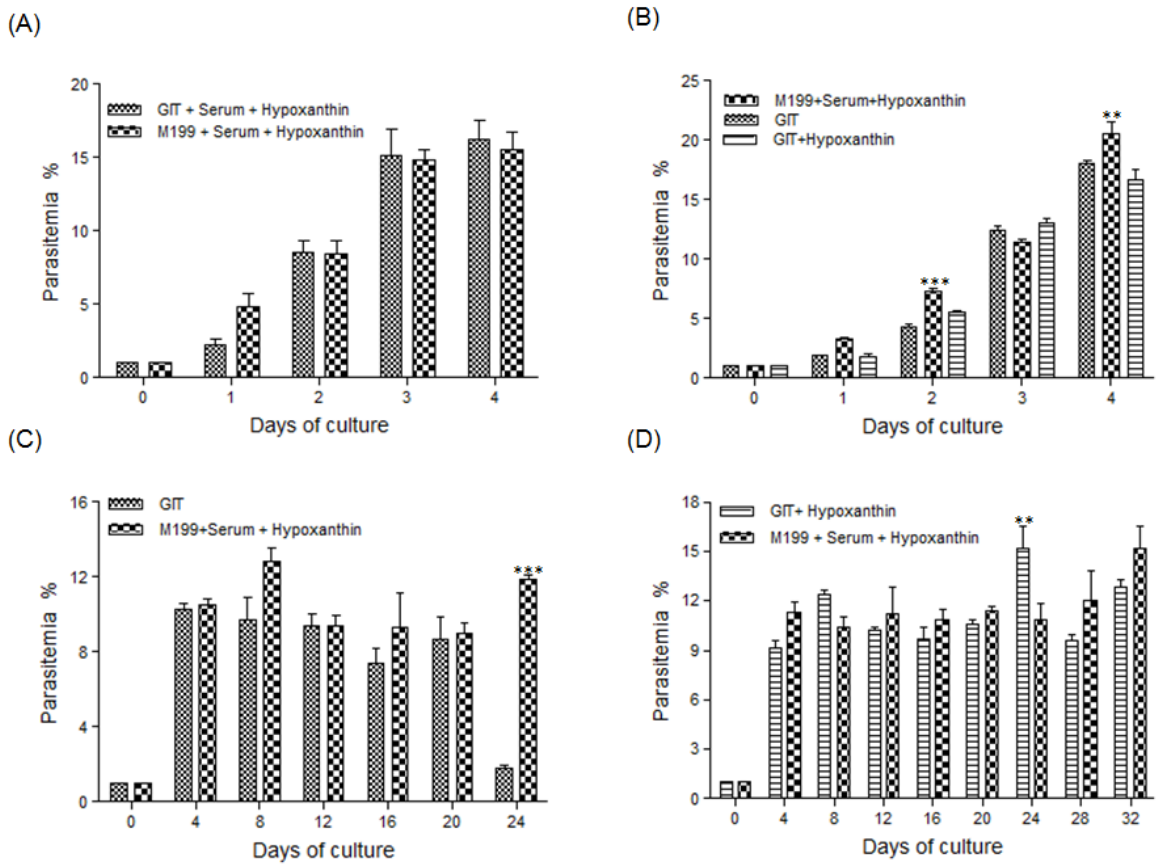

Fig. 5. *In vitro* growth of *Theileria equi* in different medium culture systems. *T. equi* growth in M199 supplemented with 40% serum plus hypoxanthine was compared with GIT supplemented with 40% serum plus hypoxanthine (A), GIT supplemented with hypoxanthine, and serum-free GIT (B) media. The experiments were repeated for three times. Continuous culture of *T. equi* in GIT supplemented with hypoxanthine (C) and serum-free GIT (D) media. Each value represents the mean ± standard deviation in triplicate (two-way ANOVA followed by Bonferroni's *post-hoc* tests). Asterisks indicate significant difference between two culture systems on same days (*$P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

