

Isolation and Purification of the Schizont Stage of *Theileria annulata* from Host Leukocytes through Novel Biochemical Techniques

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ABSTRACT: The intracellular protozoan parasite, *Theileria annulata*, induces uncontrolled proliferation and transformation in bovine B lymphocytes and monocytes in blood circulation and lymph nodes of host cells. This uncontrolled replication happens in the macroschizont stage of the life cycle of the parasites. The development of a rapid and efficient technique is likely to necessitate for isolation of purified schizonts from host cells. This is necessary for the isolation of highly purified RNA, protein or glycoproteins of schizonts from host cells. This study aimed to isolate the purified schizont based on the aerolysin–nocodazole technique. Aerolysin which was purified from gram-negative pathogen *aeromonas hydrophila* has an ability to form discrete channels and unstable eukaryotic cell membranes in 0°C and low concentration. Nocodazole used for parasite separation from the microtubule network of infected lymphocytes and monocytes. In purified schizonts, no large nucleus of host cells visualized in giemsa and DAPI staining. The isolated schizonts were free and intact from host cells. Intact *T.annulata* schizonts obtained from this study are suitable for purification of RNAs, proteins, glycolipids and glycoproteins of schizonts free of host cell debris.

KEYWORDS: Aerolysin; Schizont; *Theileria annulata*; Nocodazole; Percoll; Leukocytes.

INTRODUCTION

Theileria annulata is an obligate intracellular protozoan parasite causes tropical theileriosis, a devastating lymph proliferative disease of cattle in developing countries (1). Schizont stage of *T.annulata* lives and

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proliferate in B lymphocytes and monocytes of cattle blood circulation and lymph nodes. Devastating changes in gene expression and protein production constrain by macroschizonts. These changes induced lymph proliferation and immortality in host cells. These alterations in infected cells facilitate the increase in cell numbers and finding of new host cells without aggressing from previous host cells. The capacity of *T. annulata* to induce leukocyte transformation implies within the infected leukocyte express 'oncogenic' substances, and as a step towards their identification, one must develop a procedure which leads to isolation of the parasite gene(s) [2-5]. Although parasite DNA can be readily obtained from infected erythrocytes due to the absence of host cell nuclei, identification of putative oncogenes requires purification of parasite-derived peptides, proteins and/or the construction of parasite-specific cDNA libraries made from the transforming stage of the parasite's life cycle. For many researches and experiments needfulness to have purified protein, RNA or DNA of intracellular parasite is obviously required. Observations of multinucleated macroschizonts are common a few hours after sporozoites are entering the leukocytes of cattle blood circulation, especially B lymphocytes and monocytes. The most critical aspect of invasiveness of *T. annulata* correlates with altered expression of downstream target genes in cell life cycle arrangement in this stage (6, 7). A strong interaction between some parasite surface proteins and microtubules of host cells is obviously important [3, 7, 8]. Microtubules are the primary components of mitotic spindles and are therefore essential for mitotic cell division. Moreover, in concert with actin and intermediate filaments, microtubules organize the cytoplasm and control trafficking, and their disruption leads to cell cycle arrest and loss of cellular architecture (9, 10).

The strong parasite-microtubule interaction prevents efficient separation of the parasite cells from their host cells so if we aim to separate schizonts from host cells efficiently, we must isolate the association between microtubules and parasites in leukocytes [6, 11]. There are various microtubule inhibitors in pharmacology and nocodazole is one of them which belongs to the family of colchicine-site-binders that interact with tubulin dimers, inhibit their assembly into microtubules, and enhance GTPase activity in the absence of polymerization. Addition of nocodazole to mammalian cells cultured

in vitro results in the loss of most of the cytoplasmic microtubules including spindles [12, 13]. At sufficient and tested concentrations to disrupt host cell microtubules by nocodazole, there is no effect on the schizont of *T. annulata* [14-16]. After schizont isolation, if nocodazole is not used for schizont purification we will have incomplete synchrony in established cell lines and there will be no clear results to obtain and therefore studies on how the parasite induces host cell proliferation which involves cell cycle analysis will be hampered [16].

