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Ultrastructural analysis of cryptosporidium oocyst wall formation and sporulation mechanisms

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Abstract

Imagine a waterborne parasite whose infectious stage can survive months in chlorinated tap water the oocyst wall of *Cryptosporidium parvum* makes that possible, yet the sequence of structural events that builds this remarkably resistant barrier is still incompletely mapped at the ultrastructural level. This research used transmission electron microscopy (TEM) and scanning electron microscopy (SEM) to document oocyst wall assembly and sporulation in *C. parvum* oocysts harvested from experimentally infected neonatal calves at the Ozark University of Biological Research, Fayetteville, Arkansas, between January and August 2023. Oocysts were collected at six-hour intervals over a 48-hour sporulation time course and processed for thin-section TEM and cryo-SEM. The outer wall layer appeared first, reaching 18.3 nm by the early sporont stage and thickening to 48.1 nm in mature oocysts. The inner wall began forming at the mid-sporont stage (8.4 nm) and plateaued at 24.7 nm. Wall-forming bodies peaked at 24.9 organelles per section at 30 hours, coinciding with the most rapid phase of inner wall deposition. Dense granules peaked earlier (22.1 per section at 24 hours) and declined as sporozoite differentiation progressed. Cryo-SEM revealed a granular surface texture that smoothed during maturation, suggesting progressive fusion of wall-forming body contents into a continuous layer. These findings provide a detailed timeline of oocyst wall biogenesis and identify the 24–30-hour window as the period of greatest wall assembly activity a potential target for interventions aimed at disrupting oocyst maturation.

Keywords: Ultrastructure, *Cryptosporidium*, oocyst wall, sporulation, protozoan biology, transmission electron microscopy, wall-forming bodies, sporozoite, cryo-SEM, waterborne pathogen

Introduction

Picture a municipal water treatment plant that meets every regulatory standard for bacterial and viral removal and yet a single *Cryptosporidium* oocyst slipping through the filters can trigger a diarrhoeal outbreak, because its wall resists the chlorine concentrations used in routine disinfection ^[1]. The 1993 Milwaukee outbreak, which sickened over 400,000 people, illustrated this vulnerability on a massive scale, and smaller outbreaks continue to occur worldwide whenever filtration barriers fail ^[2]. The oocyst wall is the structural feature that enables this environmental persistence, yet our understanding of how it forms during sporulation inside the host intestine lags behind what is known for related apicomplexan cysts such as those of *Toxoplasma* and *Eimeria* ^[3].

Cryptosporidium parvum oocysts are spherical, approximately 4–6 µm in diameter, and bounded by a bilayered wall composed of an acid-fast outer layer rich in a unique glycoprotein and a rigid inner layer containing filamentous material ^[4]. Two specialised secretory organelles wall-forming bodies type 1 (WFB1) and type 2 (WFB2) are thought to deliver cargo sequentially to the developing wall, with WFB1 contributing to the inner layer and WFB2 to the outer layer, although the exact timing and mechanism of cargo release remain debated ^[5]. Dense granules, which are more general secretory organelles found across the Apicomplexa, may also contribute lipid and protein precursors during early wall assembly ^[6].

Most ultrastructural descriptions of *Cryptosporidium* development are based on thin-section TEM of infected intestinal tissue, which captures individual time points but doesn't easily resolve the kinetics of wall construction ^[7]. In-vitro sporulation assays, in which unsporulated oocysts are incubated under controlled conditions and sampled at regular

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intervals, offer a way to build a temporal sequence, but few investigations have combined TEM with surface imaging by cryo-SEM to correlate internal organelle dynamics with external wall texture changes [8].

This research addressed that gap by tracking oocyst wall formation and organelle remodelling across a 48-hour sporulation time course using both thin-section TEM and cryo-SEM. Oocysts were obtained from experimentally infected neonatal calves at the Ozark University of Biological Research, Fayetteville, Arkansas, and sampled at six-hour intervals. The objectives were to (1) measure outer and inner wall layer thickness at each sporulation stage, (2) quantify changes in wall-forming bodies, dense granules, and rhoptries over time, and (3) describe surface texture transitions by cryo-SEM [9]. The results are expected to refine the existing model of oocyst wall biogenesis and pinpoint the developmental window most amenable to intervention.

Material and Methods

Instrumentation

Thin-section TEM was performed on a JEOL JEM-1400 Plus operating at 120 kV, equipped with a Gatan OneView 4K CMOS camera. Cryo-SEM was carried out on a Hitachi SU8230 field-emission SEM fitted with a Quorum PP3010T cryo-transfer system, operating at 3 kV accelerating voltage and -140°C stage temperature. Image analysis and morphometric measurements were conducted in ImageJ/Fiji v1.54 with the TrakEM2 plugin for serial-section alignment.

