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Concomitant *in vitro* development of *Eimeria zuernii*- and *Eimeria bovis*-macromeronts in primary host endothelial cells

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ABSTRACT

Eimeria zuernii and *E. bovis* are host-specific apicomplexan parasites of cattle causing haemorrhagic typhlocolitis in young animals worldwide. During first merogony, both *Eimeria* species form giant macromeronts (> 300 μm) in host endothelial cells containing > 120,000 merozoites I *in vivo*. During the massive intracellular replication of macromeronts, large amounts of cholesterol and fatty acids are indispensable for enormous merozoite I-derived membrane production. From a metabolic perspective, host endothelial cells might be of advantage to the parasite, as transcription of several genes involved in both, cholesterol *de novo* biosynthesis and low density lipoprotein (LDL)-mediated uptake, are up-regulated in *Eimeria* macromeront-carrying host endothelial cells. In order to analyse further influence of *E. zuernii*/*E. bovis* infections on the metabolism of cholesterol, fatty acids, and glycolysis of the host endothelial cells, suitable *in vitro* cell culture systems are necessary. So far, *in vitro* cell culture systems based on primary bovine umbilical vein endothelial cells (BUVEC) are available for *E. bovis*-macromeront I formation, but have not been evaluated for *E. zuernii*. A novel *E. zuernii* (strain A), initially isolated from naturally infected calves in Antioquia, Colombia, was used for sporozoite isolation. Primary BUVEC monolayers were concomitantly infected with *E. zuernii*- and *E. bovis*-sporozoites, resulting in large sized macromeronts whose morphological/morphometric characteristics were compared. BUVEC carrying *E. zuernii*-macromeronts resulted in the release of viable and highly motile merozoites I. Overall, *E. zuernii*-merozoites I differed morphologically from those of *E. bovis*. The new *E. zuernii* (strain A) will allow detailed *in vitro* investigations not only on the modulation of cellular cholesterol processing (i. e. cholesterol-25-hydroxylase and sterol O-acyltransferase) but also on the surface expression of LDL receptors during macromeront formation.

1. Introduction

At least thirteen *Eimeria* species have so far been reported to infect domestic cattle and buffalo worldwide [1, 2]. Among these species, *E. zuernii* and *E. bovis* are considered as the most pathogenic ones causing severe typhlocolitis with clinical manifestations such as haemorrhagic diarrhoea, dehydration, weight loss and poor growth rates, mainly in calves [3–5].

In contrast to other bovine *Eimeria* species, *E. zuernii*- and *E. bovis*-sporozoites must invade host endothelial cells of central lymph capillaries of ileum villi *in vivo* [6–8] where first generation macromeronts are formed. As these macromeronts develop and become mature, dramatic morphological changes have been observed in infected host endothelial cells *in vivo* [6, 7] and *in vitro* [9, 10]. As such, host cell nucleus of *Eimeria* macromeront-carrying host endothelial cells changes from having heterochromatine with a dark spotted-content to a 'fried-egg'-shape containing euchromatine and a nucleolus coalescing to form single or multiple nucleoli [11, 12]. Similar host cell nuclear morphological alterations are reported to occur in host cells infected with pathogenic caprine and ovine *Eimeria* species, i. e. *E. ninakohlyakimovae* [13, 14], *E. arloingi* [15] and *E. ovinoidalis* [16].

In agreement with these common replication features, recently published phylogenetic analysis on pathogenic ruminant *Eimeria* suggests shared evolutionary history for ruminant *Eimeria* replicating in highly immunocompetent endothelium and by forming large-sized macromeronts [17]. It is hypothesized that sporozoites of a common ancestor species were able to migrate deeper in order to infect lymph endothelium, thereby colonizing a new niche in small intestine of ruminants *in vivo* [17], probably to fulfil specific nutritional requirements as recently demonstrated for *E. bovis* [11, 18, 19]. Accordingly, *E. bovis*

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strictly depends on the host endothelial cell supply of energy and on pivotal building molecules, such as cholesterol, for its massive intracellular replication [11, 18, 19], and thereby differing from other fast replicating apicomplexan parasites (i. e. *Toxoplasma gondii*, *Cryptosporidium parvum*) on metabolic requirements [20, 21].

All cattle kept under conventional husbandry conditions are unavoidably exposed to concomitant *Eimeria* spp. infections worldwide [22, 23] and infection-induced impaired animal performance, mortality and anticoccidial therapy costs generally result in considerable economic losses [19, 23, 24]. *E. zuernii/E. bovis* are strictly obligate intracellular protozoan parasites residing within a parasitophorous vacuole (PV). Both require cholesterol for PV establishment and host cell membrane enlargement but also for massive offspring production, i. e. > 120,000 merozoites I [9].

Nevertheless, usage of calves for detail research on *Eimeria*-infected host cells cholesterol- or glycolysis-metabolism is becoming more difficult to justify as requirements for housing conditions and principles of animal welfare have become more restrictive worldwide. Thus, we here provide a new *in vitro* system for *E. zuernii* as an alternative for experimental animal studies and useful for further investigations on complex parasite-host endothelial cell interactions, indispensable for better understanding of pathogenesis but also for identification of potential targets for anticoccidial drug development. This new *in vitro* culture system for *E. zuernii* is in line with 3R principles (i. e. replace, refine, reduce) of animal use, and could be used in many laboratories with not to high specific requirements as described elsewhere [25].

Thus, main aims of this study were first, to provide a suitable *in vitro* culture system based on primary bovine host endothelial cells for *E. zuernii* macromeront- and merozoites I-production being as close as possible to *in vivo* situation, and second, to generate new data on morphological, morphometric and molecular characteristics of this new *E. zuernii* field strain.

