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A cell culture platform for *Cryptosporidium* that enables long-term cultivation and new tools for the systematic investigation of its biology

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ABSTRACT

Cryptosporidium parasites are a major cause of diarrhoea that pose a particular threat to children in developing areas and immunocompromised individuals. Curative therapies and vaccines are lacking, mainly due to lack of a long-term culturing system of this parasite. Here, we show that COLO-680N cells infected with two different *Cryptosporidium parvum* strains produce sufficient infectious oocysts to infect subsequent cultures, showing a substantial fold increase in production, depending on the experiment, over the most optimistic HCT-8 models. Oocyst identity was confirmed using a variety of microscopic- and molecular-based methods. This culturing system will accelerate research on *Cryptosporidium* and the development of anti-*Cryptosporidium* drugs.

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Cryptosporidiosis causes a significant number of deaths in children and immunocompromised individuals (Kotloff et al., 2013). It is caused by species of the genus *Cryptosporidium*, in humans typically by *Cryptosporidium parvum* and *Cryptosporidium hominis*. The *Cryptosporidium* spp. belong to the phylum Apicomplexa and it has recently been proposed for the species to be reclassified as a member of the subclass of gregarine (Ryan et al., 2016). They are waterborne pathogens, and cryptosporidiosis has commonly been associated with disease in developing countries. However, more recent molecular epidemiological studies suggested that the disease is also an increasing health concern in developed countries and may have reached epidemic levels (Kotloff et al., 2013; Checkley et al., 2015). Only one moderately effective drug (nitazoxanide) is available for the treatment of cryptosporidiosis. More effective drugs are urgently needed (Checkley et al., 2015).

Cryptosporidium is a parasite that invades host cells, within the boundaries of the host cell membrane, residing intracellularly yet extra-cytoplasmic, sometimes referred to simply as epicellular (Ryan et al., 2016). *Cryptosporidium* typically infects epithelial tissues of the upper intestinal tract, accompanied by localised deterioration of microvilli. In immunocompromised individuals, the parasite can also be found in other epithelial tissues including most of the upper stages of the digestive and respiratory tracts as well as other unrelated organ systems (Sponseller et al., 2014). The *Cryptosporidium* life cycle is complex and involves a number of intracellular/extracytoplasmic and extracellular stages, resulting in oocysts that contain the infective sporozoites (Supplementary Fig. S1).

A cell culture system that enables continuous *Cryptosporidium* cultivation and systematic elucidation of the *Cryptosporidium* life cycle, especially the endogenous phases, is missing. Previous approaches have been hampered by problems including rapid senescence of primary cell cultures, incomplete parasite life cycles, and insufficient production of sporulated infective oocysts (Karanis and Aldeyarbi, 2011; Checkley et al., 2015). The current methods used to produce infective *Cryptosporidium* oocysts, aside from

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small-scale cultures in vitro, require continuous infection of animals, typically neonatal cows or sheep and sometimes mice (Vinayak et al., 2015). Due to a lack of cryopreservation methods, oocysts cannot be stored and need to be freshly prepared on a continuous basis. A recent publication tackled the challenge of cell culture-based oocyst production using a hollow fiber technology that mimics the gut (Morada et al., 2016). However, specialised equipment is needed and the required cell culture media supplements are expensive. In addition, the system does not enable study of the *Cryptosporidium* life cycle and biology in real time at a cellular level in the context of a host cell.

