The 100 Faces of Cryptosporidium parvum

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This thesis is presented for the degree of Doctor of Philosophy at Murdoch University, 2012
Declaration

I declare that this thesis is a true account of my own research and contains work, which has not been submitted for a degree at any other educational institution.

______________________

Hanna Edwards
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Thank you
Abstract

*Cryptosporidium parvum* is a protozoan enteric parasite of humans and livestock. *C. parvum* infection mainly affects the ileum, where it has the potential to cause severe enteric disease. Drugs for the treatment of cryptosporidiosis are still not available and the biology and life cycle of *C. parvum* remain incompletely understood. The present study gives new insight into the parasite’s morphology, life cycle and host cell relationship.

This study utilised light microscopy, scanning electron microscopy, transmission electron microscopy and labeling of *C. parvum* surface receptors to examine infected cell cultures, cell-free cultures and oocyst stocks of *C. parvum*. Hence, this study compared different culture and different microscopic examination methods to determine the most suitable way to examine *C. parvum*’s morphology. Cell-free culture did not provide additional information to this study. However, it served as a valuable comparison for life cycle stages detected in the supernatant above cells which are expected to occur in the intestinal lumen of infected hosts. Scanning electron microscopy was the most suitable tool for obtaining information on the parasite’s morphology, whereas transmission electron microscopy enabled a view into the interior of stages. Employing light microscopy in this study was essential to progressively monitor live samples and visualise stages in the supernatant above cells, which were not attached to host tissue. In the course of this study a protocol was developed, which enabled the visualisation of *Cryptosporidium* receptors on the surface of parasites and/or host cell material via immunogold labeling with scanning electron microscopy.
For the first time, the entire range of *C. parvum*’s life cycle stages has been morphologically characterised (including their interactions with host cells) and presented in one study. A better understanding of the parasite’s biology, proliferation in host tissue and interactions with host cells will aid the drug development process.

Recent electron micrographs acquired in the course of this study revealed new life cycle stages, provided new information about the parasite’s morphology and its relationship with host cells. New insight into the host cell invasion process of *C. parvum* sporozoites as well as merozoites I and II was obtained. Features of gliding motility of the invasive stages were visualised and explained. Phenomena including binary fission - commonly employed by bacteria for the production of two identical daughter stages from one parent stage - and syzygy - the pairing of gamonts to exchange genetic material, described in gregarines - , was observed and described. Extracellular gamonts and gamont-like stages were also characterised; developing from intracellular trophozoites to finally break host cell contact and take their place in the life cycle of *C. parvum*, travelling free in the intestinal lumen. The morphology of the two different types of oocysts has been described and findings on receptor expression in their outer membranes are presented. Furthermore, *C. parvum* receptors were identified in the apical membranes surrounding parasite stages. *C. parvum* surface receptors were also found on host cell microvilli in proximity to invading and/or gliding parasites.
Additionally, the present study observed the effect that a *C. parvum* infection exerts on host tissue with respect to necrosis and apoptosis. This study also poses new ideas regarding the parasite’s host-dependent feeding behaviour.
Publications

Part of the work presented in this thesis has been accepted for publication or presented in scientific conferences as described below:

Publications in Journals


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List of Abbreviations

a: apical
ACBP: fatty acid acyl-CoA binding protein
AGS: human stomach adenocarcinoma
AIDS: autoimmune deficiency syndrome
AP: arginine aminopeptidase
AQP1: aquaporin 1
ARP2/3: actin related proteins 2 and 3
ASM: acidic-sphingomyelinase
ATCC: American type cell culture collection
ATP: adenosine triphosphate
b: rudimentary body
BSD: backscattered electron detector/detection
c: cyst
Caco: human Caucasian colon adenocarcinoma cell line
C. andersoni: Cryptosporidium andersoni
C. baileyi: Cryptosporidium baileyi
CD: cluster of differentiation
Cdc42: cell division cycle 42
C. muris: Cryptosporidium muris
cm: centimetre(s)
cn: contact region
CoA: Coenzyme A
CP: Cryptosporidial protein
Cpa: Cryptosporidium parvum
CpABC: C. parvum ATP-binding cassette protein
C. parvum: Cryptosporidium parvum
CpATPase: C. parvum adenosine triphosphatase
CPS: C. parvum sporozoite protein
CSL: C. parvum sporozoite ligand
c-Src: cellular Src
db: dense band
DMSO: dimethyl sulfoxide
DNA: Desoxyribonucleic acid
EM: electron microscopy
ER: endoplasmatic reticulum
et. al.: and others
f: feeder organelle
F-actin: filamentous actin
Fas: apoptosis stimulating fragment
FasL: Fas ligand
FCS: foetal calf serum
Fig.: figure
Figs.: figures
G: gram(s)
G: gamont
Gal: Galactose
Gal/GalNAc: N-acetylgalactosamin
GDP: guanosine diphosphate
GEFs: guanine nucleotide exchange factors
GP: glycoprotein
GTP: guanosine triphosphate
GTPase: guanosine triphosphatase
gz: gametocyte
H: hole
HCT-8: human ileocecal tumor (adenocarcinoma) epithelial cell line
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV: human immunodeficiency virus
hrs: hours
lg: Immunoglobulin
INF: interferon
j: junction
k-Da: kilo-Dalton
kV: kilo-Volts
kx: thousand times
l: litre(s)
mAB: monoclonal antibody
m: membrane
M/Ma: macrogamont
Mi: microgamont
min: minute(s)
MDCK: Madin-Darby Canine kidney epithelial cell line
mf: membrane folds
mg: milligram(s)
ml: milliliter(s)
mm: millimetre(s)
mv: microvillus, microvilli
mz: merozoite
n: nucleus
NK: natural killer
nm: nanometer
no: nucleolus
O: oocyst
p: protein
P: parasite
P₃: phosphatidylinositol 3,4,5-trisphosphate
p34-Arc: subunit protein with 34 k-Da of the actin-related protein complex (Arp2/3)
PBS: phosphate buffered saline
PCR: polymerase chain reaction
pH: power of hydrogen
Ph: pleckstrin homology domains
PI-3K: phosphoinositide 3-Kinase
PM: parasite membrane
psi: pounds per square inch
PTPase: protein tyrosine phosphatase
PV: parasitophorous vacuole
PVM: parasitophorous vacuole membrane
r : residual body
rhp: rhoptries
rpm: revolutions per minute
RPMI: Rapid prototyping and Manufacturing Institute
RT: room temperature
s: stork
SE: secondary electron
sec: second(s)
SEM: scanning electron microscopy
SEMs: sphingolipid-enriched membrane microdomains
SGLT1: sodium/glucose cotransporter
sp./spp.: species
sPLA2: secretory phospholipase A2
Src: a group of non-receptor tyrosine kinases
T: trophozoite
TEM: transmission electron microscopy
TKGFR: tyrosine kinase growth factor receptor
TNF: tumor necrosis factor
TRAP: thrombospondin-related adhesive protein
TRAP-C1: thrombospondin-related adhesive protein 1 of C. parvum
TSP: thrombospondin
VASP: vasodilator-stimulated phosphoprotein
VPSEM: variable pressure scanning electron microscope
μg: microgram(s)
μl: microlitre(s)
μm: micrometer(s)
x: times
z: zoïte
1. General Introduction

1.1. Introduction

The topic of the present thesis was the apicomplexan parasite Cryptosporidium parvum. The driving force behind this study was to gain a better understanding of the parasite’s life cycle, morphology and parasite-host relationship. Experiments were based on light microscopy, scanning (SEM) and transmission electron microscopy (TEM) of infected cell cultures, cell-free culture and oocyst stocks. Labeling of C. parvum surface receptors for SEM was employed to gain specific information on the parasites biology and host cell interactions. This study offers novel insight into the life cycle, morphology and host-cell relationships of all C. parvum life cycle stages known to date, as well as highlighting the existence of previously unknown life cycle stages and features. Novel information about the pathology of cryptosporidiosis is also presented.

As a prerequisite for this study, the host-parasite interactions leading to host cell invasion were reviewed and brought into context to understand the host cell interaction and invasion process.
1.2. Active Invasion and/or Encapsulation? A Reappraisal of Host Cell Parasitism by *Cryptosporidium*

*Cryptosporidium* species

*Cryptosporidium* is a protozoan enteric parasite of humans and other vertebrates (Fayer *et al.*, 1997). Cryptosporidiosis is recognised as one of the most common human enteric infections (Griffiths, 1998). It has the highest prevalence in young children and immunocompromised patients (Chalmers and Davies, 2009). The parasite affects the epithelial lining of the ileum, resulting in self-limiting diarrhoea in immunocompetent individuals, through to life threatening diarrhoeal disease in immunocompromised individuals. Belonging to the apicomplexan parasites, *Cryptosporidium* shares common life cycle features and morphological characteristics with other members of this phylum (Tetley *et al.*, 1998). According to phylogenomic analysis, *Cryptosporidium* species are closely related to gregarines (Barta and Thompson, 2006). Indeed, *Cryptosporidium* shows many similarities to gregarines in a number of characteristics, including an epicellular location connected to the host cell via a myzocytosis-like feeding mechanism. The only difference between the two modes of feeding is that *Cryptosporidium* has evolved a way to induce the host cell to overlay it with the apical membrane (Barta and Thompson, 2006; Butaeva *et al.*, 2006). The parasite resides on the surface of cells, within a parasitophorous vacuole (PV) between the cytoplasmic membrane and the apical membrane, as described by Huang *et al.*, (2004). To date, more than 14 different species of *Cryptosporidium* are known (Smith *et al.*, 2005), most of which are specific to their vertebrate host. The species *C. parvum* is of medical
and economic relevance as it affects both humans and cattle, with its primary site of infection being the gastrointestinal tract. The life cycle of *C. parvum* is shown in Fig. i and a short summary of the infection process is given in Box i. Critically, the mechanisms of pathogenesis are not fully understood. Parasite stimuli as well as host immune responses are thought to play critical roles. To date, no drugs are available to effectively treat *Cryptosporidium* infections. A better understanding of the parasites’ biology will help in finding specific drug targets and aid the drug development process.

Fig. i: Life cycle of *C. parvum* (modified from Barta and Thompson, 2006). Thick walled oocysts of *Cryptosporidium* are transmitted via the faecal oral route. Ingested oocysts pass through the gastrointestinal tract of the host to excyst in the ileum. Four infective sporozoites are released leaving an empty oocyst wall.
with a residual body. Sporozoites attach to the apical membrane of host cells to invade the cell and form a trophozoite. The trophozoite undergoes merogony to transform into the meront I stage, which is the next intracellular stage, with six or eight merozoites arranged parallel to each other. Merozoites (of both types) are also one of the invasive stages of Cryptosporidium occurring extracellularly in the lumen of the gut. Merozoites type I hatch from meronts I to initiate the following events:

i) An auto-infective cycle in which merozoites type I invade enterocytes to form further meronts I.

ii) A sexual cycle in which merozoites type I invade enterocytes to form a meront II that is smaller than the meront I stage and housing four merozoites type II. The merozoites type II infect further enterocytes to form micro- and macrogamonts that are respectively the male- and female gamont stages in Cryptosporidium’s life cycle.

Microgamonts release a number of microgametes. One microgamete fertilizes one macrogamont:

j) To form a thin walled oocyst that which measures 5 x 5 μm in diameter and re-infects the same host.

jj) To form a thick walled oocyst approximately 5 x 7 μm, which is passed in faeces.
Box i. **Summary of the C. parvum Infection Process**

When *C. parvum* oocysts are ingested, oocyst attachment to host tissue is inhibited until the target tissue is reached. The acidic environment of the stomach exerts the first trigger for excystation of the oocyst that is protected from this environment by its thick wall. Further stimuli are thought to be exerted through pH changes, bile and pancreatic fluids after passage through the stomach. In addition, *Cryptosporidium*’s own enzymes are important for disrupting oocyst walls to allow excystation of sporozoites. These excysted sporozoites are one of the infective forms of the parasite. Parasite and host derived enzymes and factors are involved in sporozoite activation for host cell invasion. Activation eventually results in the expression of apical organelles which are required for gliding motility and host cell invasion. Apical organelle discharge results in the expression of sporozoite surface receptors that are important for host cell contact, attachment and invasion. Specific *C. parvum* enzymes are involved in the surface expression of receptors, while others play a role in disrupting host cell membranes for invasion. Upon contact, host - parasite interactions include an increase in cell volume which facilitates invasion, intracellular signalling events which result in actin remodelling to establish the parasite in its extracytosolic niche below the host cell apical membrane, and the formation of the feeder-organelle, a tunnel through which *C. parvum* is thought to obtain vital nutrients from the host cytoplasm. Once the parasite has completed merogony, *C. parvum* enzymes are thought to rupture the apical membrane so that the parasite can progress through its life cycle.
Prerequisites for Host Cell Contact: Excystation

Thick walled oocysts are the infective stage of Cryptosporidium (Fayer et al., 1997). Lectin receptors are present on the surface of oocysts which, after passage through the gastrointestinal tract and exposure to trypsin, are altered in such a way as to enhance adherence to the epithelial lining (Kuznar and Elimelech, 2006). The thinning of the oocyst wall through exposure to trypsin also facilitates the transduction of excystation signals to sporozoites, mediated by lectin receptors. Studies by Snelling et al., (2007) showed that the expression of a variety of sporozoite proteins was increased during excystation. Such proteins are located in apical complex organelle structures of C. parvum, which demonstrates a possible role for C. parvum sporozoite surface molecules in the excystation process (Table i). Independent of host triggers, parasite derived enzymes are known to be involved in sporozoite excystation. Studies by Forney et al., (1996) measured a significant increase in protease activity during peak periods of oocyst excystation. Serine and cysteine protease inhibitors in combination blocked excystation by up to 95%, indicating that C. parvum cysteine and serine proteases also play a major role in the excystation process. A previously identified integral membrane protein of C. parvum sporozoites, arginine aminopeptidase (AP), may also be involved in sporozoite excystation as it has been found to be functionally associated with sporozoites during excystation (Okhuysen et al., 1994). Another enzyme identified within C. parvum, the secretory phospholipase A₂ (sPLA₂), is a known virulence factor in pathogenic micro-organisms (Pollok et al., 2003). sPLA₂ was identified as a rhoptry protein and thought to be involved both in excystation and expression
of receptors for host cell contact (See Table ii for a summary of C. parvum enzymes).

