POSSIBILITY OF ELIMINATION OF PATHOGEN FROM *Babesia* INFECTED ANIMALS BY MEANS OF EMBRYO TRANSFER

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ABSTRACT

Babesia infections occur mainly in animals, and are transmitted by ticks. The severity of the diseases varies considerably depending on the species of Babesia involved as well as the immune response of the infected animal. In Mongolia infection produced by Babesia parasites is widely spread, provoking severe damage to the agricultural and economic sectors. Currently, strategies to control and prevent the infection are inefficient. Indeed, the necessity to look for suitable and accessible strategies to obtain animals free from the infection is needed. Currently, assisted reproductive technologies (ART) are used for the improvement of productivity in livestock. Moreover, embryo transfer seams to be useful approach to obtain clean embryos obtained from infected animals. Therefore, by using a mice model (ICR) infected with Babesia microti, an alternative method to obtain animals free from infection was examined. ICR mice at 8 weeks old were challenged with 0.2 ml of $1x10^7$ IRBC/ml by i.p injection. After infection, superovulation was induced and then embryos were obtained and washed. Then, their development stage along with their morphological characteristics were monitored. In vitro embryos obtained from uninfected mice were used as a control group. The results indicate that the infection does not have any influence on pre-implantation embryonic development and morphological characteristics. Thus, we suggest that embryos obtained from infected animals might be useful for embryo transfer in order to improve productivity of livestock and reduce the risk of congenital infection. In summary, ART such as embryo transfer might be an useful technique in countries where Babesiosis is an endemic disease.

KEY WORDS: *Babesia microti*, mice, in vitro development, superovulation

INTRODUCTION

Assisted reproductive technology (ART) is the application of laboratory or clinical technology to gametes and/or embryos for the enhancement of reproduction (David et al., 2012). Embryo transfer is a step in the process of assisted reproduction in which embryos are placed into the uterus or oviduct of female with the intent to establish a pregnancy. This technique may be used in humans or in animals. Embryo transfer is a biotechnological method that uses female reproductive capacity at maximum (Gordon., 2005). If proper procedures are followed, the risk of transmitting infectious diseases via embryo transfer is lower than with natural mating or artificial insemination (Hare et al., 1986). The general epidemiological aspects of embryo transfer indicates that transfer of embryos provides an opportunity to introduce genetic material into

populations of livestock while greatly reducing the risk for transmission of infectious diseases (Stringfellow et al., 1991).

Babesiosis is a disease especially of domestic and wild mammals mainly prevalent in the tropics and subtropics areas. It has a great economic importance due to the loss of animals or their products (Krauss et al., 2003). *Babesia* infections are transmitted mainly by ticks (Malagon et al., 1994). The parasites live and reproduce in red blood cells of the vertebrate host. The severity of the diseases varies considerably depending on the species of *Babesia* involved, as well as the immune response of the host. The most frequent outcome of *Babesia* infection is anemia as the immune system destroys infected red blood cells. However, *Babesia* can have other effects throughout the body as well (Michael et al., 2012). *Babesiosis* is one of the infectious diseases that widely spread in Mongolia. The main species of *Babesia* present in Mongolia are *B.equi*, *B.caballi*, and *B.bigemina* (Boldbaatar et al., 2005). It is well known that this infection provoke severe damage to agricultural and economic sectors (Battsetseg et. al., 2012). Moreover, the cattle industry considered to be one of the major sectors that influence the country's economic development is seriously affected (Sivakumar et al., 2012). Indeed, it is important to maintain healthy animals to improve the quality and quantity of products from livestock farming (Altangerel et al., 2012). Recently the occurrence, treatment and diagnosis of *Babesiosis* has been addressed (Altangerel et.al., 2012; Boldbaatar et al., 2005). However, the diseases still remain a problem in the country. Currently, strategies to control and prevent *Babesiosis* are inefficient; indeed the necessity to look for suitable and accessible strategies to obtain animals free from the infection is needed. In this study, by using a mouse model (ICR) infected with *B.microti,* an alternative method to obtain animals free from infection by means of embryo transfer was examined.

MATERIALS AND METHODS

Mice and parasite

ICR female mice at eight weeks old were obtained from the Specific Pathogen Free (SPF) room of the National Research Center for Protozoan Diseases. Common daily manage was carried out throughout the experiment. Briefly, the light-dark cycle was 14:10hr (lights on 07:00 a.m. to 19:00 p.m.). The room temperature and humidity were maintained at 68 to 70°F (24.0±1°C) and 50% to 60%, respectively. Four mice were housed in a polycarbonate cage and were allowed to free access to standard laboratory chow and tap-water. Cages were changed once a week. The parasite used in these experiments was *B.microti,* obtained from the Research Unit for Functional Genomics which was kept at -80°C.