Aerolysin is a channel-forming cytolysis toxin secreted as a proaerolysin form by a gram-negative rod shape microorganism called *Aeromonas hydrophila* [17-19]. The ability of destruction of membrane and permeability barrier of aerolysin is due to the induction of 3nm holes of pro-aerolysin polymerization [20, 21]. Lower concentrations of lethal concentration of aerolysin on eukaryotic cells cause sensitive cells to osmotic changes without entire destruction [16, 20, 21]. In the present study, we used aerolysin which was isolated from *A. hydrophila* to disrupt the *T. annulata* schizont infected leukocyte cell membrane. The aerolysin concentration which was used in this study was a lower concentration than can lyse RBC of cattle in the hemolysis test. The isolated schizonts were free and intact from host cells and could be further used for suitable purifications of parasite biological molecules. Intact *T. annulata* schizonts obtained from this study are suitable for purification of RNAs, proteins, glycolipids and glycoproteins of schizonts free of host cell debris.

EXPERIMENTAL SECTION

Parasite strain and culture conditions

The vaccine cell line, strain S15 Iran, of *T. annulata* used in this study was obtained from the Razi Vaccine and Serum Research Institute, Karaj, Iran. The parasites were cultured in RPMI-1640 medium (Sigma, Chemical Co., St. Louis, U.S.A.) supplemented with 10% FBS (Sigma, WKG, Germany), 292 µg/ml L-glutamine (Sigma, WKG, Germany), 4.5 mg/ml glucose, 100 µg/ml penicillin (sigma, WKG, Germany), and 100 µg/ml streptomycin (Sigma, WKG, Germany).

Microtubule destruction

2×10^8 S15 cells were grown to a density of approx. 8×10^5 cells/ml. 3µM nocodazole (Sigma, WKG, Germany) used to destruct microtubules of a host cell for 2h at 37°C.

Cells were harvested by centrifugation of 200g for 5 min at 37°C. After washing one time by ice cold PBS, cells were re-suspended at 5×10^7 cells/0.9ml on ice cold 1xHEPES buffer (10 Mm HEPES, 150 Mm NaCl, 20 Mm KCl, pH 7.4) containing 1 Mm CaCl_2 . Re-suspended cells in HEPES buffer were transferred into 1.5ml eppendorf microtubes.

Aerolysin

Activated aerolysin destructs infected cells and schizonts completely by direct treatment. But at 0°C Aerolysin only bind to receptors on the plasma membrane of target cells and is not active. Therefore incubation on icy aerolysin causes the enzyme to binds to the cell membrane but prevents rapid cell lysis of the host cell and subsequently destruction of parasite cells.

After isolation of aerolysin from *A.hydrophila*, with a hemolysis test, we calculated that 1mg isolated enzyme must be used for 2×10^8 cells. 1mg activated enzyme is soluble in 1.5 mL ice cold PBS. Cells in HEPES buffer were added to the activated enzyme in ice cold PBS and the tubes were incubated under rotation on ice for 30 min. After wards, the enzyme binds so tightly that washing the cells is not able to remove the enzyme from receptors. By two gentle washing (1200 rpm, 5min at 4°C) in ice cold PBS, the unbound enzyme was removed. Cells were re-suspended in 1xHEPES containing 1 Mm CaCl_2 and was incubated at 37°C for 30 min (after each 10 min incubation we tested trypan blue exclusion and when trypan blue is penetrated to cells, we stopped incubation). Finally by centrifugation at 200g, pelleted cells (by the sensitive membrane) and 5×10^7 cells/mL were re-suspended in 1xHEPES containing 5 Mm EDTA.