*In vitro* excystation protocols mimic passage through the gastrointestinal tract. Excystation can be achieved under *in vitro* conditions by incubation of thick walled oocysts in an excystation medium at a pH between 2.5 - 3, with 0.5% trypsin at 37°C, followed by transfer into nutrient media at 37°C, to effectively trigger sporozoite release (Hijjawi *et al.*, 2001). Temperature and pH therefore seem to be the major host-derived triggers for excystation, being sufficient on their own, whereas the importance of other host-derived components, such as bile salts, reducing agents or proteases remains debatable. However, studies by Hijjawi *et al.*, (2004) described the complete development of C. parvum in cell-free medium, which suggests that parasite derived triggers are sufficient on their own to initiate excystation. As such, sporozoites that excyst from oocysts in cell-free media may not have received sufficient signals for cellular invasion. Future comparative studies could show whether sporozoite receptors (Table i), known to be essential for host cell contact and invasion, are expressed in the same fashion on the sporozoite surface in the absence of host cells, as in the presence of host cells.

Although the trigger(s) for sporozoite activation still remain unclear, it is known that sporozoites initiate the process of invasion immediately after exiting the oocyst, as interactions such as actin aggregation occur from the earliest contact between parasite and host cells (Forney *et al.*, 1999).
A Barrier before Host Cell Contact: Mucus

The first barrier *C. parvum* has to pass to establish itself within its target tissue, the ileum, is intestinal mucus. The exact mode of *C. parvum* attachment to and penetration of, intestinal mucus is not understood. It is possible that the macromolecules expressed on the oocyst surface play a role in mediating oocyst adherence to intestinal mucus; otherwise oocysts would probably pass through the gut and be excreted in the faeces. Once the oocysts have reached their target tissue, infective sporozoites are released and initiate the process of host cell invasion. *Cryptosporidium* sporozoites may express enzymes that degrade intestinal mucus to facilitate the penetration of this barrier and enable sporozoite-host cell contact. Further, it is suggested that Gal/GalNAc surface lectins on the surface of excysted sporozoites adhere to mucus via receptor-ligand interactions (Bhat *et al.*, 2007) and thus mediate sporozoite attachment to intestinal mucus as a prerequisite to the penetration of this barrier (see Fig. ii). A number of *C. parvum* surface receptors, that may mediate attachment to mucus, have been identified to date (Petersen *et al.*, 1992a). These receptors, shown in Table I, are heavily O-glycosylated glycoproteins, characterised as mucin-like receptors. Two surface receptors of infective zoites (sporozoites and merozoites), GP900 and GP40, contain carbohydrate residues that are bound by αGalNAc-specific lectins, which suggests that αGalNAc residues of GP900 and/or GP40 are involved in mediating zoite attachment to host cells via adherence to intestinal mucus.
Fig. ii: Sporozoite penetration of the mucus barrier. Sporozoites of *C. parvum* have to penetrate intestinal mucus to establish host cell contact. Ingested oocysts adhere to ileal mucus via surface lectins (a). Oocysts excyst and release four sporozoites (b). Sporozoites express mucin-like surface receptors that mediate attachment to the ileal mucus lining (c). Sporozoites discharge enzymes to degrade intestinal mucus (d). The penetration of mucus is thus facilitated. Sporozoites penetrate the mucus lining and establish host cell contact via specific receptor-ligand interactions (e).
Gliding Motility Mediates Host Cell Contact

Fig. iii: *C. parvum* gliding trails. In the current study, gliding trails were observed on the surface of HCT-8 cells (as referred to earlier as antigen trails, Arrowood *et al.*, 1991). Sporozoites that excyst from the oocyst possess gliding motility. Gliding movement along the host cell surface leaves a visible gliding trail. Sporozoites invade cells to form a trophozoite. a) Excysted oocyst. b) Gliding trail of atrophic microvilli. c) Trophozoite. Gliding trails become visible as *C. parvum* interacts with microvilli, which leads to microvilli extension and atrophy.
Host cell selection and contact, invasion and internal establishment of apicomplexan organisms relies on the secretion of molecules from organelles within the apical complex (Dubremetz et al., 1998; Preiser et al., 2000). Excysted sporozoites of Cryptosporidium exhibit apical organelle discharge (Chen et al., 2004b) and gliding motility (Wetzel et al., 2005) to initiate host cell invasion. Apical organelle discharge triggers the expression of receptors on the zoite surface that mediate gliding motility. Gliding requires host cell attachment via sporozoite receptor-host cell ligand interactions. In the current study, gliding trails were seen on the surface of host cells of *C. parvum* in *in vitro* cultures (Fig. iii), whereas in studies by Wetzel et al., (2005) antigen trails by gliding zoites were monitored. Studies by Arrowood et al., (1991) utilising immunofluorescence microscopy identified trails left behind by gliding sporozoites. Conclusively, gliding motility is dependent on sporozoite surface receptors, a process in which trypsin may be involved. Trypsin does not only play a role in the excystation process, but is also known to enhance the motility of released sporozoites to facilitate host cell invasion (Smith et al., 2005). How trypsin facilitates gliding motility is not understood. One possibility may be that trypsin promotes exposure of sporozoite receptors and thus facilitates gliding motility of this zoite stage.

Additionally, various zoite surface receptors of the apical region have been associated with gliding motility (Riggs et al., 2002; Tossini et al., 2004; O’Connor et al., 2007a; Wanyiri et al., 2007). Most of these receptors are micronemal proteins that are shed by gliding sporozoites. For more detailed information, please refer to Table i. The TSP-related adhesive protein of *Cryptosporidium*-1, TRAP-C1, is a well studied micronemal protein that is
thought to link *C. parvum*'s actin-myosin motor to cellular surfaces and thus promote gliding of infective zoites (Spano *et al.*, 1998; Deng *et al.*, 2002; Putignani *et al.*, 2008). Other micronemal receptors known to be involved in gliding motility are GP900 (Petersen *et al.*, 1992a), and Cpa135 (Tossini *et al.*, 2003 and 2004) which co-localise with GP900 on the surface of infective zoites. GP40 (O’Connor *et al.*, 2007a and 2007b), which is involved in gliding motility is localised to the apical region of zoites, whereas CPS-500 (Riggs *et al.*, 1999) localises to the zoite pellicle. Some receptors involved in gliding motility are more uniformly expressed on the sporozoite surface. For example GP15 (Cevallos *et al.*, 2000b) and P23 (Perryman *et al.*, 1996; Bonafonte *et al.*, 2000) are expressed during host cell invasion on the entire surface of infective zoites. Antibody labeling revealed that GP900, GP15, GP40, Cpa135 and CPS-500 are all shed during gliding motility of infective zoites (Riggs *et al.*, 2002; Tossini *et al.*, 2004; O’Connor *et al.*, 2007a; Wanyiri *et al.*, 2007).

**Attachment and Invasion**

Host cell attachment is mediated by sporozoite receptors expressed on the apical surface during the course of apical organelle discharge (Fig. iv). The exact mechanisms of apical organelle discharge by *C. parvum* are unclear. As already mentioned, the *C. parvum* enzyme sPLA₂ triggers the release of rhoptry proteins in the apical complex region. It must be assumed that also other, yet unidentified *C. parvum* enzymes, may be involved in the process of apical organelle discharge. It is known that the intestinal environment can trigger the discharge of molecules. Intracellular calcium was also found to trigger organellar discharge by cytoskeletal remodelling (Chen *et al.*, 2004b). Indeed, a
C. parvum calcium transporter (CpATPase 1) was identified in earlier studies by Zhu and Keithly (1997), which functions as a metabolic signal rather than a membrane transporter. An important factor for apical organelle discharge is the temperature of the intestinal environment. A temperature of 37°C was observed to trigger the discharge of molecules from the apical organelles of C. parvum sporozoites in the absence of host cells. GP900, GP40 and CP2 are examples of proteins found to be discharged (Chen et al., 2004b). These and a variety of other discharged molecules have a described role in host cell attachment and invasion (See Box ii). Receptors that mediate initial host cell attachment locate apically, whereas receptors that play a later role in the invasion process are not necessarily in an apical position but can locate elsewhere on the surface of infective zoites or within zoite structures (Table i).

It can be expected that apical surface receptors of infective zoites that have the ability to adhere to intestinal mucus, are the first to be expressed. GP900 and GP40/15 have been identified as mucin-like glycoproteins and are thought to exert a major function in the infection process of C. parvum via binding of mucus and establishing initial host cell contact and attachment (Barnes et al., 1998; Cevallos et al., 2000a; Strong et al., 2000).

Other C. parvum zoite surface receptors associated with the early host cell attachment/invasion process are Cpa135, TRAP-C1, CSL, CP47 and CP12 (Table i), whereas the sporozoite surface receptor CP2, although shown to have a role in sporozoite infectivity, appears to be primarily associated with sexual development of the parasite (Chen et al., 2004b).
Fig. iv: Sporozoite drawn from photomicrograph of Tetley et al., (1998). Host cell selection, contact, invasion and internal establishment of apicomplexan organisms relies on the secretion of molecules from apical complex (ac) organelles (Elliott and Clark, 2000). The secretory organelles of Cryptosporidium consist of a single rhoptry (r), multiple micronemes (mn) and dense granules (dg) (Tetley et al., 1998). Micronemes and rhoptries are known to secrete their contents apically whereas dense granules secrete their contents elsewhere on the zoite surface (Chen et al., 2004c). Micronemal proteins enable
gliding motility of zoite stages on host cells, whereas rhoptries are more important in parasitophorous vacuole formation while dense granules are predominantly involved in modifying the host cell after invasion. Some Cryptosporidium receptors involved in host cell invasion have been located on the zoite pellicle (p) or the entire zoite surface.

Box ii. **Major C. parvum Receptors Involved in Host Cell Invasion**

The mucin-like micronemal protein GP900 is thought to play an essential role in initial host cell contact via glycoprotein portions of the protein that mediate specific receptor-ligand interactions (Barnes et al., 1998). The two mucin-like glycoproteins GP15 (also known as C17) and GP40 are both proteolytic fragments of the same precursor (Cevallos et al., 2000a; Strong et al., 2000), but nevertheless antigenically distinct proteins that are differentially localised on the surface of sporozoites and merozoites (Cevallos et al., 2000a). As both proteins are heavily glycosylated like GP900 they are associated with initial host cell contact. Both GP15 and 40 exhibit a high degree of polymorphism between isolates, which may account for host specificity via specific receptor-ligand interactions (Strong et al., 2000). A monoclonal antibody against GP900 and GP15/40 reduced invasion of C. parvum into host cells, supporting the conclusion that discharge of respective Cryptosporidium antigens is involved in host cell invasion (Cevallos et al., 2000a, 2000b). Cpa135 is another micronemal protein that is thought to play a role in the initial parasite-host interaction via a ricin-b domain, but is also found in intracellular stages of the parasite, defining the boundaries of the parasitophorous vacuole in infected cells (Tossini et al., 2004). A well characterised adhesive protein of C. parvum is the thrombospondin TRAP-C1. TRAP-C1 is structurally related to micronemal
proteins of the apicomplexan parasites *Toxoplasma, Eimeria* and *Plasmodium* (Spano *et al.*, 1998). TRAP-C1 has the ability to bind to sulfated glycoconjugates via a thrombospondin type 1 repeat (Spano *et al.*, 1998; Chen and LaRusso, 2000). Thus, TRAP-C1 is a major candidate protein in the host cell invasion process by *C. parvum* sporozoites. CSL is a soluble zoite exoantigen for which a host cell receptor has been identified that specifically binds CSL and rapidly internalises it within two minutes upon binding (Lumb *et al.*, 1998). These findings suggest a major role for CSL in sporozoite infectivity, and make it a major target for immunisation therapies. CP47 is the only other zoite surface receptor for which a host cell receptor has been reported (Nesterenko *et al.*, 1999). It is postulated that this receptor is more highly expressed in ileal tissue than elsewhere, explaining the binding preference of *C. parvum* to ileal cells. These findings show a role for CP47 in host cell invasion. CP12 is a newly identified 12 k-Da adhesion protein on sporozoites and oocysts of *C. parvum*. Antibody labeling located the protein to the entire surface of oocysts, but especially the apical region of sporozoites, inferring a role in the host-parasite interaction (Yao *et al.*, 2007). The *C. parvum* sporozoite surface antigen CP2 (O'Hara *et al.*, 2004) was found to localise to the parasitophorous vacuole membrane (PVM) of intracellular stages, with its highest expression during sexual development of the parasite, located at amylopectin-like granules and in the cytoplasm of macrogametocytes, as well as on the oocyst wall and sporozoites of developing sporulated oocysts. Thus, it may be integrally involved in the infection process. An antibody to CP2 was shown to decrease parasite invasion (Chen *et al.*, 2004b) suggesting that CP2 is involved in the invasion process.
As mentioned previously, enzymatic activity appears to play a role in the processing of *C. parvum* surface receptors, from the translation of DNA to the successful surface expression of respective receptors. Both host and parasite derived protease activity cleaved recombinant *C. parvum* GP40/15 precursor protein into its two peptides. However, the *C. parvum* enzymes responsible for this activity have not been identified (Wanyiri *et al.*, 2007). As GP40/15 is processed early in the infection process it is most likely to occur through parasite-derived protease activity. A role for parasite derived proteinases in the processing of other *C. parvum* surface receptors is possible but has not been investigated to date. Studies by Marshall and Flanigan (1992) reported a significant reduction in *C. parvum* infection *in vitro* with paromomycin, an aminoglycoside that inhibits protein synthesis at the ribosomal level, and it was hypothesised to have a similar inhibitory effect upon protozoan parasites. Together, these results suggest that parasite derived protease activity is involved in processing of *C. parvum* surface receptors that are involved in the early infection process. On the other hand, recent studies by O’Connor *et al.*, (2007a) suggest another function for *C. parvum* enzymes in the processing of parasite surface receptors; in which *C. parvum* enzymes link parasite surface receptors like GP40/15 as complexes to the host cell surface.
**Active Invasion and/or Host Cell Encapsulation**

Sporozoites attach to the apical membrane of host cells and induce host cell membrane protrusion that encapsulates the parasite in a PV underneath the host cell apical membrane. Dynamic membrane protrusion is a common way for intracellular microbes to enter host cells (Elliott and Clark, 2000). The overall rate of membrane protrusion depends on the actin polymerisation rate as well as an increase in localised cell volume.