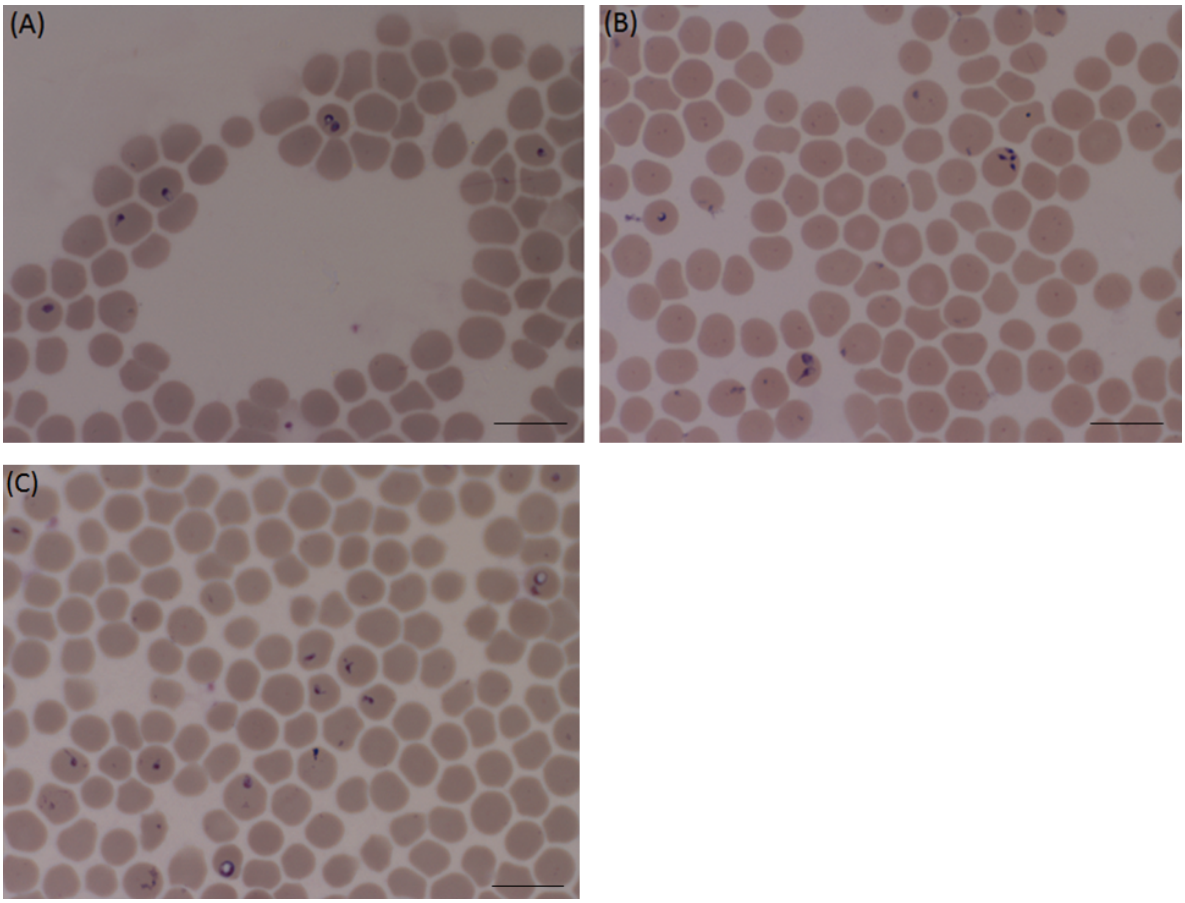

Fig. 6. Light micrographs of *Theileria equi* from M199 supplemented with 40% serum plus hypoxanthine (A), serum-free GIT supplemented with hypoxanthine (B), and serum-free GIT (C) medium culture systems. Micrographs were taken on the days twenty four (B) and thirty two (A and C) of *in vitro* cultivation. Bars, 10 μm.

DISCUSSION

Serum-free GIT medium was selected for the replacement of standard media used for the *in vitro* culture of *B. bigemina*, *B. divergens*, and *T. equi* due to its previous use in the cultures of *B. caballi* (Ikadai *et al*., 2001) and *B. bovis* (Bork *et al*., 2005). *B. bigemina* and *B. divergens* were continuously grown in the serum-free GIT medium without any supplementations. The parasitemia levels and the morphological appearances of *B. bigemina* and *B. divergens* cultured in the serum-free GIT medium were also identical to those cultured in 40% serum containing M199 medium and 10% fetal calf serum, respectively, indicating that the GIT medium may be resourceful for the parasites as basic culture medium. Furthermore, serum-free GIT medium might be suitable for assessing the drug effects in the *in vitro* growth inhibition assay of *B. bigemina* and *B. divergens*. The results of this study are in agreement with the previous results of Ikadai *et al*. (2001) and Bork *et al*. (2005) who successfully grew *B. caballi* and *B. bovis*, respectively, in serum-free GIT medium without any supplements, indicating that the components of GIT are sufficient for the growth of *Babesia* parasites. This was also in agreement with our unpublished observations of culturing *B. bigemina* in GIT with or without serum for about four months. We did not continue the culture of *B. divergens* using M199 medium due to

low parasitemia achieved and changes in the parasite morphology compared with RPMI1640 and serum-free GIT media.

Serum-free GIT medium showed similar growth rates to serum-free GIT with either horse serum or hypoxanthine and the standard M199 medium in the four-day culture system, indicating that serum-free GIT medium might be suitable for studying the drug effects on the parasite in the four-day *in vitro* growth inhibition assay of *T. equi*. On the other hand, serum-free GIT supported *T. equi* continuous culture for up to 20 days, and, later, the parasitemia dramatically decreased to its lowest level on day 24, while the supplementation with hypoxanthine improve the growth for 32 days; this agreed with the previous work on *T. equi* (Zweygarth *et al*., 1995), which indicated that hypoxanthine is an essential supplement for *T. equi* culture. Therefore, serum-free GIT supplemented with hypoxanthine might be suitable for the *in vitro* culture of *T. equi*.

In conclusion, this study indicated serum-free GIT medium for short-term *in vitro* cultivation of *B. bigemina* and *B. divergens* or supplemented with hypoxanthine for the *in vitro* culture of *T. equi*. Our next goal is to assess this system in the *in vitro* drug inhibition assay of *B. bigemina*, *B. divergens*, and *T. equi*.

