Use of flow cytometry to separate *Leucocytozoon caulleryi* gametocytes from avian blood

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SUMMARY

The highly pathogenic avian protozoan *Leucocytozoon caulleryi* infects host chicken cells, and interference by the host genome results in difficulty in obtaining protozoal DNA for genetic analysis. We used flow cytometry analysis to separate expelled *L. caulleryi* gametocytes from infected chicken blood and to analyse cell populations and sorting by FACS efficiency. Infected blood cells stained with SYTO-24 showed a specific area on 2-dimensional scattergrams compared to uninfected blood. The specific area was sorted, and approximately 85% of the sorted cells were identified as *L. caulleryi* gametocytes by microscopic observation. DNA was also extracted from the sorted fraction, and a clear increase in polymerase chain reaction (PCR) amplification of protozoal DNA was observed compared to infected blood without sorting. Host-derived DNA was also detected by PCR; however, its amplification was decreased compared to that in unsorted infected blood. This is the first report of the separation of *L. caulleryi* gametocytes from infected host blood using flow cytometry. This method may be applied to further genetic analyses such as studies of the dynamics of stage-specific *L. caulleryi* gene expression.

Key words: *Leucocytozoon caulleryi*, gametocyte, flow cytometry, separation.

INTRODUCTION

Infection with *Leucocytozoon caulleryi*, the pathogen in chicken leucocytozoonosis, induces severe illness and results in high mortality in chicken farms in Japan (Morii, 1992). *L. caulleryi* has a life cycle similar to that of *Plasmodium* spp. causing malaria; it is transmitted by *Culicoides* biting midges (Akiba, 1960) and has 3 growth stages, schizogony and gametogony in chickens and sporogony in *Culicoides* (Morii, 1992). However, unlike the malaria protozoan, *L. caulleryi* is not easily transmitted by blood because it does not undergo schizogony in circulating blood cells. Thus, *in vitro* culture of *L. caulleryi* in chicken blood has not been successful (Morii, 1992). *L. caulleryi* infection in chickens causes listlessness, green feces, anorexia and often death from haemorrhage by second-generation schizonts. Survivors suffer from severe haemolytic anaemia, owing to erythrocyte destruction by gametocytes, and haemorrhagic anaemia owing to vascular endothelial cell destruction (Morii, 1992).

Several vaccination methods for *L. caulleryi* have been reported (Isobe *et al*. 1991; Itoh and Gotanda, 2002; Ito and Gotanda, 2004; Ito *et al*. 2005). A recombinant antigen of chicken leucocytozoonosis has been developed (Ito and Gotanda, 2004), but the gene(s) has not been identified because of the lack of sufficient genetic data for this protozoan. Recombinant R7 antigen is derived from second-generation schizonts and can be produced in *Escherichia coli* in large quantities (Itoh and Gotanda, 2002; Ito and Gotanda, 2004; Ito *et al*. 2005). However, several problems regarding the safety of and difficulty in production of vaccines have prevented their practical use. For example, the period of protective immunity is only 5 months in chickens (Itoh and Gotanda, 2002); it is therefore desirable to lengthen the period of defensive immunity by methods such as booster vaccination. In addition, there are many difficulties in the development of protozoan vaccines, given their complex life cycle and the host/parasite interaction. Each developmental stage of the parasite is

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characterized by different sets of expressed antigens, eliciting different types of immune responses (Carvalho *et al*. 2002). Thus, molecular analysis of *L. caulleryi* is required to develop more effective vaccines.