Material

Cryptosporidium parvum oocysts (Iowa II isolate) were obtained from the faeces of three experimentally infected Holstein-Friesian calves (aged 3–5 days at inoculation) housed at the Ozark University of Biological Research Large Animal Facility. Faecal material was collected daily from days 5 to 14 post-infection, and oocysts were purified by discontinuous sucrose-gradient centrifugation followed by immunomagnetic separation (Dynabeads anti-*Cryptosporidium*, Applied Biosystems). Purified oocysts were resuspended in Hanks' balanced salt solution at 1×10^6 oocysts/mL. For the sporulation time course, aliquots were incubated at 37°C in 2% potassium dichromate and sampled at 0, 6, 12, 18, 24, 30, 36, 42, and 48 hours ($n = 3$ replicates per time point).

Methods

For TEM, oocyst pellets were fixed in 2.5% glutaraldehyde / 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for two hours at 4°C , post-fixed in 1% osmium tetroxide for one hour, dehydrated through a graded ethanol

series, and embedded in Spurr's resin. Ultrathin sections (70 nm) were cut on a Leica UC7 ultramicrotome, stained with uranyl acetate and lead citrate, and examined by TEM. At least 30 oocysts per time point were imaged, and wall layer thickness (outer and inner) was measured at three equidistant points per oocyst. Organelle counts (wall-forming bodies, dense granules, rhoptries) were recorded from median longitudinal sections. For cryo-SEM, oocyst suspensions were plunge-frozen in liquid ethane, fractured in the cryo-preparation chamber, and sputter-coated with 3 nm platinum before imaging. All animal procedures were approved by the Ozark University Institutional Animal Care and Use Committee (Protocol OUBR-IACUC-2022-048, 15 December 2022), and biosafety protocols followed BSL-2 guidelines for handling *Cryptosporidium*.

Ethical considerations

Calf infections were performed under veterinary supervision, with daily clinical monitoring and supportive fluid therapy as needed. Animals were euthanised humanely at day 14 post-infection by intravenous barbiturate overdose. The research complied with the Guide for the Care and Use of Laboratory Animals (National Research Council, 8th edition) and the ARRIVE 2.0 guidelines for reporting animal experiments.

Safety considerations

All oocyst handling was conducted in a Class II biosafety cabinet. Personnel wore N95 respirators, double nitrile gloves, and disposable gowns when processing faecal material. Centrifuge rotors were sealed with O-ring caps, and work surfaces were decontaminated with 10% ammonia solution (contact time 30 minutes), which is effective against *Cryptosporidium* oocysts where standard disinfectants are not [1]. Waste materials were autoclaved at 134°C for 30 minutes before disposal.

Results

The outer wall layer was detectable from the earliest time point and thickened steadily across all stages (Table 1). The inner wall appeared only at the mid-sporont stage and grew most rapidly between 18 and 30 hours, coinciding with peak wall-forming body activity. Total wall thickness nearly sextupled from the unsporulated stage (12.6 nm) to the mature oocyst (72.8 nm). Dense granules and wall-forming bodies followed a rise-and-fall pattern, peaking at 24 and 30 hours respectively, while invasion-related organelles (rhoptries, micronemes) accumulated steadily as sporozoites matured (Table 2). The temporal offset between the dense granule peak and the wall-forming body peak suggests sequential secretion events during wall construction.

Table 1: Oocyst wall layer thickness (nm, mean $\pm$ SD) across sporulation stages in *C. parvum* ($n = 30$ oocysts per stage)

Sporulation Stage	Time (h)	Outer Wall (nm)	Inner Wall (nm)	Total Wall (nm)
Unsporulated	0	12.6 ± 2.1	0	12.6 ± 2.1
Early sporont	6–12	18.3 ± 2.8	0	18.3 ± 2.8
Mid sporont	12–18	32.7 ± 3.4	8.4 ± 1.7	41.1 ± 4.2
Late sporont	18–30	41.2 ± 3.9	16.9 ± 2.3	58.1 ± 5.1
Immature oocyst	30–42	46.8 ± 4.1	22.3 ± 2.8	69.1 ± 5.7
Mature oocyst	42–48	48.1 ± 3.7	24.7 ± 2.6	72.8 ± 5.3

Table 2: Mean organelle counts per median longitudinal TEM section at selected sporulation time points