Schizont separation from host cells

Host cell debris and nuclei were separated from the schizont of *T.annulata* by percoll centrifugation essentially as described by Baumgartner et al, with several changes. In brief, a stock solution of percoll was prepared by mixing 8.5 parts of Percoll with 0.5 parts of 20 xHEPES (200 Mm HEPES, 3 M NaCl, 400 Mm KCl, pH 7.4) and one part of 50 Mm EDTA (pH 7.4). In this study, we did not use ultracentrifuge so we made a solution in 2ml eppendorf microtubes. The cell lysate (138µL) was added to 520 µl of this percoll stock solution and the volume was adjusted to 680µl by the addition of 1x HEPES containing 5 Mm EDTA, giving rise to

64.6% (vol/vol) final percoll concentration. The percoll-cell lysate mixture was transferred into 2mL microtubes and carefully overlaid with a 45% percoll solution in 1xHEPES and 5 Mm EDTA. The mixture was thereafter centrifuged at 12000rpm for 1h at 10°C. During centrifugation, a gradient was established that separates parasites by density from cellular debris and nuclei. After centrifugation, we observed 2 bands and parasite cells were between two layers. This can be collected with a Pasteur Pipette. Next, the parasites from the individual samples were collected in 2ml microtubes. In this stage, in giemsa staining, there were a lot of percoll crystals so it was necessary to wash once in a large volume of PBS and pelleted by centrifugation at 5 000 rpm, for 10min at 4°C.

RESULTS AND DISCUSSION

In order to visualize the purified schizont, we employed Giemsa and DAPI staining procedures. Giemsa is a simple method to evaluate purified isolated schizonts from infected leukocytes. In giemsa staining, nuclei of host cells and schizonts were visible in infected leukocytes. After purification, in giemsa staining, there were no visible nuclei of host cells (Figs. 1 & 2). The most important point that draws attention in the image of purified schizonts in giemsa and DAPI staining, is the lack of large nuclei of host cells. The macroschizonts were very clear in DAPI and giemsa staining within miniature nuclei of microschantos. These purified schizonts are ready for any experiment with the aim of purification of biological molecules such as nucleic acids or protein from schizont without impurity and debris of host cells.

A simple analysis to check the quality of purified parasites is staining the parasite fraction with DAPI, which is a DNA intercalating agent. In DAPI staining, nuclei is stained and under a mixture of UV and standard illumination, nuclei of purified free schizont is seen without nuclei of host cells (Figs. 3 & 4).

In this study, nocodazole elevated the quality and quantity of purification. In the early phase of the study, nocodazole was not used in the purification process and in comparison to samples that used nocodazole, acceptable results were not obtained. There is a close association between *Theileria annulata* and the host cell microtubule network. In mitotic cell division, a task of microtubules is the separation of chromosome pairs. In infected host cells with *T.annulata*, schizonts bind to microtubules and divide between two daughter cells. Using the colchicine

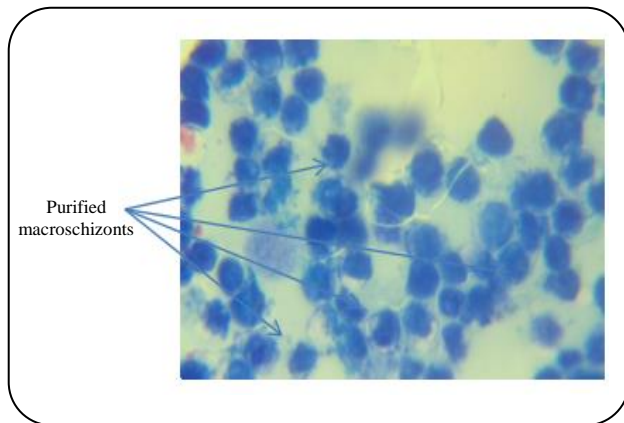


Fig. 1: In the light microscopic image of Giemsa staining of intact schizont of *Theileria annulata*, free of host nuclei (magnification of the image is 1000x)

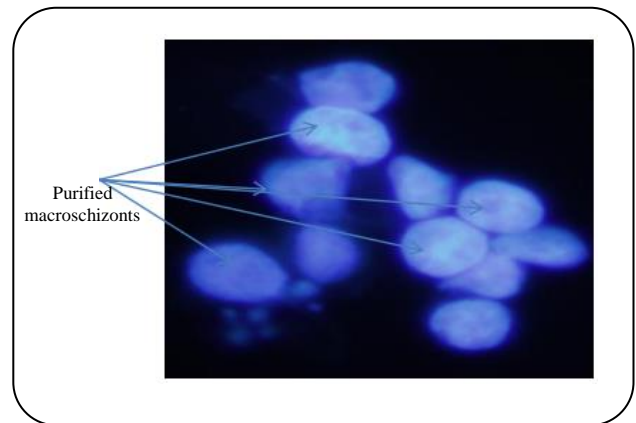


Fig. 3: DAPI staining of purified schizont of *Theileria annulata* free of host nuclei under UV light (magnification of the image is 1000x)