Stage-specific gene expression in *L. caulleryi* has not been characterized because of the difficulty in preventing host genome contamination. Avian apicomplexan protozoa, including *Leucocytozoon* spp., parasitize bird erythrocytes intracellularly. The result is that extracted DNA from infected avian blood includes 2 sets of genomes, protozoan and host. Therefore, separation of *L. caulleryi* from infected avian blood cells is needed. Unlike methods for isolation of mammalian blood protozoa, there are no reports of such methods in birds. Attempts to isolate *L. caulleryi* gametocytes from infected bird blood by gradient centrifugation have resulted in host bird cells still remaining in the target fraction (T. Isobe, personal communication). Flow cytometry is one potential method to detect or separate protozoa from infected blood. Several flow cytometric methods have been applied for the detection of mammalian malarial parasites in infected blood (Saito-Ito *et al*. 2001; Xie *et al*. 2007). To the best of our knowledge, flow cytometric analysis of *L. caulleryi* has not been reported. Only 1 species of avian protozoa, *Eimeria tenella*, has been analysed by flow cytometry to detect sporozoites and oocysts (Crane *et al*. 1988; Fuller and Mcdougald, 1989). Ficoll density-gradient centrifugation was used in an attempt to separate the blood stage of the avian protozoan for *Leucocytozoon smithi* (Henry and Dick, 1978). Thus, there are no published data for comparison of dot-plot patterns between infected and uninfected blood for *L. caulleryi*. Although *L. caulleryi*-infected chicken blood can be used as a first step to investigate stage-specific gene expression of this protozoan, the presence of host blood cells may contaminate DNA/RNA extraction procedures.

Light and electron microscopy studies have shown that mature gametocytes of *L. caulleryi* are enclosed by the cytoplasm of the host cell and are surrounded by a distinct 3-layered pellicle in the peripheral blood of infected chickens. However, the nucleus of the host cell is not observed around mature gametocytes (Morii *et al*. 1981, 1984). The outer membrane of the gametocyte may originate from the host cell; this is supported by the fact that the outer membrane disappears together with the cytoplasm of the host cell after withdrawal or ingestion by biting midges (Morii *et al*. 1984). Mature *L. caulleryi* gametocytes exit from host erythrocytes 18–24 days after infection (Morii, 1992), suggesting that the protozoa and host cells clearly separate.

The aim of the present study was to obtain purified *L. caulleryi* DNA and/or RNA from infected chickens. We used flow cytometry to separate *L. caulleryi* gametocytes from infected avian blood cells.

MATERIALS AND METHODS

L. caulleryi-*infected chicken blood*

L. caulleryi-infected chicken blood was obtained from experimentally infected chickens at day 20 post-infection (Scientific Feed Laboratory Co. Ltd, Tochigi, Japan). Collected blood was stored in an equal volume of Alsever solution (Nippon Biotest Laboratories Inc., Tokyo, Japan) at 4 °C until analysis. Uninfected chicken blood (Nippon Biotest Laboratories Inc.) was used as a negative control. Wright-Giemsa-stained blood smears were produced before flow cytometric analysis, and *L. caulleryi* gametocyte parasitaemia (gametocytes only) was calculated 3 times ($n = 3$ for each sample) by microscopic observation of 1000 blood cells on each slide (× 400 magnification).

Flow cytometric analysis

A volume of 5–10 μl of whole infected or uninfected chicken blood was added to 500 μl of sterile distilled water and incubated at 4 °C for 1–5 min to induce erythrocyte lysis. A volume of 500 μl of sterile 1·8% (w/w) saline was added to terminate lysis, and SYTO-24 nucleic acid stain was added at a final concentration of 10 ng/ml (Dyer and Day, 2003) and incubated at room temperature for 20 min. The cells were then centrifuged at 1500 ***g*** for 5 min at 4 °C for collection. The supernatant was removed by aspiration, and the cells were resuspended in 200 μl of Alsever solution. The suspension of stained cells was analysed using an EPICS ALTRA HyPerSort (Beckman Coulter, Inc., Fullerton, CA, USA) equipped with a 488-nm argon ion laser. Green fluorescence was detected in the corresponding FL-1 photomultiplier through a 530/30 bandpass filter. The parasite area, in which gametocytes were plotted, was defined in a 2-dimensional scattergram of green fluorescence (GF) and forward scatter (FSC) primarily by comparing the patterns of events in the 2 scattergrams for uninfected and infected chicken blood.