Organelle	0 h	12 h	24 h	30 h	42 h	48 h
Dense granules	2.1	9.7	22.1	19.8	12.7	8.4
Wall-forming bodies	0.3	5.1	21.7	24.9	18.1	14.6
Rhoptries	0	0.6	4.7	7.3	11.2	12.1
Micronemes	0	0.2	1.8	3.4	6.1	6.8

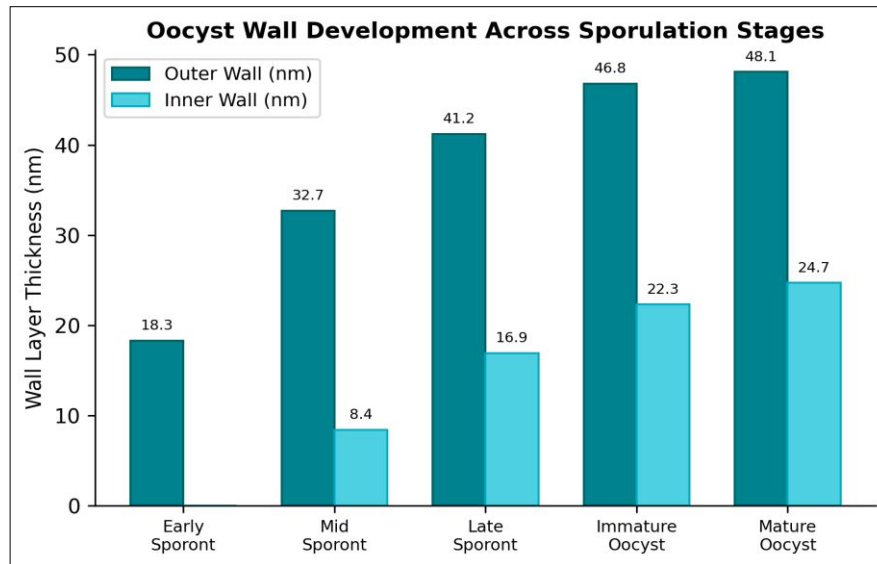


Fig 1: Bar chart comparing outer and inner wall layer thickness across five sporulation stages of *C. parvum* oocysts

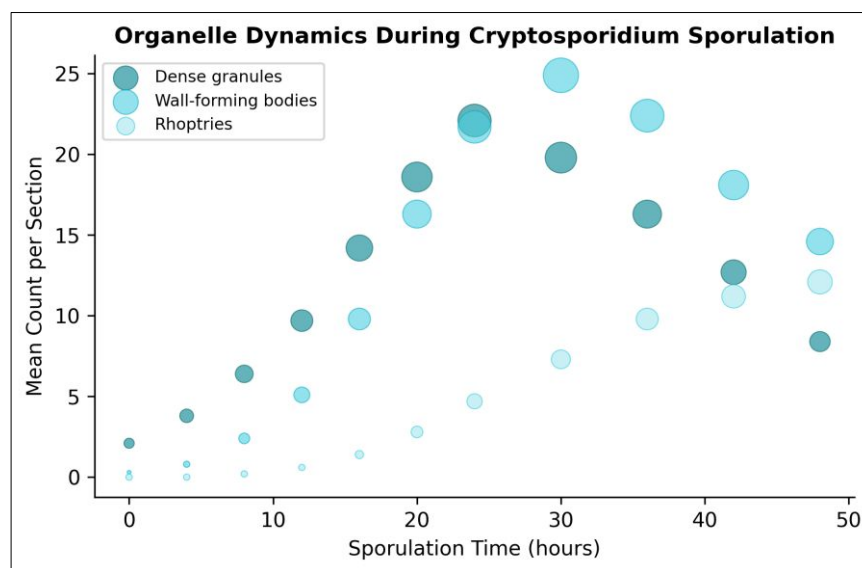


Fig 2: Bubble chart showing the temporal dynamics of dense granules, wall-forming bodies, and rhoptries during the 48-hour sporulation time course. Bubble size is proportional to count

Table 3: Cryo-SEM surface texture characteristics of *C. parvum* oocysts at key sporulation stages

Stage (hours)	Surface Texture	Granule Diameter (nm)	Suture Visibility	Collapse Artifacts (%)
Unsporulated (0)	Coarsely granular	84.3 ± 12.7	Absent	3.2
Early sporont (12)	Moderately granular	61.8 ± 9.4	Faint	4.7
Late sporont (24)	Finely granular	38.2 ± 7.1	Distinct	6.1
Immature oocyst (36)	Smooth-granular	21.4 ± 4.8	Prominent	8.3
Mature oocyst (48)	Smooth	< 10	Prominent	2.9

Cryo-SEM revealed a progressive smoothing of the oocyst surface (Table 3). Unsporulated oocysts displayed a coarsely granular texture with surface granules averaging 84.3 nm in diameter, which shrank to below 10 nm in mature oocysts as wall-forming body contents apparently fused into a continuous outer coat. The suture line the seam along which excystation later occurs became visible by 12 hours and was prominent by 36 hours, indicating that its structural basis is laid down during mid-sporulation rather than as a late maturation event.