Here, we show that inoculation of COLO-680N cultures with *C. parvum* produced sufficient amounts of infective oocysts to enable sustainable propagation of the parasite in standard tissue culture at a laboratory scale. We tested a panel of seven human cancer cell lines (using methods described in Supplementary Data S1) for their capacity to support *C. parvum* propagation including COLO-680N (oesophageal squamous-cell carcinoma), DLD-1 (colon adenocarcinoma), KYSE-30 (oesophageal squamous-cell carcinoma), HCT-15 (colorectal adenocarcinoma), SJSA-1 (osteosarcoma), MKN-1 (gastric carcinoma), and the colon adenoma carcinoma cell line HCT-8, which has most commonly been used for the investigation of *Cryptosporidium* in cell culture (Hijjawi et al., 2001). However, *Cryptosporidium*-infected HCT-8 cultures do not produce enough infective oocysts to maintain infected cultures (Muller and Hemphill, 2013), which also raises concerns about the suitability of HCT-8 for the study of *Cryptosporidium* biology. The cell lines were infected with the *C. parvum* strain Moredun (Girouard et al., 2006) using a total input of 5×10^5 of excysted oocysts per 10 mL of medium (25 cm^2 flask). After an incubation period of 2 weeks, COLO-680N cultures were the only ones that had produced substantially more oocysts (approximately 40-fold higher) than the number of input oocysts (Fig. 1A, Supplementary Table S1). While HCT-8 cells died after a few days of infection, COLO-680N cultures remained viable and produced oocysts for almost 8 weeks without sub-culturing, requiring only weekly medium exchange (Fig. 1B). As a result, total *Cryptosporidium* oocyst production in the COLO-680N cell line (number of oocysts produced) exceeded the HCT-8-mediated oocyst production (2.5×10^5 oocysts/mL of culture medium) by 20 times (5×10^6) after 10 days of incubation (Fig. 1C). At day 60, COLO-680N cells had produced an accumulated number of 1.2×10^7 oocysts/mL of culture medium obtained from weekly harvests. Given that the initial oocyst count was 1×10^5 oocysts/ml, this represents a 50-fold increase in oocyst numbers at 10 days p.i. and a 120-fold increase by the end of the culture. Also of note, oocysts derived from the supernatants of COLO-680N cell cultures, but not from the supernatants of HCT-8 cell cultures, enabled the infection of novel cell cultures (Supplementary Fig. S2C). Infection of COLO-680N cells with cattle-derived *C. parvum* oocysts resulted in similar amounts of infective oocysts in 25 independent experiments. In addition, we performed three rounds of infection using COLO-680N culture-derived oocysts without noticing changes in oocyst production efficacy, showing that COLO-680N cells are suited for the continuous long-term cultivation of *C. parvum* oocysts. Continuous *C. parvum* infections of COLO-680N cells were confirmed using PCR primers specific to *C. parvum* DNA, which displayed the presence of parasite DNA in both the cell monolayer (Fig. 1D), and media fractions of the two-dimensional (2-D) cultures (Fig. 1E). *Cryptosporidium*-specific primers did not produce bands in non-infected COLO-680N cells (Fig. 1D and E; Supplementary Fig. S3). The amplified DNA regions were sequenced to confirm their identity. In addition, purified COLO-680N-produced oocysts were visualised by scanning electron microscopy (Fig. 1F). To evaluate our results even further, we repeated the infection experiments using freshly excysted and purified sporozoites; the purity of the sporozoites (absence of oocysts in the sample) was validated using bright-field microscopy. Quantitative PCR (qPCR) has demonstrated the production of 2.4×10^6 oocysts (from an initial inoculation of 1×10^6 sporozoites, Fig. 1I), 9 days p.i., and fluorescence microscopy confirmed the presence of newly produced oocysts in the medium (Fig. 2E). The discrepancy in the numbers of oocysts produced (from the other experiments) could be a result of the oocyst treatment/purification, the detection method using qPCR (Shahiduzzaman et al., 2009), or the presence of a high amount of host cell material (debris and RNA) that could inhibit the reaction. The produced oocysts were used in two rounds of infection. The first round of infection was done in triplicates in 12×25 cm^2 flasks format and the presence of oocysts was assessed by standard PCR analysis using Heat shock protein 70 (Hsp70)-specific primers (Supplementary Table S2). Then crudely purified oocysts were used to re-infect fresh COLO-680N cells, and the production of fresh ones was further evaluated (Supplementary Fig. S4).

The identity of the COLO-680N-produced *C. parvum* oocysts was further confirmed using different specific staining methods. Crypt-a-glo (Waterborne™; an antibody that recognises the oocyst cell wall), *Vicia villosa* lectin (VVL, Vector laboratories, UK); binds to O-glycan mucin repeats on *C. parvum* sporozoites), a mucin-like glycoprotein that contains a C-type lectin domain (CpClec; binds to surface of the apical region and to dense granules of sporozoites and merozoites (Bhalchandra et al., 2013)) and direct sporozoite staining using propidium iodide and Sporo-glo (Waterborne™) resulted in virtually identical staining patterns in *C. parvum*-infected COLO-680N cells, indicating the presence of oocysts and other non-extracellular life stages of *Cryptosporidium* (Fig. 2A; Supplementary Figs. S5–S7; Supplementary Movie S1). Crypt-a-glo staining did not reveal any significant differences between COLO-680N- and cattle-produced oocysts (Fig. 2B and C). Closer examination of the produced oocysts did, however, appear to demonstrate two morphological populations, which has been observed in *C. parvum* cultures previously (Thompson et al., 2005) (Fig. 2D; Supplementary Movie S1). The comparison of Crypt-a-glo staining of *C. parvum*-infected COLO680N- with HCT-8 cells further confirmed that *C. parvum*-infected COLO-680N cultures are characterised by enhanced numbers of infected cells compared with *C. parvum*-infected HCT-8 cultures (Fig. 1G; Supplementary Fig. S5). To finally confirm the production of fresh oocysts, Crypt-a-glo stained oocysts were excysted (Supplementary Fig. S2A) and used for the infection of COLO-680N cultures. Then, cell cultures were washed to remove remaining Crypt-a-glo stained oocysts. Upon harvesting, neither the infected cultures nor the newly produced oocysts displayed Crypt-a-glo staining. However, oocysts were detected using DAPI, indicating that indeed new oocysts were produced (Supplementary Fig. S2). We also subsequently have been able to propagate successfully the alternative *C. parvum* Iowa strain in COLO-680N cells (Supplementary Fig. S7).

In addition, we have attempted to resolve the issue of lacking of a cryopreservation system that enables the long-term storage of infective *Cryptosporidium* parasites. Here, *C. parvum* strain Moredun-infected COLO-680N cells were cryopreserved, stored for 2 weeks at −80 °C, and resuscitated by standard protocols used for cell cultures. Three days after resuscitation, the cultures started to produce oocysts similar to freshly infected COLO-680N cultures (Fig. 1H). This demonstrates that *C. parvum*-infected COLO-680N can be cryo-conserved, providing the first known long-term storage system for *Cryptosporidium*.

Next, we compared *C. parvum*- and non-infected cell cultures by a MALDI-MS-based fingerprinting approach. Principal Component Analysis (PCA) of the pre-processed data, as described in Supplementary Data S1 and in more detail in Povey et al. (2014), resulted in separate groupings of the COLO-680N, but not the HCT-8 samples (Supplementary Fig. S8A). We found substantial alterations

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