Until recently, molecular mechanisms regulating local cell volume associated with membrane protrusions remained unclear. *In vitro* studies by Chen *et al.*, (2005) have now demonstrated the recruitment of a host-cell aquaporin 1 (AQP1) and a Na⁺/glucose cotransporter (SGLT1) to the host-parasite interface. Aquaporins are channels that specifically allow water and associated small non-ionic molecules to rapidly cross membranes along an osmotic gradient. SGLT1 establishes the osmotic gradient to allow transport by aquaporins. The two transporters were found in the PVM and along the host-parasite interface. Recruitment of AQP1 and SGLT1 to the parasite attachment site results in a localised increase of glucose uptake and a subsequent AQP1-mediated water influx (Chen *et al.*, 2005). The localised water influx seems to enhance *C. parvum*-induced host cell membrane protrusion and thus facilitates cellular invasion. Functional inhibition of AQP1 and/or SGLT1 significantly reduced the invasion rate, whereas overexpression of AQP1 increased the invasion rate.

The other factor essential for successful host cell membrane protrusion is actin polymerisation, which is independent of cell volume regulation (Chen *et al.*, 2005). In the polymerisation process, actin filaments assemble and organise as a dendritic network by actin-associated signalling pathways, to engulf the
invading parasite. Many parasite-host cell signalling pathways are involved in the process of establishing the parasite within the host cell, including actin polymerisation, and these are now well characterised (Perkins et al., 1999; Elliott and Clark, 2000; Pollok et al., 2003; Chen et al., 2004a, 2005; Hashim et al., 2006) (see Box iii).

**Box iii. Actin Polymerisation Events in the Course of C. parvum Host Cell Invasion**

*C. parvum* invasion of host cells requires host cell tyrosine phosphorylation as an essential prerequisite for actin polymerisation. Studies by Abrahamsen et al., (2004) identified an acid phosphatase in the *C. parvum* genome that might be involved in tyrosine phosphorylation of host cells (Aguirre-Gracia and Okhyusen, 2007). Host tyrosine phosphorylation was found to occur within 30s of inoculation (Forney et al., 1999). Host cell tyrosine phosphorylation activates the first component of a further signal pathway mediated by phosphoinositide 3-kinase (PI-3K). PI-3K activity was observed during *C. parvum* invasion (Forney et al., 1999). Thus, phospholipid-mediated signal pathways play a role in the early infection process of *C. parvum*. The activation of PI-3K is thought to be linked to the activity of tyrosine kinase growth factor receptors (TKGFRs), and results in the production of phosphatidylinositol 3,4,5-trisphosphate (P₃) at cell membranes (Forney et al., 1999). P₃ is bound by frabin, which is also found to be recruited to the attachment site (Chen et al., 2004c). Frabin then stimulates the formation of the GTP-bound active form (Chen et al., 2004c), which is essential for the activation of the next component in the signalling pathway. The cell division cycle 42 (Cdc42) is a key regulator of actin cytoskeletal remodelling induced by extracellular signals. Actin-regulating downstream effectors of
Cdc42 are the neural Wiskott Aldrich syndrome protein (N-WASP) and p34-Arc, a subunit of the host cell Arp2/3 complex, which are recruited to the host-parasite interface, together with Cdc42, during invasion (Chen et al., 2004a). Expression of constitutively active host cell Cdc42 promoted *C. parvum* invasion. Conversely, inhibition of Cdc42 or blockage of N-WASP inhibited *C. parvum* invasion (Chen et al., 2004a). The host cell actin related protein (Arp2/3) complex is important for actin binding and the initiation of actin polymerisation, whereas N-WASP stimulates nucleation and branching of actin filaments (Chen et al., 2004a).

Inhibitors of PI-3K prevented frabin recruitment and Cdc42 activation in infected cells, thereby inhibiting actin remodelling and cellular invasion (Chen et al., 2004c). Therefore, an important component of the complex invasion process of *C. parvum* into target epithelial cells results from the ability of *Cryptosporidium* to trigger host cell PI-3K/frabin signalling, via phosphorylation of a tyrosine residue to activate the Cdc42 pathway via frabin.

Host cell sphingolipid-enriched membrane microdomains (SPEMs), which are involved in the clustering of membrane receptors and activation of downstream intracellular signalling pathways, are thought to play an important role in *C. parvum* attachment to, and invasion into host cells (Grassme et al., 2003). Multiple SPEMs cluster into larger platforms, which results in re-organisation of molecules in the cell, bringing signalling molecules closer together. It has been shown that these processes can be stimulated by pathogenic microbes (Langer et al., 2001; Grassme et al., 2003). Disruption of SPEMs or blockage of SPEM-associated enzymes decreased *C. parvum* host cell attachment and invasion by...
up to 88% (Nelson et al., 2006). The activation of PI3-K/Cdc42 pathways was blocked by up to 75%. It is assumed that SPEMs facilitate invasion by the clustering of membrane-binding molecules and subsequent activation of PI3-K/Cdc42 pathways which result in actin polymerisation (Nelson et al., 2006). For an overview over the whole infection process see Fig. v.
Fig. v: Summary of the infection process.

Despite swallowing oocysts, C. parvum enzymes are involved in excystation and in expression of sporozoite surface proteins during excystation.

With host cell contact, sporozoites initiate host cell membrane protrusion that encapsulates the parasite.

Ingestion of thick-walled oocysts: Lectin receptors on oocyst surfaces mediate adherence in the ileum.

The acidic pH of the stomach, bile and pancreatic fluids trigger excystation.

Apical organelle discharge: Sporozoite surface lectins mediate attachment to ileal epithelium.
**C. parvum's Niche**

In contrast to other apicomplexans, *Cryptosporidium* does not invade the cytoplasm of host cells, but resides within a PV, which has an extracytosolic location between the apical plasma membrane and the host cytoplasm (Huang *et al.*, 2004).

The PV is the space between the apical membrane, with the PVM separating the two structures, except at the annular ring where the membranes of these two structures fuse (Lumb *et al.*, 1988). The exact composition of these structures and the process of formation are yet to be determined. It is thought that a branched network of actin filaments grows by actin assembly to engulf the parasite (Lumb *et al.*, 1988). Host cytoskeletal rearrangement is thought to contribute to the formation of long, branched microvilli clustered around the cryptosporidial vacuole. Indeed, microvilli are observed to cluster in long branched forms around developing trophozoites (Fig. v) (Lumb *et al.*, 1988).

The microvilli associated with the parasite are particularly thick (Fig. vii) (Lumb *et al.*, 1988). In previous studies it was assumed that the apical membrane and PVM are derived from the host cell membrane (Tzipori and Griffiths, 1998). Studies by Bonnin *et al.*, (1999) observed that the parasitophorous vacuole wall contains at least two microvillous-derived components, villin and ezrin, as well as a low amount of filamentous actin (f-actin). Conflictingly, other studies which utilised monoclonal antibodies found that *Cryptosporidium* antigens were localised to the "host derived" PVM (Robert *et al.*, 1994; Bonnin *et al.*, 1995; McDonald *et al.*, 1995; O'Hara *et al.*, 2004), leading to the conclusion that the apical membrane and PVM are composed of host material, but also contain parasite derived elements.
Once the parasite is encapsulated at the apical surface of an epithelial cell an electron-dense band is formed, which separates the parasite from the host cytoplasm (Fig. viii) (Huang et al., 2004). Cryptosporidium employs the actin binding protein villin, a cross-linking protein that stabilises f-actin bundles in microvilli, and α-actinin, an actin-cross-linking protein, to organise f-actin at the host parasite interface (Elliott and Clark, 2000). This attachment zone contains a folded membranous structure known as the feeder organelle (Fig. vii) (Perkins et al., 1999). The feeder-organelle is a tunnel-like formation, through which presumably nutrients are accessed from the host cell cytoplasm, as Cryptosporidium lacks several de-novo synthesis pathways (Huang et al., 2004). Thus, the feeding dependency of Cryptosporidium species is closely related to that of gregarines, which also lack de-novo synthesis pathways, occupy a similar epicellular niche and show a similar feeding-behaviour (Barta and Thompson, 2006; Butaeva et al., 2006). Indeed, an integral membrane protein, the C. parvum ATP-binding cassette protein (CpABC), was localised at the host-parasite boundary in intracellular stages of C. parvum and also found in sporozoites (Perkins et al., 1999). As ABC proteins transport substrates it is possible that the CpABC forms the feeder organelle and serves to uptake nutrients from host cells. Although the modes of feeding are virtually the same, Cryptosporidium has an advantage over the gregarines. In contrast to gregarines, Cryptosporidium has found a way to induce the host cell to overlay it with the host cell apical membrane. This encapsulation of invasive Cryptosporidium stages, which can be readily observed using electron microscopy (see Figs. vi-ix), appears to be induced by the parasite itself (Box 3,
Fig. v). This unique niche presumably conferred great advantage during evolutionary selection, particularly as a means to escape the host immune response and to prevent stages of the parasite being voided with the faeces. This same biology may now be a factor which could frustrate drug targeting to the parasite, possibly avoiding exposure to drugs. As a consequence of the protective membranes, the encapsulated stages of the parasite are dependent on their feeding connection to the host cell. Despite the advantages that *C. parvum*’s intracellular niche offers, surprisingly, extracellular development was also observed *in vitro* (see Chapters 3 and 5). Extracellular development of *C. parvum* appears to be a rudimentary event as explained in Box iv.
Fig. vi: *C. parvum*’s niche. a, b) In the current study, *C. parvum* was seen to cause atrophy of microvilli. Microvilli cluster in long branches around developing trophozoites as indicated by arrows. c) The parasite is engulfed by the host cell apical membrane (arrow). d) A tunnel like feeding connection is established to the host cell cytosol: the feeder organelle (left arrow). Thick microvilli are observed in close proximity to developing parasites (right arrow). e) Once the parasite is encapsulated, an electron dense band is formed, which separates the parasite from the host cytosol (left arrow). The right arrow indicates the PV. f) Formation of daughter stages directly within a parent meront, in the HCT-8 cell line without prior host cell contact of each single daughter stage. g) Extracellular formation directly from within the oocyst in the HCT-8 cell line, 4 days after the incubation with *C. parvum*. The four sporozoites have probably developed into trophozoites directly within the oocyst (arrow) without prior host cell contact of parent or daughter stages. The oocyst wall is perforated for the release of intracellular stages.
Studies by Hijjawi et al., 2004 showed that Cryptosporidium can complete its life cycle extracellularly in cell-free media. Extracellular stages of C. parvum have also recently been observed in the current study using the human ileo-caecal cell line HCT-8 as an in vitro model (See images in Figs.10 and 11; unpublished observations). These observations raise the question as to how Cryptosporidium chooses its intracellular niche? The abundance of the extracellular stages was very low in contrast to intracellular stages developing in the HCT-8 cell line. Thus it can be assumed that the occurrence of extracellular stages in host cells is a rudimentary vestige from the evolution of the parasite from an extracellular to an intracellular life-style. Cryptosporidium probably adapted to an intracellular life-style in host tissue, to escape the immune response. The fact that there are asymptomatic human and animal carriers of Cryptosporidium, for which recrudescence occurs from time to time, supports that Cryptosporidium developed a way to escape the host defence mechanisms by adaptation to an intracellular life-style.

Clinical Pathology of Cryptosporidiosis

It has been recognised recently that human cryptosporidiosis is usually caused by Cryptosporidium parvum genotype I (C. hominis), which is not found in animals (Hashim et al., 2004). Cryptosporidium parvum genotype II (C. parvum) however is of medical and economical importance as it affects both humans and life stock and can be transmitted from one to the other. The mode of C. parvum transmission is most commonly waterborne, but other sources of
infection, including foodborne and person-to-person transmission, have been
documented (Leav et al., 2003). The environmental form of the parasite is the
thick walled oocyst, which is resistant to most water purification methods,
including chlorination (Leav et al., 2003).

The clinical pathology underlying an infection with C. parvum is still not fully
understood. Cryptosporidium infection usually causes a self-limited diarrhoeal
illness but can be life-threatening in immunocompromised individuals (Leav et
al., 2003), with mortality rates of 52% – 68% (Rose, 1997). Severe C. parvum
infections can not only infect the small intestine but also other organs such as
the gall bladder (Owen, 1998) or the lungs (Dupond et al., 1996). Studies by
Elliott and Clark (2003) identified in an MDCK cell model that the host cell is
killed upon parasite egress; this death is necrotic, rather than apoptotic (as the
current study has shown), possibly causing the release of toxic substances from
the cell, which might contribute to the pathology associated with
cryptosporidiosis. Studies by Widmer et al., (2000), however, suggest that the
host might employ controlled cell death (apoptosis) itself as a means to
eradicate Cryptosporidium infections. Both humoral and cellular immunity play a
role in combating this infection, but the latter plays the major role, mainly in the
intestinal mucosa. IgG, IgM and IgA have been detected in serum and mucosa
of humans and animals with the resolution of the infection (Gomez Morales and
Pozio, 2002). Studies on the mechanisms of immunity to cryptosporidiosis
indicate the importance of the T-cell response. The spectrum and severity of
disease in immunocompromised individuals with cryptosporidiosis reflect this
importance since the most severe disease is seen in individuals with defects in
the T-cell response (Hunter and Nichols, 2002). Diseases that are associated
with increased risk of severe cryptosporidiosis are HIV, immunodeficiencies and leukemia, due to their associated T-cell defects (Hunter and Nichols, 2002). Studies in mice by Aguirre et al., (1994) showed a major role for CD4 T-cells but not CD8 T-cells in the immune response to Cryptosporidium infections. In clinical trials, CD4 T-cells have been shown to be required to prevent the establishment of the infection. IFN-gamma and CD4 T-cells were also found to limit the duration and the clinical manifestations of the infection. The importance of IFN-gamma becomes clear, as the inability to produce INF-gamma has been associated with the severity of the infection (Gomez Morales and Pozio, 2002). Other studies in mice identified a role for TNF signalling in the pathology following infections with Cryptosporidium (Ponnuraj and Hayward, 2002). Recent studies in mice by Barak et al., (2009) identified a role for NK cells and IFN-gamma in the innate immunity against C. parvum, with NK cells possibly functioning independent of the production of IFN-gamma.