Cell sorting, observation of sorted cells and DNA extraction

Cell sorting was performed using an EPICS ALTRA HyPerSort (Beckman Coulter, Inc., Fullerton, CA, USA). Sample preparation and cell staining was performed as described above. Stained cells were filtered with a 35μm filter with cell-strainer tubes (Becton Dickinson, Tokyo, Japan) before sorting. We sorted 4000–10 000 cells in the gate specifically identified as infected blood. Sorted cells were collected in 1·5ml tubes containing Alsever solution plus 50% (v/v) fetal bovine serum to observe cell morphology or in 1·5ml tubes containing DNA extraction solution.

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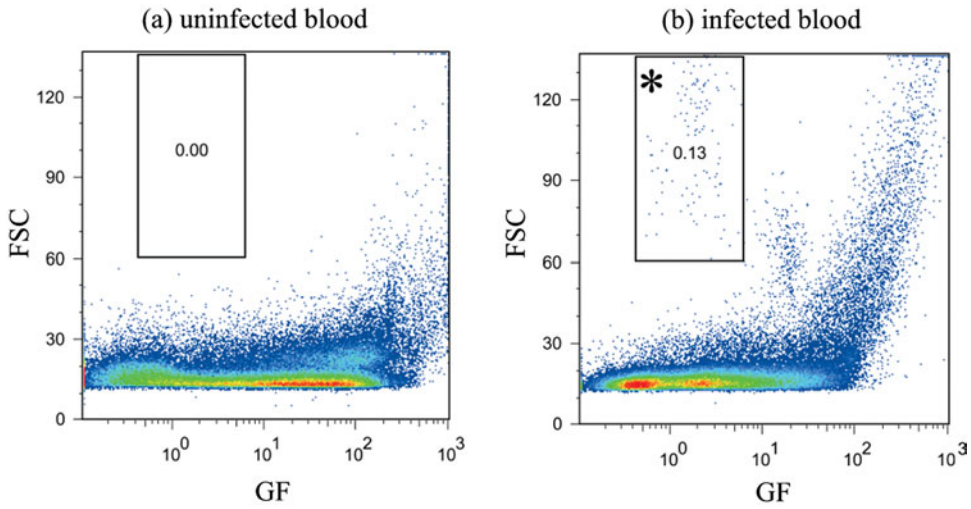

Fig. 1. Flow cytometric analysis of *Leucocytozoon caulleryi*-uninfected (a) or -infected (b) chicken blood. Lysed chicken blood was stained with SYTO-24. Compared to uninfected blood, a specific area was identified in dot plots of infected chicken blood (indicated by asterisk). Morphological analysis of sorted cells from the gated area (*) of infected blood showed that the cells were *L. caulleryi* gametocytes (see Fig. 2). The number in each gate indicates the percentage of gametocytes. GF, green fluorescence; FSC, forward scatter.

To observe cells, they were centrifuged onto glass slides using a Cytospin 3 cytocentrifuge (Shandon, UK); cells were centrifuged at 1000 ***g*** for 5 min at room temperature, smear preparations were made and stained with Wright-Giemsa and cells were analysed. Each set of 3 Cytospin slides ($n=3$) was counted in triplicate, and the percentage of *L. caulleryi* gametocytes was calculated by microscopic observation of 1000 cells (×400 magnification). DNA of sorted cells was extracted using a QIAamp DNA mini kit (Qiagen Ltd, Crawley, UK) according to the manufacturer's instructions.

Evaluation of separation by cell sorting

To evaluate the efficiency of cell sorting of infected blood, we performed polymerase chain reaction (PCR) of extracted DNA from infected whole blood and sorted cells. A total of 1 ng of DNA was used as a template to amplify the *L. caulleryi clpC* gene (primer: F, 5′-GAT TTG ATA TGA GTG AAT ATA TAT GGA AAA AC-3′; R, 5′-AAT ATA TTA GTT AAT CTA TTT AAT AAT TC-3′) as described previously (Omori *et al*. 2007, 2008) and the avian host 16S rRNA gene (primers and PCR condition were described previously by Imura *et al*. 2010). We then compared the degree of amplification of each gene between whole blood and sorted cells.