Experimental infection

Frozen *B.microti* was recovered by intraperitoneal (i.p) injection into the ICR mice (n=2). When the parasitemia was about 22.4%, 50 µl of infected blood obtained from the tail vein was mixed with 500 µl of phosphate buffer saline (PBS). Then, three ICR mice were challenged with 0.2 ml of this preparation by i.p injection. Parasitemia was monitored every two days. When parasitemia reached about 26.7 %, one of these mice was sacrificed and blood was obtained from the heart. Then, a fixed infection dose of $1x10^7$ IRBC/ml was prepared. Thereafter, twelve female ICR mice were challenged with 0.2 ml of $1x10^7$ IRBC/ml by i.p injection. Then, from day two after infection a drop of blood was collected by cutting the tip of mouse tail to make a smear for each mouse. This procedure was repeated every two days. Blood smears were stained by Giemsa method and parasitemia quantification was carried out using a light microscope (Nikon, YS2-H, Japan) at 100x magnification in five consecutive fields which include about 200 cells per field.

Superovulation and mating

Twenty female ICR mice were superovulated by i.p injection with 5 I.U. of pregnant mare serum gonadotropin PMSG (Teikoku Hormone MFG Co. Ltd., Tokyo, Japan). Forty eight hours later they were treated with 5 I.U. of human chorionic gonadotropin (hCG) (Teikoku Hormone MFG Co. Ltd., Tokyo, Japan). Thereafter they were mated with males of the same strain.

Embryo culture

Embryo culture was performed according to the protocol described by Quinn et al., 1985. Briefly, two drops (200µl / drop) of modified Whitten's medium (mWM) (Ark resource Co., Ltd., Tokyo, Japan) was placed into a dish and covered with liquid paraffin and then incubated at 37°C, 5% CO_2 in air. Then, 2-cell stage embryos were flushed from oviducts from plug positive females to this medium for washing. Then, embryos were

placed into new mWM medium and incubated at 37°C, 5% CO_2 in air. Cultures were monitored every day to follow embryonic development using a stereomicroscope (Nikon, SMZ1500, Japan). Culture media composition and flushed media composition are described in Table 1.

Table 1

Media composition

	Modified Whitten's Medium (mWM)
NaCl	640.0
KCL	35.6
KH2PO4	16.2
MgSO4	29.4
CaCl2	-
NaHCO3	190.0
Ca- lactate	46.0
Na-pyruvate	2.5
Glucose	100
Penicillina	7.5
Streptomycina	5.0
0.5% Phenol red(µl)	0.2
20mM $^{\beta}$-ME(ul)	0.1368
BSA	300.0
100 mM EDTA 2Na(mg)	1.86

Materials are in mg/100ml

Statistical Analysis

The data presented in Table 3 were analyzed using Chi-square test. p value < 0.05 was considered significant.

RESULTS

1.1. Infection course of *B.microti* in ICR mice

Parasites were detected in all mice from Day 4 (1.5 %) and increased from Day 6. Peak of parasitemia was observed on Day 10 post infection (38.7 %), and from Day 12 parasitemia decreased significantly. After one month parasites were almost totally cleared (Figure 1 A and B).

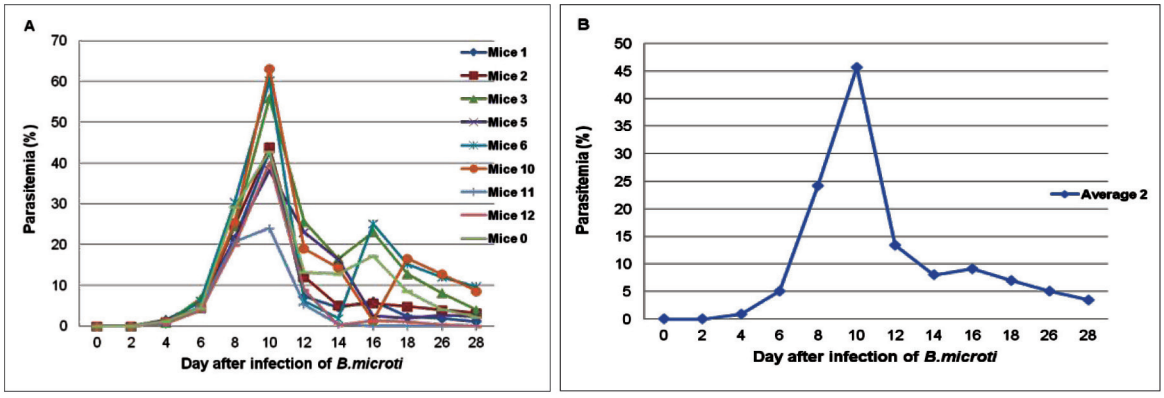

Figure 1. Parasitemia kinetics in ICR mice infected with 0.2 ml of 1x10^7 IRBC/ml of *B.microti* (A) Individual data. (B) Average data.