Treatment of Cryptosporidiosis

A treatment for the complete eradication of Cryptosporidium infections is not yet available though several attempts have been made. Due to the diminished immune system and absence of T cells in the course of HIV infections, a large number of clinical trials have been performed in patients with AIDS.

Early studies by Flanigan et al., (1991a) identified hyperimmune bovine colostrum to be highly inhibitory for C. parvum infections in vitro. In studies by Hunt et al., (2002), cryptosporidiosis was decreased in calves through oral bovine serum. Similar attempts in humans however yielded controversial results. In studies by Ungar et al., (1990) hyperimmune bovine colostrum
proved effective in clinical trials on humans. Likewise, oral bovine immunoglobulins were identified as a promising treatment in AIDS patients (Greenberg and Cello, 1996). However the treatment of HIV+ patients with bovine hyperimmune colostrum was not a successful therapy according to Nord et al., (1990). Hyperimmune colostrum also failed to help a 6 month old infant (Tzipori et al., 1986). It can be concluded that it does not represent a promising treatment option. Studies by Gomez Morales and Pozio (2002) finally lead to the suggestion that hyperimmune bovine colostrum therapy is not worth pursuing.

Monoclonal antibodies have been successfully used to reduce, but not eliminate, Cryptosporidium infections in mice (Riggs et al., 2002). This path however has not been pursued any further. Likewise the antiviral agent indinavir has been shown to directly interfere with C. parvum’s life cycle and thus decrease infections in mice (Mele et al., 2003), however, it did not appear to be a promising treatment against cryptosporidiosis as it did not completely eliminate the infection.

While cryptosporidiosis is self limiting in otherwise healthy individuals, Cryptosporidiosis in HIV+ patients is a heterogeneous disease with poor survival rates (Blankhard et al., 1992). In studies by Blankhard et al., (1992) Cryptosporidiosis remained unaffected by any drugs apart from zidovudine. First attempts with the antibiotic paromomycin in in vitro studies showed success in inhibiting Cryptosporidium infections (Marshall and Flanigan, 1992). In early medical trials with paromomycin the mean number of diarrheal episodes decreased from 10.9 to 1.7 daily. Thus, paromomycin appears to be a promising agent for treatment of acute cryptosporidiosis (Fichtenbaum et al.,

Nitazoxanide is an antiprotozoal agent used for the treatment of metronidazole-resistant giardiasis (Rossignol et al., 2009). In early studies by Blagburn et al., (1998) paromomycin showed excellent activity against Cryptosporidium in mice whereas nitazoxanide showed only moderate activity. In contrast, nitazoxanide showed significant improvement in immunocompetent humans. However, an intact immune system is needed as results from a HIV+ patient group showed (Amadi et al., 2002). Still, nitazoxanide efficiently reduced diarrhoea in clinical trials (Rossignol et al., 2001).

Studies with the antibiotic azithromycin showed excellent success with cure rates of 91% and a 99% oocyst reduction (Allam and Shehab, 2002). In different studies, azithromycin effectively treated cryptosporidiosis in all patients, however, in a few patients the infection was not eradicated (Kadappu et al., 2002). In stark contrast, HIV+ patients with disseminated cryptosporidiosis responded much better to nitazoxanide (67.2 % decrease of parasite count) and paromomycin (63.4 %), in contrast to azithromycin (26.5 %) (Giacometti et al., 1999).

Altogether, azithromycin still appears to be the most promising treatment option. However, a treatment must be adjusted to the patient’s situation such as the state of the immune system, age and underlying diseases, as studies by Giacometti et al., (1999) showed.
### Table i. *C. parvum* Receptors and their Role in Host Cell Parasitism

<table>
<thead>
<tr>
<th><em>C. parvum</em> receptors</th>
<th>Location</th>
<th>Characteristic</th>
<th>Possible Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Localization</td>
<td>Description</td>
<td>Function</td>
<td>References</td>
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<tr>
<td>GP15 (C17)</td>
<td>Entire zoite surface, and colocalises to apical region with GP40</td>
<td>Mucin-like glycoprotein, O-glycosylated, Proteolytic fragment of the same 60 kDa precursor protein as GP40, GPI anchored</td>
<td>Shed during gliding motility, Adherence to host cells</td>
<td>Cevallos et al., 2000; Jakobi &amp; Petry, 2006; O’Connor et al., 2003 and 2007a; Sestak et al., 2002; Strong et al., 2000; Tilley &amp; Upton, 1994; Wanyiri et al., 2007</td>
</tr>
<tr>
<td>GP40</td>
<td>Apical region of zoite surface</td>
<td>Mucin-like glycoprotein, O-glycosylated, Proteolytic fragment of the same 60 kDa precursor protein as GP15, soluble</td>
<td>Shed during gliding motility, Zoite cytoplasmic membrane, Adherence to host cells</td>
<td>Bhat et al., 2007; Cevallos et al., 2000; O’Connor et al., 2003a and 2007; Sestak et al., 2002; Wanyiri et al., 2007</td>
</tr>
<tr>
<td>P23</td>
<td>Zoite surface</td>
<td>Possibly involved in gliding motility</td>
<td></td>
<td>Bonafonte et al., 2000; Jakobi &amp; Petry, 2006</td>
</tr>
<tr>
<td>Cpa135</td>
<td>Apical region of zoites, presumably micronemes</td>
<td>Colocalises with GP900</td>
<td>Secreted during zoite gliding, Initial Parasit-host interaction</td>
<td>Tosini et al., 2003 and 2004</td>
</tr>
<tr>
<td>CPS-500</td>
<td>Zoite pellicle</td>
<td>Polar glycolipid, Insoluble, requires adhesion</td>
<td>Depositioned during gliding motility</td>
<td>Riggs et al., 1999</td>
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<tr>
<td>CSL</td>
<td>Apical region of zoite</td>
<td>Soluble exoantigen</td>
<td>Zoite infectivity</td>
<td>Langer &amp; Riggs, 1999 and 2001; Riggs et al., 2002; Schaefer et al., 2000</td>
</tr>
<tr>
<td>CP47</td>
<td>Apical region of zoite</td>
<td>Membrane protein</td>
<td>Host cell invasion</td>
<td>Nesterenko et al., 1999</td>
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<td>CP12</td>
<td>Oocyst surface, Apical region of Sporozoites</td>
<td></td>
<td>Host cell adhesion</td>
<td>Yao et al., 2007</td>
</tr>
<tr>
<td>CP2</td>
<td>Sporozoites, Found in host-parasite attachment region, PVM of intracellular stages, cytoplasm of gametocytes</td>
<td>Integral protein, Highest expression during sexual development of the parasite</td>
<td>Host cell invasion, most likely integrally involved in infection process</td>
<td>Bonafonte et al., 2000; Chen et al., 2004b; O’Hara et al., 2004; Tilley &amp; Upton, 1994; Tossini et al., 2004</td>
</tr>
</tbody>
</table>

**Table ii. C. parvum Enzymes and their Role in Host Cell Parasitism**

<table>
<thead>
<tr>
<th>C. parvum enzymes</th>
<th>Location</th>
<th>Characteristic</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine and</td>
<td>Oocyst excystation</td>
<td></td>
<td></td>
<td>Forney et al., 2004</td>
</tr>
<tr>
<td>Enzyme Name</td>
<td>Membrane Type</td>
<td>Function</td>
<td>Host Cell Function</td>
<td>Reference</td>
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<tr>
<td>-------------</td>
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</tr>
<tr>
<td>Serine proteases</td>
<td>Integral membrane protein</td>
<td>Functionally associated with sporozoites during excystation</td>
<td>Oocyst excystation</td>
<td>Okhysen et al., 1994</td>
</tr>
<tr>
<td>Arginine Aminopeptidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Furin-like protease</td>
<td>Membrane-bound</td>
<td>Cleavage of GP40/15 in its two peptides</td>
<td></td>
<td>Wanyiri et al., 2007</td>
</tr>
<tr>
<td>Protein tyrosine phosphatase</td>
<td>Membrane-bound</td>
<td>Acid phosphatase, hydrolyses phosphomonoesters and phosphoproteins</td>
<td>Possibly repressing respiratory burst of host cell. Possibly modulation of host cell tyrosine phosphorylation for host cell entry</td>
<td>Aguirre-Garcia &amp; Okhysen, 2007</td>
</tr>
<tr>
<td>sPLA2</td>
<td></td>
<td>Haemolytic activity</td>
<td>Host cell invasion. May activate release of rhoptry proteins Membrane fusion</td>
<td>Pollok et al., 2003</td>
</tr>
</tbody>
</table>
1.3. Hypothesis

The hypothesis leading to this study was that the life cycle of *C. parvum* is incompletely understood and that behaviours and host cell interactions of *C. parvum* life cycle stages have been incompletely described. Thus, it was thought that life cycle stages may exist that have not been described and that many life cycle stages accepted to date may not have been optimally characterised morphologically.

With the literature review leading into this thesis, the molecular pathways underlying the host cell interaction and invasion process have been brought into context. The host cell invasion process is not fully understood and requires further examination. The hypothesis that *C. parvum* actively invades the cell by inducing a host cell response, directed this study. With that, this study hypothesises that the apical membrane surrounding invaded *C. parvum* stages contains parasite elements as well as host cell material. In the introduction to this thesis it has already been mentioned that *C. parvum* induces a host cell response to utilise host cell material for its own purpose. Adding onto that, this study hypothesises that *C. parvum*’s main host cell interaction occurs via microvilli-*C. parvum* surface receptor contact.

Further, it was found that the host-dependant feeding behaviour of the parasite has not been explained. This study hypothesises that intracellular stages remodel host cell intracellular mechanisms to satisfy their own feeding-dependency.

Last but not least, the host cell death caused by *C. parvum* is still not fully understood. This study is based on the belief that research until today has focused on apoptosis to the neglect of necrosis, disregarding the pathology that
infection by *C. parvum* causes. This study hypothesises that *C. parvum* causes necrosis rather than apoptosis.

### 1.4. Aims of this Thesis

*C. parvum* research commonly concentrates on the host cell invasion process or specific *C. parvum* stages only, to the exclusion of the entire picture. The whole life cycle as such has never been extensively studied at the EM level, as it has been done in this study. It is essential to fully understand the parasite’s life cycle to maximise the chance of finding a target for drug therapy.

The first aim of this study was to morphologically characterise the HCT-8 cell line with the same microscopic methods that would later on be used to examine the parasite. With this information as a reference point, the impact of a *C. parvum* infection on the host cell line could be determined.

Further, this study tested the suitability of cell-free culture systems, to serve as a model for *C. parvum* infection and to examine the morphology of parasite stages, as compared to cell culture. Stages observed in cell-free culture were compared to stages observed in cell culture.

Additionally, this study aimed to identify and characterise hypothesised, previously unknown intra- and extracellular life cycle stages. The main aim however remained to morphologically characterise and present the entire range of *C. parvum* life cycle stages in one study. Special interest was aimed towards the host cell contact and invasion process of *C. parvum* infective stages, as well as the host-parasite relationship of other stages in the parasite’s life cycle. Parasite-microvilli interactions and involvement of microvilli material in *C. parvum* processes were closely observed.
The involvement of C. parvum receptors in all of these processes was included into the interest of this study. This study aimed to identify C. parvum receptor expression on oocyst surfaces and the dependency of their expression on host cell presence. Another aim was to examine the expression of surface receptors on intracellular and extracellular C. parvum stages in context with their place in C. parvum’s life cycle. Finally, this study aimed to determine the composition of the apical membranes engulfing invaded parasite stages and whether, and to what extent, the membrane contained parasitic elements.

1.5. Study Design

To perform this study, the parasite C. parvum was cultured with HCT-8 cells, which is a human ileoceleal tumor (adenocarcinoma) epithelial cell line. Cell-free cultures were performed as well, to test their suitability as an infection model. Ideal culture conditions were tested and monitored with light microscopy. Light microscopy also served to identify C. parvum stages in the supernatant above cells and for comparison with stages observed in cell-free cultures. Apart from cell and cell-free culture, oocyst stocks used for the inoculation of samples were microscopically examined.

Once ideal culture conditions were established, both infected monolayers and oocyst stocks were examined with SEM and TEM at various time points to identify and morphologically characterise C. parvum life cycle stages, their interactions with host cells and the impact an infection exerts upon host cells.

To examine the parasite-host relationship, C. parvum surface receptor expression and the composition of the apical membrane, a protocol was developed that enabled labeling of C. parvum surface receptors and
visualisation by SEM. This protocol also served to identify life cycle stages and morphological features of *C. parvum*. 
2. General Materials and Methods

2.1. Cell Culture

2.1.1. C. parvum Isolates and Preparation
The C. parvum cattle isolate used during this study was originally obtained from the Institute of Parasitology in Zurich and was subsequently passaged through mice as described by Meloni and Thompson (1996). C. parvum oocysts of the cattle type were purified from infected ARC/Swiss mice as described by Meloni and Thompson (1996).

2.1.2. Cell Line
HCT-8 CCL224 cells used in this study were originally obtained from the American type cell culture data base (ATCC).

2.1.3. Cryopreservation of Cells
HCT-8 cells were cryopreserved for storage. Confluent monolayers were trypsinised and resuspended in appropriate amounts of the cryopreservation mixture (10% dimethyl sulfoxide (DMSO) and 90% foetal calf serum (FCS)) then aliquoted into 2 ml cryopreservation vials for cryopreservation in liquid nitrogen. It was important to routinely cryopreserve many stocks, as passaging a cell lines too long has a negative impact on the cells and lowers their susceptibility to infection with C. parvum considerably.
2.1.4. Cell Culture

For routine passaging, HCT-8 cells were cultured in RPMI medium with 2 g/l sodium bicarbonate, 0.3 g/l L-glutamine, 3.574 g/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer) (15 mM) and 10% FCS at a pH of 7.4. For maintenance purposes, cells were cultured in 75 cm² flasks and incubated at 37°C, 5% CO₂. For routine splitting the RPMI medium was aspirated and confluent monolayers were washed with 1 x phosphate buffered saline (PBS) and then incubated with 2 ml trypsin at 37°C until the monolayer detached. The cells were resuspended in RPMI medium and split into fresh 75 cm² flasks, which were topped up to a total of 20 ml RPMI medium.