RESULTS AND DISCUSSION

L. caulleryi gametocyte parasitaemia was calculated in 3 independently collected samples at 20 days post-infection and was found to be approximately 0·33% to 0·87%. Results of flow cytometry are shown in Fig. 1. A 2-dimensional scattergram of gametocyte-positive blood showed a unique area, not shown in uninfected blood, as indicated by an asterisk (Fig. 1b). Gametocytes of *L. caulleryi* were observed in the sorted cell fraction, as shown in Fig. 2; approximately 85% of sorted cells were gametocytes. Host erythrocytes and lymphocytes were found mixed with gametocytes (Fig. 2b and c).

The effect of cell sorting on PCR is shown in Fig. 3. Compared to infected blood without sorting, amplification of the protozoa *clpC* gene was notably higher in sorted cells, and amplification of the 16S rRNA host gene was lower (Fig. 3; compare band intensity between lanes 3 and 9 (*clpC*) and lanes 4 and 10 (16S rRNA)). We did not quantify amplification results in the present study; quantitative PCR may be applied for more precise investigation of the effects of sorting.

In the present study, we used flow cytometry to create 2-dimensional scattergrams of chicken blood infected with the avian protozoa *L. caulleryi* for the first time. A protozoa-specific area was identified in dot plots of infected blood samples, as shown in previous flow cytometric analysis of mammalian blood protozoa (Saito-Ito *et al*. 2001). Henry and Dick (1978) separated the blood stage of *L. smithi* by Ficoll density-gradient centrifugation; however, morphologic findings of microscopic observation of the separated fraction were not shown in that report. Here, we clearly show that the sorted fraction of the specific area in the flow cytometry dot plot contained *L. caulleryi* gametocytes. The difference in flow cytometry pattern between infected and uninfected chicken blood might be associated with morphological features among gametocytes and other host blood cells. The collected fraction of sorted cells

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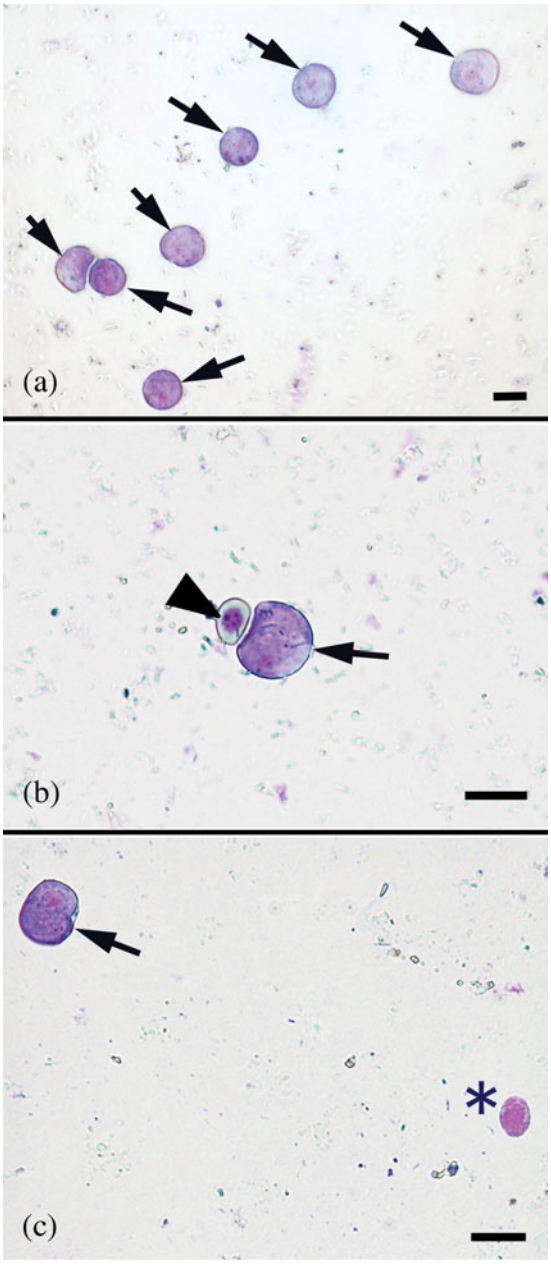

