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# A PRELIMINARY STUDY FOR ELIMINATION OF CONTAMINATED *BABESIA MICROTI* FROM THE MODEL OF HUMAN PLASMA FRACTION

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## ABSTRACT

The physical and chemical treatments for elimination of pathogens from human plasma fraction (HPF) are very important for transfusion therapy. In this study, we evaluated the safety by the sterile filtration, virus removal filtration, heat inactivation and alcohol treatment of human albumin fraction containing *Babesia microti*. Following treatments, mice were challenged infection to establish a model for detection of *B. microti* in HPF. We chose the same methods to process the globulin and albumin fractions, as with the treatments of the samples. As a result, we did not detect parasites in mice inoculated with all treated samples when Giemsa-stained thin blood smears from the challenged mice were examined for 51 days. However, by immunofluorescent antibody assay, we found some parasites in the sample which had been filtrated by a sterility filter (0.2μm). This suggests that B. microti might be able to pass through the routinely employed sterile filter, and babesiosis might occur when the manufacturing process includes the sterile filtration only. It is suggested that blood plasma products which have been introduced with the multiple processes of virus removal and/or inactivation such as alcohol fractionation, pasturization, chemical treatment, virus removal filtration to their manufacturing process, might be safe against protozoa infections.

**Key words**: *Babesia microti*, human plasma fraction, filtration.

## INTRODUCTION

*Babesia microti* is a hemoprotozoan parasite of rodents which is transmitted by ticks and has been recognized as the etiological agent of human babesiosis (Shih and Liu. 1997; Dammin et al. 1981). Hundreds of human babesiosis have been reported in the north-eastern and upper mid-western areas in the U.S.A., and most cases were reported to be caused by *B. microti* (Gorenflot et al. 1998).

The possibility of transmission of babesiosis by transfusion has been of deep concern (Gerber et al. 1994). Blood and human plasma fraction (HPF) for transfusion are checked for viral or bacterial infection and treated to prevent etiological agents from transmission to patients. HPF is treated with alcohol for fractionation, following multiple processes such as chemical treatment, pasteurization, virus removal filtration and sterile filtration to remove and/or inactivate bacteria, viruses and the other infectious microorganism. Cohn alcohol fractionation and pasteurization, namely heating at 60C for 10 hrs, have been used as a plasma protein manufacturing process for many years, and have been demonstrated effective for virus removal and/or inactivation. Recently, treatment with filtration (35nm filter) of blood plasma products to remove viruses has been introduced for many blood plasma products.

Although any of these treatments can not exclude the possibility that *B. microti* might exist in

HPF or its products after these treatments, there is no program of national surveillance for human babesiosis in Japan, as well as in the U.S.A and Europe. In present study, we utilized the same methods as the treatment of samples to examine whether contaminated *B. microti* could be eliminated or inactivated by filtration, heat inactivation or alcohol treatment. The effects of these treatments were then examined by challenge infection in mice.

## MATERIALS AND METHODS

***Mice:***Female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). Seven week old mice were used for the present experiments.

***Parasite:***The Munich strain of *B. microti* (Igarashi et al. 1994) was maintained by blood passages in mice. When parasitemia reached about 30%, parasitized blood was used for the experiment.

***Treatment of parasite:****B. microti*-infected red blood cells (RBC) were collected from infected mice with a heparinized syringe, treated with 0.83% $NH_4Cl$ solution for 10 min at $37^0$C, and then centrifuged at 2,000 *g* for 10 min at 4C. The obtained pellet was washed by 9.6mM phosphate-buffered saline (PBS, pH 7.4) twice, and parasites ($6.2\times10^7$/ml) were suspended in PBS containing 5% albumin. The parasite suspension (untreated sample) was filtrated either by large (Pall, 0.2μm, filtration area is $5.3cm^2$) or small (Asahikasei, 35nm, filtration area is $0.001m^2$) pore membrane filters and designated as LPF or SPF, respectively. The original sample ($4.5\times10^6$/ml) was also treated with 40% alcohol adjusted pH 6.8 with acetic acid for 5 hrs on ice and named alcohol-treated sample (ATS). To remove the possibility that serum protein prevent parasites from heat inactivation 25% albumin fraction containing the parasites (3×106/ml) were treated at $60^0$C for 10 hrs and designated as heat-treated sample (HTS).