2.1.5. Pretreatment of Oocysts

Before cell line infection, C. parvum oocysts were bleach treated for sterilisation and excysted to facilitate cell invasion. Oocysts were incubated for 30 min at room temperature (RT) in 10 ml of water containing 200 µl of household bleach. Bleach treated oocysts were spun down at 3500 revolutions per minute (rpm) for 10 min and then resuspended in 10 ml of excystation medium that consisted of 0.5% trypsin in acidic water at a pH of 2.5. For excystation, oocysts were incubated in excystation medium for 30 min in a 37°C waterbath with shaking every 5 min. Excysted oocysts were spun down at 3500 rpm for 10 min and then resuspended in an appropriate amount of maintenance medium.

Maintenance medium consisted of the RPMI medium described in chapter 2.4 with 3 g/l sodium bicarbonate, 0.2 g/l bovine salt, 1 g/l glucose, 250 µg/l folic acid, 1 mg/l 4 amino benzoic acid, 500 µg/l calcium pentothenate and 8.75 mg/l
ascorbic acid. To prevent overgrowth of cell lines, 1% of FCS was used in maintenance medium instead of 10% FCS for routine cell passaging. The pre-treated oocysts were then ready to be applied to cell lines or cell-free culture for infection studies.

2.1.6. Infection

HCT-8 cells were split into 24 well plates (1 cm² per well) 24 hrs prior to an infection of cells with *C. parvum*. The HCT-8 cells of a confluent 75 cm² flask were trypsinised and resuspended in 20 ml of RPMI medium and 150 µl of the resuspended HCT-8 cells were applied to each well and the well then topped up to a total of 1 ml RPMI medium. Cells were allowed 24 hrs before an infection in RPMI medium in order to form a monolayer. It was important that the monolayer was only 80% confluent, still showing a few gaps between cells, so that the cells did not overgrow during the first days of the study.

For an infection, the RPMI medium was aspirated and cells were washed with PBS. Cells were infected with 15,000 *C. parvum* pre-treated oocysts per cm² in 1 ml maintenance medium per well. Incubation was done at 37°C and 5% CO₂.

To examine infected cell monolayers with electron microscopy, cells were grown on thermoax coverslips of 13 mm diameter that had been placed on the bottom of each well. For each infected well a non-infected well was included to serve as a negative control. To observe cells over a course of 5 days or more, culture plates were incubated in a candle jar under depletion of oxygen.
2.1.7. Cell-Free Culture

To monitor cell-free development of *C. parvum*, pre-treated oocysts were cultured in maintenance medium in 24 well plates that did not contain HCT-8 cells, with 15,000 to 20,000 oocysts per well, each containing 1 ml of media. Excysted oocysts were inoculated into maintenance medium with 500,000 oocysts per 20 ml. To obtain high resolution images, samples were taken and centrifuged at 3500 rpm after 4 days.

2.1.8. Light Microscopy

Cell culture and cell-free cultures were observed alive in culture plates directly, without modification. Images were viewed using a Nomarski phase-contrast microscope and digitally photographed at 200x and 400x magnification. Some samples of cell-free cultures were concentrated by centrifugation and placed onto coverslips and viewed at 1000x magnification.

2.2. Electron Microscopy

All work was performed on conventionally fixed samples. High pressure freezing could not be used due to the infective nature of *Cryptosporidium* and the unwillingness of the CMCA to allow viable infectious parasites to be brought into the common access preparatory laboratory.

2.2.1. Fixation for Scanning (SEM) & Transmission (TEM) Electron Microscopy

To monitor the infection process of *C. parvum*, infected and uninfected cells were fixed at time points of interest. The medium was aspirated and the
coverslips were washed in 1 x PBS buffer, taken out of the well plates with forceps and placed into 2.5% glutaraldehyde in 1 x PBS. 

To monitor *C. parvum* stages in the supernatant of infected cells or cell-free culture, 1 ml medium of incubated cells or of cell-free culture was aspirated from the well and added to 1 ml of 5% glutaraldehyde in 1 x PBS to achieve a total concentration of 2.5% glutaraldehyde. 

Coverslips and supernatant could be fixed up to a maximum of 5 days only before the cells started to overgrow. Fixed samples were stored refrigerated until further processing.

### 2.2.2. Microwave Processing for SEM & TEM

For faster preparation samples were processed in a Pelco Biowave Microwave Processor. 

Supernatant and cell-free samples were centrifuged and concentrated samples were placed onto poly-l-lysine coated glass coverslips. The coverslips were left for 15 min at room temperature (RT) to allow contents of the supernatant or cell-free culture to adhere to the surface. After the incubation period the coverslips were rinsed with 1 x PBS and placed into plastic petri dishes containing 1 x PBS buffer for microwave processing. 

Coverslips containing infected HCT-8 cells were taken out of the fixative and placed into plastic petri dishes containing 1 x PBS buffer. 

The first microwave processing step was a 40 sec PBS buffer wash with 250 Watts and no vacuum. The PBS was removed and samples were fixed in 1% *OSO₄* in PBS at 80 Watts under vacuum in a 2 min on, 2 min off, 2 min on cycle. After the second fixation, the samples were stepwise dehydrated with an
increasing series of ethanol for 40 sec at 250 Watts, with no vacuum. The stepwise dehydration was done with an initial concentration of 50% ethanol followed by 70% ethanol, 95% ethanol, and three times 100% ethanol. Samples were kept in 100% ethanol until further processing.

2.2.3. Critical Point Drying for SEM

For SEM studies dehydrated samples were then transferred into a critical point dryer. The boat containing the samples was filled with 100% ethanol as a substitution solvent and then placed in the apparatus. The chamber was flushed for 1 – 2 min with liquid CO₂, with its level always remaining above the samples. The samples were then incubated for 15 min at RT at approximately 400 pounds per square inch (psi). Subsequently, a second flushing step was undertaken. Again the samples were incubated for 45 min. After a third flushing, the samples were dried by slowly raising the temperature above 32°C and the pressure above 1200 psi. Once the critical temperature and pressure was reached and all liquid was removed from the chamber, the drying process was complete and the chamber was vented slowly to remove the samples.

2.2.4. Coating of SEM Samples

Coverslips were mounted on pin type SEM mounts with carbon tabs and coated with 3-4 nm platinum for high resolution SEM imaging.

2.2.5. Resin Infiltration for TEM

For TEM studies, dehydrated samples were transferred into 100% acetone and microwave processed for 40 sec at 250 Watts, with no vacuum. Resin infiltration
was carried out with increasing concentrations of Spurr’s Resin (vinyl cyclohexene dioxide 10 g, diglycidyl ether of polypropylene glycol 6 g, nonenyl succinic anhydride 26 g, dimethyl amino ethanol 0.4 g) in 100% acetone. Samples were initially infiltrated with a ratio of 3:1 of acetone : resin, then 1:1 acetone to resin, then 1:3 acetone : resin and finally with 100% resin, using microwave processing at 250 Watts, and no vacuum for 3 min for each infiltration step. After the final infiltration step, samples were transferred into fresh 100% resin and polymerised over night at 70°C. Before microtoming, the thermanox coverslips were removed and samples re-embedded.

2.2.6. Microtoming and Staining of TEM Samples

Resin embedded samples were cut with a Leica Microtome to a thickness of 80-100 nm (silver-gold sections) using a glass and/or diamond knife. Sections were mounted on 100 mesh copper grids and dried overnight.

2.2.7. Image Capturing for SEM

Images were taken with a Supra variable pressure SEM (VPSEM ZEISS 1555FEG). A 30.00 µm aperture was used with an electron beam of 3 kv, a 4 mm working distance and an in lens detector.

2.2.8. Image Capturing for TEM

Sections were viewed unstained at 120 kV using a transmission electron microscope (TEM JEOL 2100). Images were digitally acquired on a Gatan Orius 1000 digital camera.
2.3. Immunogold SEM

2.3.1. Antibodies

Antibodies were obtained through Abacus ALS (USA).

The polyclonal primary antibody used was an unconjugated *C. parvum* rat IgG antibody (Waterborne Inc.) This antibody was prepared against purified sporozoites (approximately 95% purity with 5% contamination by oocysts) of bovine source *C. parvum*. The epitope reactivity of this antibody is known to be broad with labeling of sporozoites, oocysts, schizonts and meronts observed (Henry Stibbs, Waterborne Inc., pers. comm.). We have deliberately taken advantage of the broad reactivity of this polyclonal antibody, utilising it to identify a wide range of *Cryptosporidium* life cycle stages within infected cultures. A secondary antibody (Jackson 10 nm Colloidal Gold-AffiniPure Goat Anti-Rat IgG) conjugated to 10 nm colloidal gold particles was used for the visualisation by field emission SEM.

2.3.2. The Protocol for Immunogold Labeling of Inoculated Parasites

A protocol was established following previous studies utilising gold-conjugated antibodies for electron microscopy (Matsubayashi *et al.*, 2008; Biggs *et al.*, 2008; Muscariello *et al.*, 2008, O’Hara *et al.*, 2007; Baharloo *et al.*, 2005; Yu 1998; Yu and Lee 1996; Yu and Chai 1995). The final protocol is detailed below.

2.3.2.1. Cell Culture

Experiments were performed in 24 well plates on thermanox coverslips. Monolayers of HCT-8 cells were infected at 80% confluence with 15,000
oocysts per well. One uninfected well was included in each experiment as one of two negative controls. Infected plates were incubated at 37˚C in a candle jar for five days.

2.3.2.2. Antibody Labeling of Infected HCT-8 Cultures

The primary antibody was obtained in a dilution of 1mg/ml. Five day old Cryptosporidium cultures were labeled alive with the primary antibody. As a second negative control one of the infected wells was not incubated with the first antibody. Supernatants were aspirated and wells were washed 5 times with 50mM glycine/PBS (pH 7.2) for 5 min. Cells were then blocked with 5% FCS/PBS (pH 7.2) for 30 min. at 37˚C. The primary antibody was diluted 1:20 in 5% FCS/PBS (pH 7.2) and applied to infected test wells and to the first negative control (uninfected well). One infected well was not incubated with the primary antibody to serve as an additional negative control. Wells were incubated for 2 hrs at 37˚C. After primary antibody incubation, cells were washed 5 times with 50mM glycine/PBS (pH 7.2) for 5 min. Wells were then blocked with 5% FCS/PBS (pH 7.2) for 30 min. at 37˚C. The second antibody was applied to every well in a dilution of 1:20 in 5% FCS/PBS (pH 7.2). Wells were incubated for 1 hr at 37˚C. Before the final fixation cells were rinsed several times with PBS (pH 7.2).

2.3.2.3. Antibody Labeling of Oocyst Stock

A modification of the protocol described above was used to label oocyst stock in a same fashion.
The primary antibody was directly added to oocyst stock to achieve a dilution of 1:20. The antibody was incubated with oocysts for 2 hrs at 37˚C. PBS (pH 7.2) was added for washing. The sample was then spun down at 3500 rpm for 8 min. The supernatant was aspirated and the pellet resuspended in PBS. This washing step was repeated three times. After the last centrifugation and aspiration step the pellet was resuspended in PBS and the secondary antibody added at a dilution of 1:20. The antibody was incubated for 1 hr at 37˚C. The same washing procedure as for the primary antibody was applied. Finally, labeled oocysts were resuspended in a few drops of PBS to be fixed with 3% paraformaldehyde/ 2% glutaraldehyde and stored refrigerated until sample processing.

Sample processing for SEM was performed in the same fashion as for infected cells. After samples were centrifuged and concentrated, samples were placed onto poly-l-lysine coated glass coverslips.

2.3.2.4. Antibody Labeling of Cell-Free Cultures

A cell-free culture was set up as described in section 2.1.7 and incubated over 5 days. At day 5 the culture was centrifuged and the pellet was resuspended in a few drops of medium. Cells were labeled with the primary and secondary antibody and consequently fixed and processed as described for the oocyst stock.

2.3.2.5. Fixation

Cells were fixed with 3% paraformaldehyde + 2% glutaraldehyde and stored refrigerated until sample processing.
2.3.2.6. Sample Processing for SEM

Samples were processed according to the protocol described in section 4.2.2. to 4.2.4.

2.3.2.7. Image Capturing for SEM

Images were taken with a Supra variable pressure SEM (VPSEM ZEISS 1555FEG). A 30.00 µm standard aperture was used with an electron beam of 10 kV and a 9 mm working distance. Structural images were acquired with both the in lens and SE2 detector. Immunogold labeling was detected with a backscattered electron detector (BSD). A combination of 77% BSD and 23% in lens signals was used to achieve co-visualisation of cellular (parasite and host) morphology and the immunogold label.

No image manipulation other than grey levels was performed.
3. Results

“Light Microscopy on Cell and Cell-free Cultures of C. parvum”

3.1. Introduction

In vitro cultivation of C. parvum has proven to be a useful tool for the examination of the parasite (Flanigan et al., 1991b; Hijjawi et al., 2001). It does not only represent a rapid and economical method but also minimises the use of laboratory animals. Cell and cell-free cultures are available for the short term maintenance of Cryptosporidium (Yu et al., 2000; Siripanth et al., 2004; Hijjawi et al., 2004). Unfortunately long term cultures have not yet been developed to immortalise the parasite in vitro. Until recently, the only way to maintain and multiply C. parvum in laboratories is by passage through laboratory animals (Meloni and Thompson 1996).

Numerous epithelial cell lines, as well as primary cell cultures, are used worldwide to examine the parasite in vitro (Flanigan et al., 2001; Hijjawi et al., 2001; Huang et al., 2004; Hashim et al., 2006). A recent development in in vitro cultivation of C. parvum is the utilisation of cell-free media as the only growth support for the parasite (Hijjawi et al., 2004). Evidence exists that the parasite may be able to complete its life-cycle in the absence of host cells (Hijjawi et al., 2004; Boxell et al., 2008). However, attempts to replicate these experiments in other laboratories have failed (Girouard et al., 2006). It is well known that Cryptosporidium can propagate and replicate in culture for a limited time (Zhang et al., 2009), yet it remains speculative whether Cryptosporidium might be able to complete its life cycle other than in the presence of host cells. To date, in
vitro observation of Cryptosporidium species in laboratories worldwide is based on host cell cultures.

HCT-8 is a human colon carcinoma cell line of epithelial cells (Barbat et al., 1998). The HCT-8 cell line had been shown to optimally support the growth of C. parvum (Yu et al., 2000; Sifuentes et al., 2007). However, studies by Yu et al., (2000) showed AGS cells to be the best suited for the cultivation of the parasite. In our own experiments HCT-8 cells supported the growth of C. parvum better than AGS cells. Therefore HCT-8 cells were chosen for the in vitro observation of C. parvum in this study.