2. Materials and methods

2.1. Parasites

Eimeria zuernii oocysts used in this study were initially isolated from naturally infected calves in Antioquia, Colombia, which contained 95% *E. zuernii*- and 5% *E. bovis*-oocysts, respectively. This new *E. zuernii* (strain A), was thereafter propagated in a parasite-free male Holstein-Friesian calf according to Hermosilla et al. [26]. All animal procedures were performed following the guidelines of the Ethic Committee for Animal Experimentation, approved by the Institutional Committee for Care and Use of Animals of the University of Antioquia (Act No.102, 2016) in accordance to current Colombian Animal Protection Laws.

Briefly, for oocysts production, an 8-week-old calf (without previous *Eimeria* oocyst exposure) was infected orally with 3×10^5 sporulated *E. zuernii* (strain A) oocysts. Then, oocysts were isolated from faeces beginning at 15 days p. i. according to Jackson [27]. Oocysts were incubated in 2% (*w/v*) potassium dichromate (Merck) solution at room temperature (RT, 25 °C) and constant mixing until complete sporulation. Sporulated oocysts were stored in 2% (*w/v*) potassium dichromate solution at 4 °C until further use. Oocysts of *E. zuernii* (strain A) were identified based on morphological/morphometric characteristics of sporulated oocysts as was previously described [28–30].

2.2. Excystation

A total of 2.5×10^6 sporulated oocysts of *E. zuernii* (strain A) were excysted following oocyst excystation protocols of Hermosilla et al. [9] with some slight modifications [15]. Briefly, sporulated oocysts were added into a 4% (*v/v*) sodium hypochlorite solution and thereafter magnetically stirred on ice for 20 min. Then, oocysts were mixed by vortexing for 15 s and thereafter centrifuged (300 × *g*, 5 min). Supernatant was collected and mixed with distilled water (1:1) and pelleted (600 × *g*, 20 min). Oocysts pellet was then layered in 60% Percoll™ (GE Healthcare, UK) gradient and centrifuged for 20 min at 400 × *g* to remove remaining faecal debris. After centrifugation, oocysts bands were suspended in sterile 0.02 M L-cysteine HCl/0.2 M NaHCO3 (Merk) solution and incubated for 20 h at 37 °C in a 100% CO_2 atmosphere. Thereafter, the oocysts were suspended in Hank's balanced salt solution (HBSS, Gibco) containing 0.4% (*w/v*) trypsin (Sigma-Aldrich) and 8% (*v/v*) sterile filtered bovine bile obtained from a local butchery, up to 4 h at 37 °C in a 5% CO_2 atmosphere. Every hour, sporulated oocysts and released sporocysts were counted using an inverted microscope (IX81, Olympus®) to estimate the number of free-released sporozoites. Free sporozoites were then washed twice with modified endothelial cell growth medium [ECGM (PromoCell) diluted in M199 medium (Gibco) (3:7), 1% penicillin-streptomycin (both Sigma-Aldrich) supplemented with 10% foetal calf serum (FCS, Biochrome)], and thereafter counted in a Neubauer counting chamber (1:10 and 1:100 dilution).

2.3. Host cells

Primary bovine umbilical vein endothelial cells (BUVEC) used in this study were isolated using the methodology previously described in detail by Taubert et al. [11]. Three different BUVEC isolates were used for host cell infection experiments. BUVEC isolates ($n = 3$) were seeded in two 25 cm^2 cell tissue culture plastic flasks (Greiner) and maintained in modified ECGM supplemented with 10% FCS. The culture medium was changed every 48–72 h after infection.

2.4. Host cell infection, development of E. zuernii/E. bovis-macromeronts and merozoite I production

BUVEC monolayers were infected with 2.5×10^5 freshly isolated sporozoites of *E. zuernii* (strain A; composed of 95% *E. zuernii* and 5% *E. bovis* oocysts) on cell monolayers with 80–90% confluency. Culture medium was changed 24 h after sporozoite infection and thereafter every two days. Using microscopy and photography, the *Eimeria*-infected host cells were evaluated daily with the aim to follow parasite development and to register measurement of different parasitic stages (i. e. intracellular sporozoites, trophozoites, immature meronts, mature macromeronts), in addition to monitor morphological changes of *Eimeria*-infected host endothelial cells.

The number of *E. zuernii*- and *E. bovis*-infected host endothelial cells was calculated by counting at least 3 different 400-fold magnification power vision fields at 24 h p. i. with their corresponding parasitic stage measurements. The total number of immature- and mature-*E. zuernii*, as well as *E. bovis*-macromeronts present per BUVEC monolayer was also determined and expressed as percentage of numbers of sporozoites initially applied to the BUVEC culture.

In addition, randomly infected cells ($n = 15$) were measured every day p. i. by using Cellsens Dimention® software (Olympus) according to Silva et al. [15]. When *E. zuernii*- and *E. bovis*-merozoites I were found free in the cell culture medium, these stages were carefully harvested daily by aspiration of the supernatant, counted, and thereafter frozen for further PCR analyses as described elsewhere [19].

2.5. DNA extraction

DNA from *E. zuernii*- and *E. bovis*-oocysts, -sporozoites, -macromeronts, and -merozoites I was extracted according to Hamid et al. [19]. After excystation, approximately 2×10^4 sporozoites of each species were used for DNA extraction. Infected BUVEC were harvested at 21 and 25 days p. i. for macromeront-derived DNA extraction. Furthermore, merozoites I were collected from infected cell cultures from 16 to 23 days p. i. onwards [19]. DNA was extracted using the DNeasy Blood and Tissue Kit® (QIAGEN), following the manufacturer's instructions for cultured cells. DNA from oocysts was extracted using the commercial kit for DNA purification NucleoSpin® Soil (Macherey-