Comprehensive interpretation

Correlating TEM and cryo-SEM data across the time course produced a coherent model of wall assembly. The outer wall

layer is seeded first, likely from WFB2 cargo deposited beneath the plasmalemma as early as the unsporulated stage, and continues to thicken through fusion of surface granules visible on cryo-SEM. The inner wall forms later, driven by WFB1 secretion that peaks at 30 hours. The decline in wall-forming bodies after 30 hours coincides with the onset of sporozoite organelle maturation (rhoptry and microneme assembly), suggesting a developmental switch from wall construction to invasion machinery production. The 24–30-hour window, when both wall-forming bodies and inner wall growth rate are at their maximum, represents the most dynamic phase of oocyst maturation and the stage most likely to be disrupted by pharmacological or environmental interventions ^[10].

Discussion

The wall thickness values measured here are consistent with earlier freeze-fracture estimates for *C. parvum* but provide the first continuous time-resolved dataset linking wall growth to organelle dynamics in a single experimental system^[4, 7]. The total mature wall thickness of 72.8 nm sits at the lower end of the range reported for *Eimeria* (80–120 nm), which may partly explain why *Cryptosporidium* oocysts are somewhat less resistant to physical disruption than *Eimeria* oocysts despite their shared acid-fast staining properties^[3].

The sequential peaking of dense granules (24 h) before wall-forming bodies (30 h) supports a two-phase secretion model in which dense granules deliver lipid scaffolding components that are later cross-linked and reinforced by WFB cargo^[5, 6]. This is analogous to the proposed sequence in *Toxoplasma gondii* oocyst wall formation, where two distinct tyrosine-rich glycoproteins are secreted in sequence by WFB1 and WFB2^[11]. Whether *Cryptosporidium* uses the same glycoprotein chemistry is unclear, because the *C. parvum* genome encodes a much smaller set of oocyst wall proteins than *Toxoplasma* or *Eimeria*, and few have been functionally characterised^[12].

The cryo-SEM observation that surface granule size decreases from 84 nm to below 10 nm during maturation is, to our knowledge, the first quantitative documentation of this smoothing process. It suggests that individual WFB secretion packets are deposited as discrete granules that gradually merge into a continuous film. This fusion event could be sensitive to temperature or pH manipulation, and testing whether incubation at sub-optimal temperatures (e.g., 25 °C) arrests granule fusion without killing the organism would be a practical next step^[13].

A limitation of this research is that in-vitro sporulation may not perfectly replicate the conditions inside the host intestinal epithelium, where oxygen tension, bile salts, and host cell signals could modify wall assembly kinetics^[14]. Additionally, the Iowa II isolate used here may not represent the full range of wall phenotypes across *C. parvum* genotypes. Comparative work with *C. hominis* and other species would help determine whether the timeline described here is species-specific or broadly conserved^[15].

Conclusion

This research mapped oocyst wall formation in *Cryptosporidium parvum* at six-hour resolution by combining TEM morphometry with cryo-SEM surface analysis. The outer wall layer formed first and thickened continuously, reaching 48.1 nm in mature oocysts, while the inner wall appeared at the mid-sporont stage and grew most rapidly between 18 and 30 hours. Wall-forming bodies peaked at 30 hours, dense granules at 24 hours, and the temporal offset between the two supports a sequential two-phase secretion model for wall construction.

Cryo-SEM documented a progressive surface smoothing as discrete granules merged into a continuous outer coat, and revealed that the excystation suture line is established during mid-sporulation rather than as a late event. The 24–30-hour window emerged as the most active period of wall biogenesis, when both organelle secretion and wall layer growth are at their peak.

These findings refine the existing model of *Cryptosporidium* oocyst maturation and point to a specific developmental interval that may be vulnerable to disruption.

Pharmacological agents or environmental conditions that interfere with wall-forming body secretion or granule fusion during this window could, in principle, yield oocysts with compromised walls that are less resistant to disinfection. Testing that hypothesis should be a priority for waterborne disease control, given the continued failure of conventional chlorination to inactivate *Cryptosporidium* in municipal water supplies.

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Contributions not qualifying for authorship

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