ACKNOWLEDGEMENTS

The study supported by the Ministry of Higher Education of Egypt and The Ministry of Education, Culture, Sports, Science and Technology of Japan

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

This manuscript is approved by all authors, and if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

REFERENCES

Avarzed A, Igarashi I, Kanemaru T, Hirumi K, Omata Y, Saito A, Oyamada T, Nagasawa H, Toyoda Y, Suzuki N. 1997. Improved *in vitro* cultivation of *Babesia caballi*. J Vet Med Sci. 59: 479-481.

Bork S, Okamura M, Matsuo T, Kumar S, Yokoyama N, Igarashi, I. 2005. Host serum modifies the drug susceptibility of *Babesia bovis in vitro*. Parasitology 130: 489-492.

Grande N, Precigout E, Ancelin ML, Moubri K, Carcy B, Lemesre JL, Vial H, Gorenflot A. 1997. Continuous *in vitro* culture of *Babesia divergens* in a serum-free medium. Parasitology 115: 81-89.

Homer MJ, Aguilar-Delfin I, Telford SR, Krause PJ, Persing DH. 2000. Babesiosis. Clin Microbiol Rev. 13: 451-469.

Igarashi I, Njonge FK, Kaneko Y, Nakamura Y. 1998. *Babesia bigemina*: *in vitro* and *in vivo* effects of curdlan sulfate on growth of parasites. Exp Parasitol. 90: 290-293.

Ikadai H, Martin MD, Nagasawa H, Fujisaki K, Suzuki N, Mikami T, Kudo N, Oyamada T, Igarashi I. 2001. Analysis of a growth-promoting factor for *Babesia caballi* cultivation. J Parasitol. 87: 1484-1486.

Jackson LA, Waldron SJ, Weier HM, Nicoll CL, Cooke BM. 2001. *Babesia bovis*: culture of laboratory-adapted parasite lines and clinical isolates in a chemically defined medium. Exp Parasitol. 99: 168-174.

James MA, Levy MG, Ristic M. 1981. Isolation and partial characterization of culture-derived soluble *Babesia bovis* antigens. Infect Immun. 31: 358-361.

Kappmeyer LS, Thiagarajan M, Herndon D, Ramsay JD, Caler E, Djikeng A, Gillespie JJ, Lau A, Roalson E, Silva J, Silva M, Suarez C, Ueti M, Nene V, Mealey R, Knowles D, Brayton K. 2012. Comparative genomic analysis and phylogenetic position of *Theileria equi*. BMC Genomics 13: 603-615.

Levy MG, Ristic M. 1980. *Babesia bovis*: continuous cultivation in a microaerophilous stationary phase culture. Science 207: 1218-1220.

Mackenstedt U, Gauer M, Mehlhorn H, Schein E, Hauschild S. 1990. Sexual cycle of *Babesia divergens* confirmed by DNA measurements. Parasitol Res. 76: 199-206.

Rothschild C, Knowles D. 2007. Equine piroplasmosis. pp. 465-473. In: Equine Infectious Diseases, Sellon, D.C. and Long, M.T. (Eds.), Elsevier Inc., St. Louis, Missouri.

Salama AA, AbouLaila M, Terkawi MA, Mousa A, El-Sify A, Allaam M, Zaghawa A, Yokoyama N, Igarashi I. 2014. Inhibitory effect of allicin on the growth of *Babesia* and *Theileria equi* parasites. Parasitol Res. 113: 275-283.

Schrevel J, Grellier P, Rigomier D. 1992. New approaches in in vitro cultures of *Plasmodium falciparum* and *Babesia divergens* by using serum-free medium based on human high density lipoproteins. Mem Inst Oswaldo Cruz 87: 71-75.

Schuster FL. 2002. Cultivation of *Babesia* and *Babesia*-like blood parasites: agents of an emerging zoonotic disease. Clin Microbiol Rev. 15: 365-373.

Vayrynen R, Tuomi J. 1982. Continuous in vitro cultivation of *Babesia divergens*. Acta Vet Scand. 23: 471-472.

Vega CA, Buening GM, Green TJ, Carson CA. 1985. *In vitro* cultivation of *Babesia bigemina*. Amer J Vet Res. 46: 416-420.

Zintl A, Mulcahy G, Skerrett HE, Taylor SM, Gray JS. 2003. *Babesia divergens*, a bovine blood parasite of veterinary and zoonotic importance. Clin Microbiol Rev. 16: 622-636.

Zweygarth E, Just MC, de Waal DT. 1995. Continuous *in vitro* cultivation of erythrocytic stages of *Babesia equi*. Parasitol Res. 81: 355-358.

Zweygarth E, Just MC, De Waal DT. 1997. *In vitro* cultivation of *Babesia equi*: detection of carrier animals and isolation of parasites. Onderstepoort J Vet Res. 64: 51-56.

Zweygarth E, Van Niekerk CJ, De Waal DT. 1999. Continuous *in vitro* cultivation of *Babesia caballi* in serum-free medium. Parasitol Res. 85: 413-416.