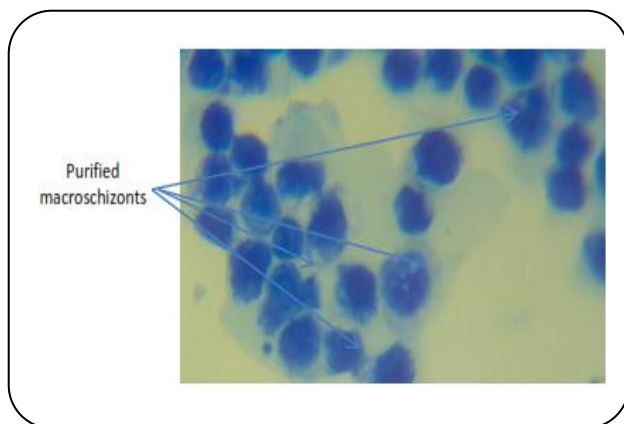


Fig. 2: In the light microscopic image of Giemsa staining of intact schizont of *Theileria annulata*, free of host nuclei (magnification of the image is 1000x)

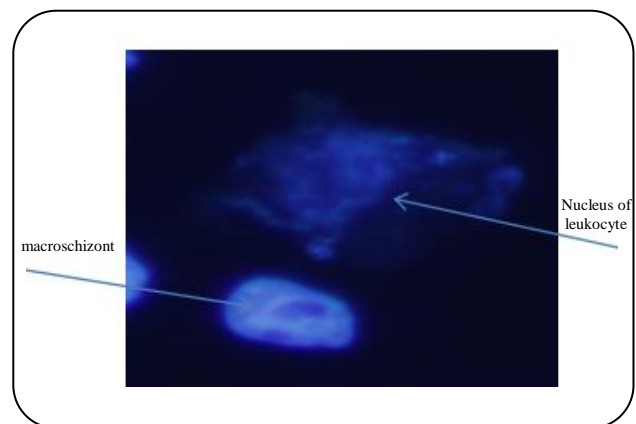


Fig. 4: This image indicate parasite residing within the host cell in DAPI staining of infected lymphocytes by *Theileria annulata* under UV light (magnification of the image is 1000x)

site-binder, nocodazole, improves schizonts separation and purification. In addition, it was confirmed that nocodazole provokes reversible growth arrest in mammalian cells so in this study we had cell cycle synchronisation of asynchronously growing *T.annulata* infected host cells.

Intact schizonts without any damage in cell structures was a principal purpose in this study thus some materials such as detergents and some enzymes were not suitable for host cell membrane permeability.

Aerolysin in lethal dose can damage the plasma membrane of the host cell and kills host cells and schizont altogether, but in a lower concentration of lethal dose and in low temperature this enzyme enables the permeable cell membrane of host cells without killing schizonts. If we

had used aerolysin directly at 37°C, not only host cells, but also parasites would have lysed, but if infected cells along with icy aerolysin is pre-incubated on ice, aerolysin binds to glycoproteins of host cells, and in the following washing step unbound enzyme is removed and there would not be free enzyme to lyse schizont cells.

CONCLUSIONS

In this study, isolation and purification of the schizont stage of *T.annulata* was performed with nocodazole and aerolysin. An ultracentrifugation is a common approach which was used in published articles dealing with isolating schizont stage of *T.annulata* but in the present study, instead of employing the expensive tools of ultracentrifugation, the schizonts were completely

isolated and purified from host nuclei and debris with conventional centrifugation in a longer time and lesser volume in only 2ml microtubes. Purified schizonts of *T.annulata* isolated by this novel approach is applicable for further studies such as nucleic acids and protein isolation as well as glycosylphosphatidylinositol (GPI) anchors purification and free GPIs isolation from the schizont cell membrane.

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