In this study Cryptosporidium was cultured in the respective cell line under standard conditions (incubator) as well as under oxygen depletion. C. parvum development, as well as the condition of the parasite and its host cell line, were observed over consequent days and compared to negative controls, which consisted of uninfected cells. Cell-free cultures were observed and compared to cell cultures to determine whether the cell free method is useful for further studies on the morphology of Cryptosporidium.

3.2. Aims

Light microscopic examination of the parasite in its host cell line was chosen initially to determine whether in vitro culture of C. parvum in HCT-8 and/or cell-free culture were suitable methods for further morphological studies. Further, optimal culture conditions and culture durations for later experiments were determined with this experiment.
3.3. Methods

Cell and cell-free cultures were prepared as detailed in sections 2.1.4 to 2.1.7 of Chapter 2. Monolayers were infected at approximately 80% confluence. Cells were grown in well plates without coverslips to enable light microscopic observation. Identical plates containing infected and non-infected wells were incubated in a standard incubator or under oxygen depletion, to compare both conditions. Light microscopy was performed as outlined in section 2.1.8.

3.4. Results

Infection of host cells did not occur ubiquitously throughout the culture plate, but distinct areas of infection were apparent within monolayers. Images shown here were taken from heavily infected areas.

After 24 hrs incubation, large gaps between HCT-8 cells were visible in infected oxygen depleted and standard incubated cells (Figs. 1a and c), as well as non-infected negative controls (Figs. 1b and d). Negative controls showed that after 24 hrs of incubation the cell line itself was in good condition (Figs. 1b and d). Small trophozoites of *C. parvum* appeared as shiny dots on the surface of their host cells (Figs. 1a and c, arrows), being approximately 1-2 µm in diameter. The numbers of trophozoites was surprisingly higher in oxygen depleted cells than in standard incubated cells. In the gaps between cells sporozoites were visible (Figs. 1c and d indicated by arrowheads and chevrons), measuring approximately 5 µm in length. The infected cells themselves still looked as healthy as their negative controls.
Fig. 1: a) Light micrograph of an infected monolayer of HCT-8 cells after 24 hrs incubation under oxygen depletion; b) Light micrograph of a non-infected monolayer after 24 hrs incubation under oxygen depletion; c) Light micrograph of an infected monolayer after 24 hrs standard incubation; d) Light micrograph of a non-infected monolayer after 24 hrs standard incubation; e) Magnification of c). Arrows indicate *C. parvum* trophozoites. Arrowheads show sporozoites easily visible between cells. Chevrons indicate sporozoites on cells.
After 48 hrs of incubation only a few small gaps remained between cells incubated under oxygen depletion and no gaps remained in between cells incubated under standard conditions. Uninfected cells incubated under oxygen depletion still looked healthy (Fig. 2b), whereas uninfected cells under standard conditions already showed the first signs of overgrowth (see right margin of Fig. 2f). Infected monolayers incubated under oxygen depletion remained in good condition, enabling easy monitoring of *C. parvum* stages. Trophozoites on the surface of host cells were considerably larger (Fig. 2c, arrow). Dependent on the plane of focus, intracellular nuclei could be seen as indicated by arrows in Figs. 2a and d. Firstly evident after 48 hrs, remainders of excysted oocysts were seen adherent to the host cell surface, as indicated by arrowheads in Figs. 2a and d. They could be identified by their shape and diameter of approximately 8 µm. The infected monolayer under standard incubation showed signs of host cell death and overgrowth (Fig. 2e), which made it difficult to examine the monolayer and visualize trophozoites which are indicated by arrows in Figs. 2a, c, d and e.
Fig. 2: a) Light micrograph of an infected monolayer of HCT-8 cells after 48 hrs incubation under oxygen depletion; b) Light micrograph of a non-infected monolayer after 48 hrs incubation under oxygen depletion; c) Light micrograph of an infected monolayer after 48 hrs incubation under oxygen depletion; d) magnification of c); e) Light micrograph of an infected monolayer after 48 hrs standard incubation; f) Light micrograph of a non-infected monolayer after 48 hrs standard incubation. Arrows indicate *C. parvum* trophozoites. Arrowheads indicate excysted oocyst shells.
From 72 hrs after inoculation signs of overgrowth could be seen clearly in negative controls of standard incubated samples (Fig. 3e, arrowhead). Standard incubated infected monolayers showed signs of host cell death, as well as overgrowth, so that the infected monolayer incubated under standard conditions had become impossible to monitor (Fig. 3d). In contrast, in infected monolayers incubated under oxygen depletion stages of *Cryptosporidium* could still be easily distinguished as indicated by arrows in Figs. 3a and b. However, these infected monolayers already showed signs of host cell death (Fig. 3a) in contrast to their negative controls (Fig. 3c). Cellular debris is indicated in Fig. 3a by an arrowhead.
Fig. 3: a) Magnification of b); b) Light micrograph of an infected monolayer of HCT-8 cells after 72 hrs incubation under oxygen depletion; c) Light micrograph of a non-infected monolayer of HCT-8 cells after 72 hrs incubation under oxygen depletion; d) Light micrograph of an infected monolayer of HCT-8 cells after 72 hrs standard incubation; e) Light micrograph of a non-infected monolayer of HCT-8 cells after 72 hrs standard incubation. Arrows indicate *C. parvum* stages. Arrowheads indicate overgrowth of HCT-8 cells.
From day 4 onwards only samples incubated under oxygen depletion were able to be monitored. Uninfected cells showed overgrowth but no host cell death (Fig. 4b). In comparison, cells of infected monolayers started to die as indicated by an arrow in Fig. 4a, though overall the monolayer still appeared to be in a condition suitable to support parasite growth and *C. parvum* stages could be monitored. Host cell death was visible especially in areas of parasite infection, compared with uninfected areas of the same monolayer. As well as the trophozoites, indicated in Fig. 3, larger stages indicated by arrowheads in Figs. 4c and d were seen after 4 days of culture. The stages measured about 8 μm in length, possessing the size of a mature stage such as an oocyst. Also, extracellular stages were detected in the supernatant above infected cells (Figs. 4e and f). The stage depicted in Fig. 4e is most likely to be an extracellular merozoite or possibly an extracellular trophozoite, as it appeared round in shape, with a diameter of approximately 2-4 μm. The stage seen in Fig. 4f is approximately 10 x 20 μm in size and resembles an extracellular merozoite accumulation previously detected in cell-free culture by Hijjawi *et al.*, (2004). This extracellular meront could be distinguished as a *Cryptosporidium* stage, as it was surrounded by a cell wall, which appeared as a halo surrounding the parasite stage under the light microscope.
Fig. 4: a) Light micrograph of an infected monolayer of HCT-8 cells after 96 hrs incubation under oxygen depletion; b) Light micrograph of a non-infected monolayer after 96 hrs incubation under oxygen depletion; c, d) Light micrographs of an infected monolayer after 96 hrs incubation under oxygen depletion; e) Light micrograph of *C. parvum* extracellular stage in supernatant; f) Light micrograph of an accumulation of extracellular stages in supernatant. The arrow indicates host cell death. Arrowheads show large *C. parvum* stages. Chevrons point to *C. parvum* stages in the supernatant.
After 5 days the monolayer was heavily overgrown (Fig. 5b) making it very difficult to monitor parasite stages (Fig. 5a). As the amount of infection had considerably increased throughout the five days, *C. parvum* stages that had not been seen earlier were observed. Figs. 5c and d show the formation of a trophozoite-like stage from a motile zoite as indicated by arrows. In Figs. 5e to 5h sporozoite invasion into host cells can be seen. Extracellular meronts that had already been described in 4 day old cultures were seen again in 5 day old cultures (Figs. 5i to k).

6 days after inoculation, extensive overgrowth (Fig. 6f, arrowhead) and host cell death (indicated by chevrons in Figs. 6b and d) of monolayers made it impossible to further monitor the parasite in culture. As the amount of host cell death had increased, the abundance of *Cryptosporidium* stages in the monolayer appeared to have diminished. The few trophozoites that could still be seen (Figs. 6a, c and e indicated by arrows) appeared small in size, compared to those described after 24 hrs of incubation.

At day 7 post inoculation small remainders of *Cryptosporidium* stages could be seen in infected monolayers (Fig. 7a). However it is unlikely that they were viable anymore, because at day 8 (Fig. 7c) no *Cryptosporidium* stages could be identified. Both negative controls were severely overgrown (Fig. 7b and d).
Fig. 5: a) Light micrograph of an infected monolayer of HCT-8 cells after 5 days incubation under oxygen depletion; b) Light micrograph of a non-infected monolayer after 5 days incubation under oxygen depletion; c, d, e, f, g, h) Light micrographs of *C. parvum* stages in a 5 day infected monolayer; I, j, k) Light micrographs of *C. parvum* extracellular stages in the supernatant of a 5 day infected monolayer. Arrows on pictures c) and d) show the formation of a trophozoite from a motile zoite stage in order. Arrowheads on pictures e) to h) show a sporozoite in the process of host cell invasion.
Fig. 6: a, b, c, d, e) Light micrographs of infected monolayers of HCT-8 cells after 6 days incubation under oxygen depletion; f) Light micrograph of a non-infected monolayer after 6 days incubation under oxygen depletion. Arrows indicate remaining *C. parvum* stages. Chevrons show cellular debris. The arrowhead points to overgrowth of HCT-8 cells.
Fig. 7: a) Light micrograph of an infected monolayer of HCT-8 cells after 7 days incubation under oxygen depletion; b) Light micrograph of a non-infected monolayer after 7 days incubation under oxygen depletion; c) Light micrograph of an infected monolayer of HCT-8 cells after 8 days incubation under oxygen depletion; d) Light micrograph of a non-infected monolayer after 8 days incubation under oxygen depletion. The arrow indicates a rudimentary C. parvum stage. Chevrons show cellular debris. Arrowheads points onto overgrowth of HCT-8 cells.
The cell-free culture was observed daily. Here, images acquired after 4 days of culture are presented. Free sporozoites could be seen in culture at every time point (Fig. 8a), measuring 5 μm in length, as described for sporozoites seen in cell culture in Fig. 1. Oval stages were frequently seen alone or accumulated in culture (Fig. 8b), which are likely to be oocysts from inoculation. But, with the low magnification of light microscopy, their morphology could not be further examined. Smaller round *C. parvum* stages (Figs. 8c to f), resembling merozoites or extracellular trophozoites were also present. The round structure depicted in Fig. 8f consisted of one round stage measuring approximately 2 μm diameter and a stage of approximately 1 μm (on its right side) which was in constant contact with the larger stage, gliding and flexing around it. Further, stages were detected that were not seen in cell culture (Figs. 8g to l). Some were elliptical in shape measuring between 2 x 4 μm (Figs. 8g and h) and 2 x 4 μm (Figs. 8i and j). In Figs. 8i and j, nuclei were visible within the parasites. Other stages were oval in shape measuring approximately 6 x 8 μm. The last novel stage shown in Fig. 8l possessed vigorous motility, swimming rapidly through the media. As in the supernatant above cells (Figs. 4f and 5i-k) extracellular meronts occurred in cell-free media (Fig. 8m). Similar to Fig. 4f, a halo was seen surrounding the merozoite accumulation, distinguishing this life cycle stage from debris or unevenness in the culture flasks themselves (Fig. 8n). A culture flask containing maintenance medium only, was included to serve as a negative control. Fig. 8n shows debris in the culture flask itself which can be clearly distinguished from *C. parvum* stages. Nothing but this debris, which is most likely pollutions in the plastic of the culture flask, was detected in any negative control observed.
As well as observing cell-free cultures directly within the culture plate, samples of 5 day old cell-free cultures were centrifuged and viewed under the light microscope at 1000x magnification under oil immersion. At this higher magnification intracellular organelles of the parasite could be distinguished. In Fig. 9a several *C. parvum* stages were visible at once. The plane of focus determined how many organelles were visible in the parasite. Also the size and shape of these organelles and the parasite itself differed, depending upon the plane of focus. For the parasite indicated with number 2 in Fig. 9a for example, one or three nuclei were visible dependent on the plane of focus. Thus, the focus was adjusted for all images to optimally visualise intracellular organelles. Parasites indicated with numbers 1, 2 and 3 appeared similar in size and shape as oocysts from inoculation (Fig. 9e). These stages (Fig. 9a [3]) may represent the transition into novel gamont like stages (Fig. 9a [4 and 5]. Parasites were observed possessing a central nucleus with other internal structures visible around the centre (Fig. 9b). Again it is possible that this stage is an oocyst left from the initial inoculation, but it may also be a newly forming oocyst-like stage. As already suggested for Fig. 9a, this stage might develop into novel gamont-like stages like those seen in Fig. 9 c and d. The parasite stage in Fig. 9d is similar to the one described in Fig. 8l, whereas parasites like the one in Fig. 9c appear similar to the ones described in Fig. 8j. Finally, accumulation of extracellular small stages was seen next to an excysted oocyst in Fig. 9e. The individual stages were round in shape and therefore could represent gametocytes or merozoites, which cannot be distinguished at this magnification.
Fig. 8: Light micrographs of a cell-free culture of *C. parvum* photographed within the culture plate after 4 days. a) Sporozoite; b) Accumulation of oocysts; c, d, e) Accumulations of merozoites or trophozoite-like stages; f) Motile *C. parvum* stages; g, h, i, j, k, l) Motile novel gamont-like stages; m) *C. parvum* formation; n) Negative control: Debris in culture plate.
Fig. 9: Light micrographs of *C. parvum* stages in cell-free culture, centrifuged after 5 days. a) *C. parvum* stages (1, 2, 3 multinucleated stages; 4, 5 novel gamont-like stages); b) Multinucleated *C. parvum* stage; c, d) Novel gamont-like stages; e) Excysted oocyst from inoculation next to a number of accumulated extracellular merozoites.
Table 1: Summary of findings over the 8 day period of cell culture. S: sample, D: day

### 3.5. Discussion

*In vitro* cultivation of *C. parvum* in the HCT-8 cell line was found to be suitable for this study, as *Cryptosporidium* proliferates in culture without interference for a sufficient number of days to monitor its life-cycle (Hijjawi *et al.*, 2001 and 2004). The parasite can easily be detected on the surface of its host cell. Light microscopy enables the monitoring of living stages. It provides the opportunity to examine the same sample progressively and monitor the effect the parasite exerts on host tissue and the host-parasite interaction. The utilisation of *in vitro*
culture in this study also satisfies ethical concerns to minimise the usage of laboratory animals.