1.2. Visible parasitemia was observed in one mouse born from one infected mother.

Two mice of group 1 delivered puppies on 21 day after mating. One puppy from group 1 (parasitemia increasing) showed visible parasitemia two weeks after delivery. The puppies delivered from the other experimental groups did not show visible parasitemia (Table 2, Figure 2). These data indicated that parasites transmitted vertically in a mouse when female was mated at early stage of *B.microti* infection.

Table 2

Number of puppies congenitally infected with *B.microti*

	No. of mice infected	No. of mice pregnant	No. of puppies born and death	Nubmer of puppies infected	Parasitemia (%)			
				Days after birth	14	16	18	20
Group 1	2	1 out of 2	4 alive, 1 death	1	3.6	19.8	16.7	14.3
Group 2	2	1out of 2	15 alive	0	-	-	-	-
Group 3	2	2	12 or 14 alive	0	-	-	-	-

Group 1, 2 and 3: Females were mated with a male on day 6, 10 and 12 post infection, respectively.

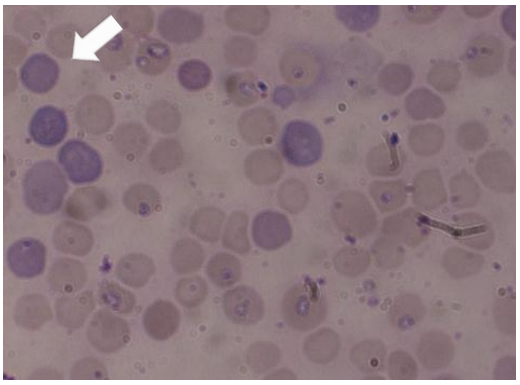

Figure 2. Blood smear obtained from the tail vein. Infected RBCs were observed in one puppy. Arrows indicate the ring form of *B.microti.*

1.1. Embryonic development was similar in the infected and uninfected mice

The percentage of embryo development was higher in the parasitemia decreasing group compared to the parasitemia increasing group. However, there were no significant different between the experimental groups.

Table 3

Development of 2 cells embryo using mWM media

	Replicant	Total 2 cell	4 cell (%)	8 cell (%)	Morula (%)	Blastocyst (%)	Hatching blastocyst (%)
Time		0h	24h	48h	72h	96h	120h
Uninfected	4	34	100.0[a]	82.3[a]	73.5[a]	70.5[a]	67.6[a]
Mating on Day 6 post infection	2	127	78.7[a]	82.6[a]	75.5[a]	63.7[a]	61.4[a]
Mating on Day 10 post infection	2	82	97.5[a]	89.0[a]	89.0[a]	82.9[a]	75.6[a]

[a] Values with different superscripts are significantly different at $p < 0.05$.

There was no difference in the morphological characteristics of embryos obtained from infected and uninfected mice. Embryos development from 2 cell stage to 4-cell, 8-cell, morula and blastocyst develop normally (Figure 3).