***Infection of mice:***For challenge infection, mice were intraperitoneally injected with 1ml of each sample. Parasitemia was monitored in thin blood smear stained with Giemsa solution.

***Immunofluorescent antibody assay (IFA):***Thin smears were prepared as antigens with infected RBC in PBS containing 3% bovine serum albumin. The antigen slides were fixed with aceton for 5 min at $-20^0$C. Mouse serum samples were added to each well as the first antibody, incubated for 30 min at $37^0$C, and washed three times with PBS. Fluorescein-conjugated anti-mouse IgG in goat IgG (ICN Pharmaceuticals) was added as the second antibody, and incubated for 30 min at $37^0$C. After three washes with PBS, coverslips were placed on the slides. Sera from *B. microti*-immune mice were used for first antibody to detect *B. microti* in smears of LPF and SPF samples.

## RESULTS

At first, the infectivity of *B. microti* after 0.83% $NH_4Cl$ treatment of infected RBC was examined by challenge infection in mice. Parasites in peripheral blood were observed in 4 out of 5 mice after infection with $6.2\times10^7$ parasites in PBS containing 5% albumin (Fig. 1). This result showed that *B. microti* kept its infectivity after treatment of infected RBC with 0.83% $NH_4Cl$.

Secondly, the effect of filtration was examined by parasite detection after filtration with 0.2μm filter or 35nm filter. By IFA, some parasites were detected in the sample filtrated with 0.2μm (LPF) but not in the sample filtrated with 35nm (SPF) after filtration.

The effect of filtration was also examined by the inoculation of LPF or SPF in mice to examine

whether *B. microti* might pass through the filter with their pathogenicity. No parasite was seen in mice injected with LPF or SPF during 51 days of observation period, although no parasitemia were seen in mice challenged with $1\times10^0$, $1\times10^1$, or $1\times10^2$ parasites until 51 days post infection (data not shown). As result of our experiments, we found that filtration can remove or reduce the number of parasites from blood products.

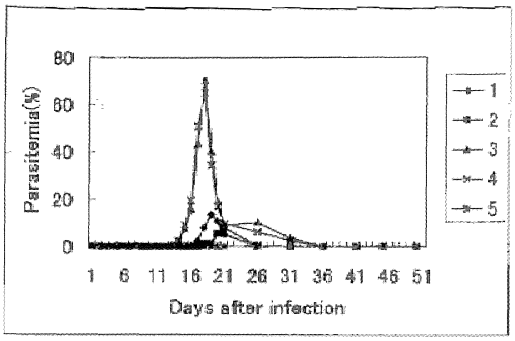


**Fig. 1. The parasitemia of mice infection with *Babesia microti*. The increase of parasites could be seen in 4 out of 5 mice.**

Thirdly, the effects of inactivation by alcohol or heat treatments were studied to examine the possibility that parasites could survive. During 51 days, no parasitemia were seen in mice injected with alcohol-treated sample (ATS) or heat-treated sample (HTS) which were not filtrated.

Finally, specific antibody response against *B. microti* piroplasms in mice was examined to see the effect of these treatments. In mice injected with LPF, the titer of specific antibody increased to 1:256 or 1:1,024 without development of parasitemia in mice. No antibody response was observed in mice injected with SPF, however, a low level of specific antibody response was observed in mice injected with ATS or HTS (Table 1).