However, light microscopy, as a method to examine *C. parvum in vitro* has its limitations. The first limitation is the magnification. A maximal magnification of 1000x was only possible under oil immersion, for which cell-free culture has to be centrifuged. Without disturbing the culture, 400x was the magnification limit. The magnification of an image has an impact on the accuracy of the scale bar. The lower the magnification, the higher the errors in scale bars will be relatively. Thus, a magnification of 400x does not allow to determine the size of the parasite as accurately as a higher magnification.

The second limitation is the limited depth of focus. Depending upon the plane of focus (compare Figs. 6c to 6d and Figs. 2a to 2c), the size and shape of the same structure could appear different. Thus, parasite stages photographed under the light microscope can be mistaken for stages, other than they actually are (see Fig. 9a). Conclusively light microscopy is perhaps not the best method to examine parasite stages in detail *in vitro* culture as other, higher resolution microscopy methods (for example electron microscopy) are available.

To obtain reliable images a magnification of 1000x is required. Images at 1000x magnification can only be obtained under oil immersion. Unfortunately cell-free samples have to be centrifuged for this purpose. During the centrifugation process at 3500rpm, forces are applied that appear to force stages of *Cryptosporidium* to clump together that may otherwise have been separate. *Cryptosporidium* accumulations like the one see in Fig. 9e were observed at 1 or 2 days of cell-free culture after the sample was centrifuged. In previous studies they were described to develop in cell-free culture after 4-5 days (Hijjawi...
et al., 2004). In addition, large accumulations of distinct *C. parvum* extracellular stages (see Fig. 9e) were only observed in centrifuged samples after 5 days of culture but not in non-centrifuged ones (compare to Fig. 8b-e). These two facts demonstrate that it is preferable to observe the parasite without the aid of sample centrifugation where it can be avoided. Consequently, cell-free culture is less suitable for microscopic observation than cell culture, as it requires centrifugation to facilitate the focusing on single parasite stages.

Oocysts do not all excyst at once but excystation occurs within the first 2-3 days. Thus, it comes as no surprise that a large number of sporozoites could be seen 24 hrs after incubation. Sporozoites similar to the ones observed in between cells (Fig. 1e) have been observed on host cells in studies by Langer *et al.*, (1999) with time-lapse video photomicroscopy, verifying the results presented. Sporozoites have been found to excyst as early as 5 minutes post inoculation (Forney *et al.*, 1999). From this, it is also no surprise that small trophozoites had formed after 24 hrs of culture.

The amount of trophozoites appeared higher in oxygen depleted cells than in standard incubated cells after 24 hrs of incubation. As oxygen depletion slows the growth of HCT-8 cells, this possibly leaves more substrate available to support the growth of *Cryptosporidium*. *Cryptosporidium* uses aerobic as well as anaerobic metabolism pathways (Barta and Thompson 2006), living in the gastrointestinal tract of its hosts. Therefore it can be expected to flourish in a low oxygen environment. Thus, oxygen supply mainly aids the growth of host cells. An environment low in oxygen is preferable for *in vitro* culture of *C.
parvum (personal observation). For further experiments, *in vitro* culture of *C. parvum* was performed in a candle jar to decrease the growth rate of the host cell line and with that reduce overgrowth and host cell death.

Because *Cryptosporidium* also utilises nutrients from the media, after 4 days of culture, infected monolayers had disintegrated, whereas uninfected cells did not show signs of host cell death. As *Cryptosporidium* infects cells, it causes host cell death, so that at any day more cellular debris can be monitored in an infected monolayer than its negative control. The nature of the cell line death induced by *C. parvum* is explained in the next chapter of this thesis.

Extracellular stages of *C. parvum* were seen in the supernatant above cells that had been infected with the parasite after 4 days of incubation (Figs. 4e and f). Merozoites might develop into trophozoites extracellularly. This would explain the stage in Fig. 4e which appeared in size and shape closest to that of a trophozoite. Further, it is thought that merozoites accumulate in cell-free culture to form extracellular meronts (Hijjawi *et al.*, 2004). The present study shows that this phenomenon described by Hijjawi *et al.*, (2004) in cell-free culture also occurs *in vitro* in the supernatant above cells (see Fig. 4f). It can be suggested that even *in vivo* such extracellular stages may form, but further studies would be needed to test this hypothesis. The isolation of respective parasite stages and inoculation into fresh media or cell cultures would be essential to monitor their progress and answer the question whether they fulfil an active function in the life cycle of *C. parvum*. 
The large intracellular stages seen in Figs. 4c and d might be mature stages of *C. parvum* that have evolved from fertilised macrogamonts within the 4 days of incubation. Yet, the nature of such stages can not be determined by light microscopy alone. Stages of *C. parvum* might occur in such a huge variety of different shapes and forms that not all stages and all events of the *Cryptosporidium* life cycle have been described to date.

That the amount of infection increased with progressive days, shows that *Cryptosporidium* replicates in culture, presumably via both, asexual and sexual life cycle (O'Donoghue, 1995). The fact that the amount of infection was considerably higher after 5 days, might account for parasite stages being observed that have not been seen before. The observation of sporozoite host cell invasion after 5 days suggests that zygotes were formed, fertilised and matured to release sporozoites after 5 days of *in vitro* culture. This observation stands in contrast to studies by Hijjawi et al., (2001 and 2004) which reported the completion of *C. parvum*’s life cycle after 7 days. However, it is unlikely that the sporozoites observed to invade host cells at day 5 of culture, had remained from the initial inoculant. Thus, it can be suggested that the life cycle of *C. parvum* might complete earlier than 7 days. This hypothesis will be backed up and further explained in Chapter 5 of the present thesis.

That the amount of *Cryptosporidium* stages in the monolayer appeared to have diminished after 6 days showed, that *C. parvum* did not continue its life cycle beyond the stage of gamont formation after 5 days of culture. It appeared to fail to reinfect the same “old” cell line. This might be due to the effect that *C. parvum* had exerted upon the host cells, as well as to the depletion of nutrients of the media. As a few trophozoites (the size of the small trophozoites
described at the initial infection 24 hrs post inoculation) were seen in culture at 6 days, some re-infection might have occurred but not succeeded any further. The small trophozoites seen may have resulted from repeated merogony. At 7 days post inoculation, only small remainders of \textit{C. parvum} stages were visible, whereas at 8 days any signs of \textit{C. parvum} had disappeared. The disappearance of \textit{C. parvum} after day 5 of culture might not be due to the state of the host cell line. Studies by Sifuentes \textit{et al.}, (2007) have shown that previously uninfected, but several week old HCT-8 monolayers still support the growth of \textit{Cryptosporidium}. Therefore it is more likely that the depletion of nutrients from the media has caused the parasite to die. It would not have been useful however, to add additional nutrients into the media as the overgrowth of host cells and cellular debris in the culture media made a monitoring of the parasite impossible after 6 days (Fig. 6). On the other hand, by replacing the old media with fresh culture media, extracellular infective parasite stages would have been eliminated, and thus not present to reinfect cells and give a complete picture of the \textit{C. parvum} life cycle.

Cell-free culture is not as easy to monitor as cell culture. Samples have to be centrifuged to view a variety of \textit{Cryptosporidium} stages at once. Without centrifuging samples, it is difficult to find parasite stages and near impossible to focus. Motile stages are particularly difficult to capture. Stages seen in cell-free sample appeared different to those described in cell culture. Thus, cell-free culture does not represent an \textit{in vivo} infection as good as \textit{in vitro} culture. Sporozoites were probably seen in cell-free culture after 4 days, because oocyst excystation occurs over several days, or they excysted earlier but had
not yet transformed into another stage. The formation of new sporozoites would require the completion of *C. parvum*’s life cycle after less than 4 days. This option is less likely, yet possible as explained in Chapter 5. Smaller, round *C. parvum* stages (Fig. 8c to f) might resemble merozoites or extracellular trophozoites. As already explained for round extracellular stages detected above host cells (Fig. 4), their nature remains speculative and would require magnifications on the EM level. The clumping of stages observed in Figs. 8b-e might occur via interactions of surface receptors which are present on *C. parvum* stages (Chen *et al.*, 2000) to facilitate attachment to host cells and possibly to each other. Further observations and discussions on surface receptor adherence will be presented in Chapter 5.

The motile stage observed in Fig. 8f could be a merozoite being released from a meront, presuming that meronts can form extracellularly in cell-free culture. However, light microscopy at 400x magnification does not allow characterisation of all *C. parvum* stages seen.

The rod shaped motile stages described in Figs. 8g-j seemed to possess single nuclei, but their size excludes them being merozoites as those described on host cells in Chapter 5. Nevertheless, *Cryptosporidium* stages in cell-free culture might look anything unlike their in vivo stages. Thus, these motile stages might represent merozoites or a new form of gamont, or a transitional stage forming a gamont. The novel stage shown in Fig. 8l moved surprisingly fast through the media. It would be interesting to find out if such large extracellular gamont-like stages also appear *in vivo* and what function they fulfil in the *Cryptosporidium* life cycle. Hijjawi *et al.*, (2002) isolated a similar looking stage of *C. andersoni* from mouse intestines. The respective stage of *C. andersoni*
was multinucleated, showing the same shape and size as the motile stage described in this study in Fig. 8l. Stages observed in cell-free culture might as well be present in cell culture and in vivo but remain undetected because of the host cell background. On the electron microscopic level such stages might be identified and observed closer.

It has been hypothesised that the accumulation of extracellular merozoites (Fig. 9e) leads to the formation of extracellular meronts (Hijjawi et al., 2004) similar to the ones in Fig. 8m. At 1000x magnification in Fig. 9e, individual merozoites appeared round and the entire accumulation showed no cell wall in contrast to the one in Fig. 8m. This raises the question, whether this so called meront is not just a random accumulation of equal stages due to centrifugation and non-specific receptor interactions.

Cell-free stages shown in Figs. 9a and b-d may show the sequential development of the parasite, with Fig. 9d showing the final gamont-like stage similar to the one seen in Fig. 8l. On the other hand, each of the stages seen in Fig. 9 might be a final stage on its own. Dependent upon the plane of focus, it might contain as many as 4 to 8 nuclei, representing a meront-like stage as those described in vitro (Hijjawi et al., 2001). Thus, stages suggested to be gamont-like stages might as well be meronts similar to those in vitro. As already mentioned earlier, the plane of focus determines the appearance of intracellular organelles and thus affects the interpretation of images. A similar problem would occur with TEM. As electron microscopy will be the method of choice for future studies, it will be desirable to employ SEM as well to obtain an overview
over the infection and a picture of the entire parasite to precisely determine its size and morphology.

3.6. Conclusions

Light microscopy is not sufficient on its own to microscopically examine *C. parvum*. Due to its magnification limit many stages cannot be characterised and/or are not identified in the first place. However, light microscopy finds its value in enabling the monitoring of live parasite stages and the progressive observation of the same culture. For future studies, both TEM and SEM will be employed to acquire information on the occurrence, morphology and host cell relationship of as many *C. parvum* stages as possible.
4. Results

“Morphology of the HCT-8 Host Cell Line”

4.1. Introduction

The HCT-8 cell line was chosen as the in vitro culture system for microscopic studies on the parasite Cryptosporidium parvum. This study served as a prerequisite for later studies, which examined C. parvum with scanning electron microscopy (SEM) in HCT-8 monolayers. Infected cells as well as the uninfected HCT-8 cell line itself were examined with SEM to distinguish between host cell line features and stages of the inoculated parasite C. parvum. To examine the effect that C. parvum exerts on its host tissue, the morphology of infected host cells was examined and compared to uninfected cells.

Like all other eukaryotic cells, HCT-8 cells multiply by mitotic division (Rosenthal et al., 1977). At the start of mitosis, the DNA of the parent cell is duplicated. Chromosome segregation splits the DNA into two equal halves and cytokinesis finally separates the parent cell into two daughter cells, each containing the same set of DNA. HCT-8 cell monolayers have been observed with phase contrast and transmission electron microscopy (TEM) in studies by Barbat et al., (1998). Post-confluent HCT-8 cells were found to be highly heterogeneous and undifferentiated with a minority exhibiting features of enterocyte-like cells, such as the expression of a microvilli brush border. In contrast to that, earlier studies by Rosenthal et al., (1977) demonstrated HCT-8 monolayers to be tightly packed with junctional complexes and a regular brush border. Karyotypic examinations of the HCT-8 cell line by Vermeulen et al., (1998) revealed that HCT-8 cells possess extra copies of chromosomes 8 and
13, whereas some cells lost the Y chromosome, or showed random structural rearrangements within the karyotype.

Apoptosis is the process of programmed cell death that occurs in all eukaryotes and as such the HCT-8 cell line. Apoptosis is essential to maintain homeostasis of cell numbers, removing old cells that are damaged beyond repair. Apoptosis was first described and differentiated from necrosis by Kerr (1965) and further described by Kerr et al., (1972). Apoptosis is the process of events leading to changes in the cellular morphology resulting in cell death. Apoptosis can be initiated by the cell itself, surrounding tissue or the immune system. Apoptosis can be triggered by a diverse range of extra- or intracellular signals. Extracellular signals can bring on or inhibit cell death. Such signals are: toxins (Fiorentini et al., 1998; Yamamoto et al., 2002), hormones (Fingrut and Flescher, 2002; Hong et al., 2008; Lucin et al., 2009), growth factors (Fu 2007), nitric oxide (NO) (Li et al., 2001; Krick et al., 2002; Du et al., 2006) and cytokines (Ensoli et al., 1999; Bose et al., 2007). Intracellular signals include nutrient deprivation (Hwang et al., 2008), glucocorticoid signalling (Horigome et al., 1997; Wang et al., 2006), viral infections (Lallemand et al., 2007; Madan et al., 2008), hypoxia (Azad et al., 2008) and elevated intracellular calcium (Mattson and Chan 2003). Apoptotic signals trigger the initiation of apoptotic pathways. They can either be tumor necrosis factor (TNF)-induced (Thomas et al., 1998; Ponnuraj et al., 2002) or Fas/FasL-mediated (Chen et al., 1999; O’Hara et al., 2007) leading to the activation of caspases (Ojcius et al., 1999) that initiate cell death. Morphological changes in the course of apoptosis include nuclear condensation and fragmentation followed by cell shrinkage. The cell membrane shows irregular buds known as “blebs” (Nusbaum et al., 2004).
Consequently the cell breaks apart into several vesicles called apoptotic bodies (Franek et al., 1992). These apoptotic bodies are round and contain the intracellular components of the disintegrated cell.