Fig. 2. Morphology of sorted cells from infected chicken blood at 20 days post-infection. Sorted cells were stained with Wright-Giemsa. Arrows denote *Leucocytozoon caulleryi* gametocytes; arrowheads indicate chicken erythrocyte; asterisk represents chicken thrombocytes. (a) *L. caulleryi* gametocytes, (b) chicken erythrocyte and *L. caulleryi* gametocyte, (c) *L. caulleryi* gametocyte and chicken thrombocyte. Scale bars = 10 μm.

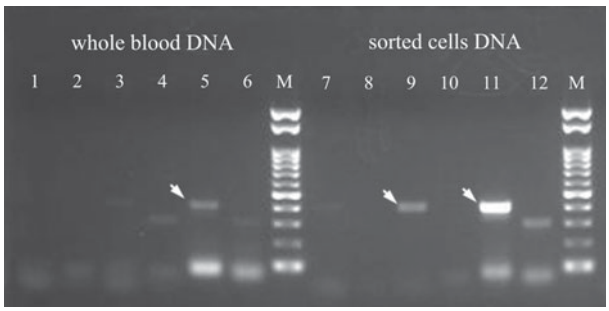

Fig. 3. Comparison of gene amplification (*Leucocytozoon caulleryi clpC* gene and avian host 16S rRNA gene) between whole blood and gametocytes by polymerase chain reaction (PCR). PCR was performed with 1 ng of each DNA extracted from infected whole blood using a kit (see Materials and Methods section) and sorted gametocytes as templates. Arrows indicate bands showing amplification of the *L. caulleryi clpC* gene. Lanes 1 to 6: infected whole blood; lanes 7 to 12: DNA extracted from sorted cells of infected chicken blood. M: molecular weight marker. Even lane numbers show 16S rRNA gene, approximately 300 bp (lanes 2 and 8, 25 PCR cycles; lanes 4 and 10, 30 PCR cycles; lanes 6 and 12, 35 PCR cycles). Odd lane numbers, show *clpC* gene, approximately 400 bp (lanes 1 and 7, 25 PCR cycles; lanes 3 and 9, 30 PCR cycles; lanes 5 and 11, 35 PCR cycles).

was expected to include only *L. caulleryi* gametocytes. However, microscopic observation and PCR examination showed remaining contamination by host erythrocytes and thrombocytes. A mix of host-derived cells was reported for the human malaria parasite by flow cytometric detection (Saito-Ito *et al.* 2001). In the present preliminary study, we attempted to compare flow cytometry patterns between *L. caulleryi*-infected and uninfected chicken blood. Methodological improvements in haemolysis, cell staining, dyes or antibodies and fluorescence-activated cell sorting (FACS) analysis may be necessary. Moreover, the precise quantification of amplified products by qPCR could clearly reveal the efficiency of cell sorting. In addition, flow cytometry analyses using anti-chicken red blood cell antibody staining could clearly confirm gametocytes without stained antibodies and the same antibody could also be used to negatively select against host cells in the FACS assay.

The period for a mature *L. caulleryi* gametocyte to exit from the cytoplasm of host erythrocytes ranges from 18 to 24 days after infection (Morii *et al.* 1992). Immature or other gametocytes may have remained in our samples because we used blood collected 20 days post-infection. For more effective FACS separation, the use of anti-chicken erythrocyte antibody may be useful to distinguish gametocytes from chicken erythrocytes.

Contamination of host DNA was reduced in flow cytometry-sorted samples compared to samples without flow cytometric sorting. The increased amount of amplified *L. caulleryi clpC* gene in extracted DNA from the sorted fraction indicates the possibility of effective analysis of protozoa-specific gene expression. Further improvements in the exclusion of host-derived DNA are needed; however, our present results provide a first step for evaluation of stage-specific gene analysis of this highly pathogenic avian protozoan and may aid in the development of genetically attenuated vaccines.

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