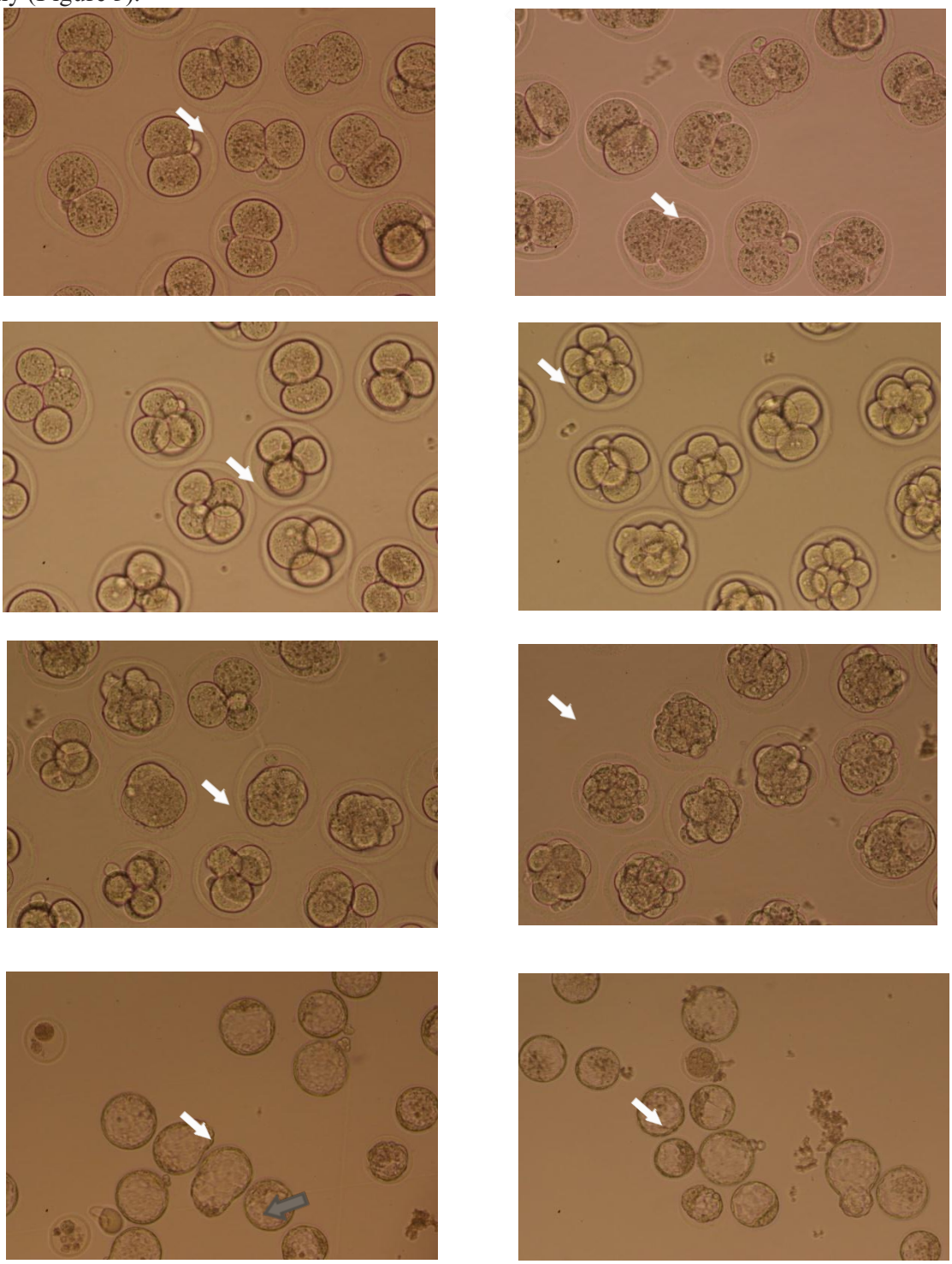

Figure 3. Phase-contrast images of mouse embryo development from day one to day six. Following fertilization, embryos undergo a series of mitotic cell divisions. Arrowheads on (d1) and (d2) indicate 2 cells and 4 cells. At day four the embryo compacts, resulting in the formation of a morula that consists of blastomeres in a compact cluster contained within the zona pellucida. The blastocyst, which forms at (d6), is a fluid-filled structure composed of an inner cell mass (white arrowhead) and trophectoderm (gray arrowhead).

DISCUSSION

This study showed that *B.microti* was vertically transmitted to the next generation. *Babesia* has a vertical transmission through the uterine route and not via the transmammary route. Moreover, there is an evidence that *Babesia* infection can be acquired by transplacental way in a dog (Fukumoto et al., 2005). When embryos implant to the uterus wall, might get infected through the blood. Therefore, embryo transfer can decrease the risk of infection. (Hare et al., 1986). There are many factors affecting ovulation including age, species, strain, hormone dosage and gonadotropin interval delivery (Byers et al., 2006, Ozgunen et al., 2001). Administration time of PMSG and hCG represents an important factor to improve fertility (Golkar Narenji et al., 2012). We confirmed that PMSG and hCG hormone treatment enhanced embryo production independently of infection. Embryos obtained from infected and non infected animals developed normally and morphological characteristics were similar (Table 3, Figure 3). Indicating that, *Babesia* infection does not have any influence on pre-implantation development. Thus, we suggest that embryos obtained from infected animals might be useful for embryo transfer in order to improve productivity of livestock and reduce the risk of congenital infection. In summary, ART such as embryo transfer might be an useful technique in countries where *Babesiosis* is an endemic disease.

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