Table 1. Detection of anti-*B. microti* antibody in serum from mivarious samples by IFA.

| Inoculated sample | Antibody titer of serum collected at indicated day post infection[a] | | | | | |
|---|---|---|---|---|---|---|
| | 7 | 14 | 21 | 31 | 41 | 51 |
| untreated[b] | 16 | 64 | >4,096 | > 4,096 | > 4,096 | > 4,096 |
| | 16 | 64 | >4,096 | > 4,096 | > 4,096 | > 4,096 |
| | 16 | 1024 | >4,096 | > 4,096 | > 4,096 | > 4,096 |
| | 16 | 1024 | >4,096 | > 4,096 | > 4,096 | > 4,096 |
| | 16 | 64 | 64 | 64 | 64 | 64 |
| LPF[c] | 16 | 64 | 64 | 64 | 64 | 64 |
| | 16 | 64 | 64 | 64 | 256 | 256 |
| | 16 | 64 | 64 | 256 | 256 | 1,024 |
| | 16 | 16 | 64 | 64 | 4 | 4 |
| | 16 | 64 | 64 | 64 | 16 | 16 |
| SPF[d] | < 4 | < 4 | < 4 | < 4 | < 4 | < 4 |
| | < 4 | < 4 | < 4 | < 4 | < 4 | < 4 |
| | < 4 | < 4 | < 4 | < 4 | < 4 | < 4 |
| | < 4 | < 4 | < 4 | < 4 | < 4 | < 4 |
| | < 4 | < 4 | < 4 | < 4 | < 4 | < 4 |
| ATS[e] | < 4 | < 4 | 4 | 4 | 16 | 16 |
| | < 4 | < 4 | < 4 | < 4 | < 4 | < 4 |
| | < 4 | < 4 | < 4 | 4 | 16 | 16 |
| HTS[f] | < 4 | 4 | 4 | 4 | 16 | 16 |
| | < 4 | 4 | 4 | 4 | 16 | 16 |
| | < 4 | < 4 | < 4 | < 4 | < 4 | 4 |

a Each serum was serially diluted from 1:4 to 1:4,096.
b Untreated sample is PBS (5% albumin) containing the parasites (6.2×107 /ml).
c LPF is the filtrated untreated sample by large pore membrane filter.
d SPF is the filtrated untreated sample by small pore membrane filter.
e ATS is the sample ($4.5\times10^6$/ml) treated with 40% alcohol for 5 hrs on ice.
f HTS is the sample ($3\times10^6$/ml) heated at $60^0$C for 10 hrs.

## DISCUSSION

Humans are at risk of infection with hemosporidia like malaria and piroplasma which can be transmitted by transfusion of blood or its products (Gerber et al. 1994). However, there have been no reports studying the removal or inactivation of the contaminated hemosporidia from human blood, as far as we could ascertain.

In this study, we tested whether the parasites contaminated in blood could be removed and/or inactivated by the treatments routinely used by blood plasma fractionation industries in the world. Filtration using 35nm filter, which can remove viruses, could eliminate parasites completely, because there was neither parasitemia nor specific antibody response in mice injected with the sample filtrated with 35nm filter. So, it is the first demonstrated case of a SPF process shown effective to remove hemosporidia, which, theoretically may be removed. Although there are some parasites in the sample filtrated with 0.2μm filter (LPF), no parasitemia were observed by the microscopical examination of blood smear from mice injected with LPF. However, relatively high antibody titers such as 256 or 1,024 were observed in two mice injected with LPF. This result implies the possibility that a small number of filtrated parasites may establish infection in some mice. It is necessary to carry out further detailed investigations in order to clarify this subject.

Parasites may be inactivated by 40% alcohol or heat treatment, because no parasites were observed in mice injected with the alcohol-treated sample or heat-treated sample. However, a low level of specific antibody response was observed in mice inoculated with these samples. The antibody response might be caused by the antigenic substance even if it was inactivated. It is believed that the parasites might be sensitive to the inactivation treatments of heat and chemicals, and may be eliminated more easily by filtration than bacterias and viruses. However, there are no reports describing the experimental data on the inactivation and elimination of parasites. As multiple virus removal and/or inactivation processes other than what we have reported here in the blood product manufacturing process have been introduced, it is considered that further studies will be required to establish the safety of their manufacturing process to parasite infections.

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