The inoculation of C. parvum will have an effect on the host cell line and might lead to increased cell death. There are two types of cell death. Apoptosis, has already been explained. The second type is necrosis. Necrosis, in contrast to apoptosis, has no benefit to an organism but is damaging or even fatal. Necrosis is the premature death of cells caused by external factors such as: infections (Peretti et al., 2000), toxins (Voth et al., 2005), trauma and inflammation (Fraser et al., 1967; Aronsky et al., 1997). Necrotic cells undergo different morphological changes than apoptotic cells, in which course it might release harmful chemicals (Ezepchuck et al., 1996). The cell swells, chromosomal digestion and DNA hydrolysis occur together with endoplasmatic reticulum vacuolation and organellar breakdown. Finally the membrane becomes disrupted and cell lysis releases intracellular components.

This chapter details morphological features of the HCT-8 cell line used here; such as cellular growths and death, as well as the effect that C. parvum exerts on cells. Infected and uninfected HCT-8 cells were cultured under the same conditions as for later studies, which aimed to examine the parasite. Monolayers of infected and uninfected cells were examined with SEM over a five day period.
4.2. Aims

This study was performed to reveal morphological features of the HCT-8 cell line that would serve as an in vitro culture system for the parasite C. parvum. It is important that one is able to identify host cell characteristics such as cellular growth and apoptosis, in order to accurately differentiate between host and parasite cells.

Further, the effect of C. parvum on host tissue was examined by comparing infected and uninfected cells.

4.3. Methods

Cells were infected and cultured as explained in sections 2.1.4 to 2.1.7 of the Materials and Methods chapter. Uninfected and infected cells HCT-8 cells were incubated under oxygen depletion, to prevent overgrowth, and to resemble negative controls of monolayers infected with Cryptosporidium for SEM studies over a 5 day period. Cultures were performed as explained in sections 2.1.4 and 2.1.6 of the Materials and Methods chapter.

4.4. Results

4.4.1. Cell Morphology of Uninfected HCT-8 Cells

To examine the host cell line that was chosen to culture C. parvum, uninfected cell monolayers were fixed over a period of five days, equivalent to a 5 day course of infection, and SEM images were taken.

An uninfected monolayer as a negative control for a 24 hrs post inoculation study for example, was 48 hrs old, as each cell culture was established 24 hrs
before infection. At this time point the monolayer was about 80 % confluent and still showed large gaps between cells (Fig. 10 b). HCT-8 cells were found to grow to a flat monolayer, with dense contact between single cells, once confluence was reached (Figs. 10a, 12a). Structures, other than flat cells were seen in non-infected cell monolayers (Figs. 10c and d). These were HCT-8 cells in the course of cellular growth (Fig. 10c) or apoptosis (Fig. 10d). Growth features of HCT-8 cells were mainly observed at places where the monolayer showed large gaps and disappeared where the monolayer was confluent. The formation of apoptotic bubbles (membrane blebs) in the course of HCT-8 cell apoptosis was commonly seen at any day of culture (i.e. Fig. 10d) and appeared to be distinct for the cell line and not associated with the parasite (see Chapter 5). As apoptotic cellular material detaches from the monolayer, cellular debris was commonly found in the supernatant of cultures (data not shown). In some cases, microvilli could still be identified on such debris. Apoptotic bubbles can be clearly distinguished in Fig. 15a. Host cell debris in the form of small round structures was commonly seen associated with host cell growth. Debris did not appear attached to the monolayer or the bottom of the culture plate, but floating away from the growing cell monolayer. This observation is particularly significant as such debris, as well as growing and apoptotic cells, must be carefully differentiated from C. parvum stages in later studies.

Host cell microvilli were not as well established at early stages of monolayer formation (Fig. 11b) than after the monolayer reached 100% confluence (Fig. 12a). Comparing Figs. 10c and 10d to for example Fig. 16b of a better established monolayer with distinguishable microvilli, it appears as if the
growing host cell (indicated by arrows labelled ‘g’) employs microvilli material to attach to substrate and spread out for growth.

Fig. 10: Scanning electron micrographs of a monolayer of HCT-8 cells at the same age as a 24hrs infected monolayer. a) Host cells grow to form a monolayer, b) however, the monolayer still shows large gaps. c, d) Features of cellular growth are abundant at this early stage as indicated by arrows labelled ‘g’. These are newly formed round cells that attach to the substrate and flatten out to establish the monolayer. Cellular apoptosis can already be monitored. Apoptotic bubbles of a host cell are indicated by an arrow labelled ‘a’.
As long as a monolayer was not confluent, features of growth and apoptosis could be seen frequently throughout the sample. At 72hrs (resembling a 48 hrs infected monolayer) monolayers had not yet reached full confluence. The contact between cells was not dense and gaps could be seen (Figs. 11a, b). Thus, cellular growth was ongoing throughout the whole sample (Fig. 11b). Apoptotic cell debris must also be carefully distinguished from *C. parvum* stages. Apoptotic cellular debris closely observed in Fig. 11c appeared perfectly round, not adherent and measuring between 0.5 μm - 1 μm in diameter. In addition, different forms of cellular debris were seen (Fig. 11c). They were spindle-shaped, wider in the centre with long spindle-like elongations at both ends, measuring up to more than 10 μm in length. Again, these host cell formations could be mistaken for parasite stages if they had not been observed previously.
Fig. 11: Scanning electron micrographs of a monolayer of HCT-8 cells at the same age as a 48hrs infected monolayer. a) The monolayer is near 100% confluence with only small gaps remaining between cells. b) Still, the monolayer is not fully established and cell growth (arrow ‘g’) can be seen throughout the whole culture. c) Apoptotic cell debris (arrows ‘d’) is found ubiquitously throughout the monolayer.
At 96 hrs (resembling a 72 hours infected monolayer), HCT-8 cells formed a flat and dense monolayer which was 100% confluent. The monolayer was better established at this time point and very little growth and apoptotic features were seen (Fig. 12a). HCT-8 cells expressed microvilli ubiquitously (Fig. 7b). Most cells were found to be densely covered with microvilli, whereas a few expressed none (Figs. 12a and b). Although less abundant, features of cellular growth were still seen (Fig. 12b). TEM images showed that cells had established themselves as a single monolayer at this point in time, forming dense contact with tight junctions between cells (Fig. 12c).

Growing host cells were observed to be initially round, measuring approximately 12 μm in diameter and already being covered with microvilli (Figs. 12b and 13b). Their size and the fact that they are covered with microvilli allowed them to be differentiated from parasite sages which are known to be smaller in size (Hijjawi et al., 2001).
Fig. 12a, b: Scanning electron micrographs of a monolayer of HCT-8 cells at the same age as a 72hrs infected monolayer. 
a) Cells are 100% confluent and form a flat monolayer. They are ubiquitously covered with microvilli apart from a few exceptions where cells express nearly none. 
b) Cellular growth becomes less obvious but still continues. Growing round HCT-8 cells are indicated by arrows.
Fig. 12c: Transmission electron micrograph of a monolayer of HCT-8 cells at the same age as a 72hrs infected monolayer. Cells have formed a single monolayer with dense contact and tight junctions between single cells; some of which are indicated by arrows.
5 day old monolayers were fully confluent and first features of overgrowth were seen (Fig. 13a). Cells started to grow on each other as no gaps were left. Such cells were visible as round cells on top of the flat monolayer (Fig. 13a). As cells started to overgrow each other, features of growth again become more abundant than in 4 day old monolayers. Dead cells (indicated in Fig. 13a) were also seen and detached from the monolayer (Fig. 13a). As they were not covered with microvilli, in contrast to newly formed HCT-8 cells (Fig. 13b), they could easily be differentiated. Once detached, dead cells were round measuring 10 μm or less in diameter (Fig. 13a); but as long as attachment to the monolayer could be seen their shape appeared different and the size was slightly larger (Fig. 13a).
Fig. 13: Scanning electron micrographs of a monolayer of HCT-8 cells at the same age as a 96hrs infected monolayer. a) HCT-8 cells are fully confluent and first features of overgrowth are visible (arrows labelled ‘g’). Dead cells detaching from the monolayer are indicated with ‘d’. b) Features of growth (‘g’) become more abundant. Newly formed round cells attach to and overgrow the existing monolayer.
Monolayers were observed to overgrow after 6 days of cell culture (Fig. 14). The image of a 6 day old monolayer shows the overgrowth in an HCT-8 monolayer (indicated in Fig. 14). At 6 days the monolayer still appears suitable to support parasite growth and microscopic monitoring. However, as previous experiments have already shown (see Chapter 3), overgrowth and host cell apoptosis make it difficult to monitor *C. parvum* in culture beyond this stage.

Fig. 14: Scanning electron micrograph of a monolayer of HCT-8 cells at the same age as a 5 days infected monolayer. Cells have overgrown each other to a degree that the overgrowth becomes visible ubiquitously throughout the monolayer (arrows).
Fig. 15a summarises morphological characteristics of the HCT-8 cell life cycle. The images show events from the formation of new cells via mitotic division, through to programmed cell death via apoptosis. As already described (Figs. 12b and 13b), new HCT-8 cells are formed by mitotic division (Fig. 15c). Two perfectly round daughter cells of exactly equal size are formed, both covered in microvilli. Fig. 15b shows an HCT-8 cell detaching from the monolayer and rounding up for mitotic division. Fig. 15c shows the mitotic division of a detached cell into two daughter cells. Fig. 15d shows how two such daughter cells attached to the HCT-8 monolayer lead to host cell overgrowth. Newly formed HCT-8 cells were found to be initially round (Fig. 15c) before they attach and spread out for growth to form a monolayer (Fig. 15d). Such cells were found to be 10 µm in size or even bigger and thus could be easily differentiated from life cycle stages of *C. parvum* which are smaller. Fig. 15a shows a high magnification image of HCT-8 cell apoptosis that has already been described in Fig. 10d. The arrow in Fig. 15a indicates the formation of membrane blebs. In the course of apoptosis, intracellular components disintegrate and leave the host cell, engulfed by its membrane.
Fig. 15: Scanning electron micrographs of examples of HCT-8 cell features from uninfected and infected cell lines. a) Host cell apoptosis. In the course of cell death, cellular components disintegrate and are excreted via membrane blebbing. Membrane blebs are indicated by an arrow. b) HCT-8 cell growth. Cells round up and detach for growth (arrowhead). c) Host cell mitotic division resulting in two daughter cells. d) HCT-8 cell overgrowth. Once the monolayer is 100% confluent, cells attach to and grow onto existent monolayers, as they continually multiply.
4.4.2. Cell Morphology of HCT-8 Monolayers Infected with *C. parvum*

Images showing the parasite were taken at areas where the monolayer was well established and thus *C. parvum* stages were easy to monitor. Infection mainly occurred in areas where the monolayer was confluent (Figs. 16a, b). Areas where the monolayer was still forming appeared to be uninfected (Fig. 16c). As features of cellular growth and apoptosis looked similar whether a monolayer was infected for a short or longer period, selected examples of HCT-8 morphological characteristics are given for particular days only.

At 24 hrs, trophozoites occurred as mainly single cells but also in pairs or more on the host cell surface (Fig. 16a). Trophozoites could be readily distinguished from host cells. The size of trophozoites detected at 24hrs varied, yet they were all smaller than 2 µm. Trophozoites were round or oval shaped with a smooth surface. The expression of microvilli was commonly enhanced around trophozoites (Fig. 16b).

Apart from the appearance of *C. parvum* trophozoites on the host cell surface, infected monolayers looked like their uninfected equivalents. The monolayers were at a similar state of confluence and showed features of growth and apoptosis (Figs. 16c, d). Host cells were in good condition with no more cell death visible in infected samples, as compared to negative controls. In areas where the monolayer was confluent an even microvilli brush border had established (Fig. 16a). Still, the monolayer showed large gaps at 24hrs. In such non-confluent areas microvilli were still expressed in an irregular fashion, irrespective of the presence of *C. parvum* infection (Fig. 16c). In non-confluent areas *C. parvum* stages could not be distinguished as easily as in confluent monolayers.
Fig. 16a, b: Scanning electron micrographs of a monolayer of HCT-8 cells, 24 hrs after infection with *C. parvum* excysted oocysts. Trophozoites of *C. parvum* are visible as round or oval stages with a smooth surface attached to the surface of host cells (arrows) in areas where the monolayer is fully established.
Fig. 16c, d: Scanning electron micrographs of monolayers of HCT-8 cells, 24 hrs after infection with *C. parvum* excysted oocysts. c) Some areas of a monolayer are not fully established. In such areas, parasite infection was difficult to monitor. Cellular growth can be monitored throughout the sample at this stage. c) Cells are rounding up in the course of mitotic division in the course of cellular growth.
48 hrs after infection, 72 hrs old monolayers were fully confluent (Fig. 17a). Infection was not evenly distributed throughout the sample, but distinct areas of infection were visible. The extent of infection had increased; so had the size of trophozoites (Fig. 17a). Trophozoites showed a slightly rounder, more extracellular appearance than a day earlier as they had further developed underneath the apical membrane (Fig. 17b). Oocysts were also found adhered to the surface of host cells, in contrast to a day earlier (Fig. 17b), the reason for which will be explained in the Chapter 5. More trophozoite accumulations were seen in comparison to 24 hrs, when the majority of trophozoites still occurred as single trophozoites (Fig. 16a).

Features of growth (Fig. 17c) and host cell death were visible in the cell line (Fig. 17d). In stark contrast to uninfected control samples most of the host cell death monitored at 48 hrs onwards appeared as shown in Fig. 17d. Such ruptured cells were exclusively observed in infected cultures and in infected areas of monolayers. They were never found in uninfected control samples or uninfected areas of a monolayer. Cell lysis is an event of necrosis not apoptosis. Thus, HCT-8 cell death as seen in Fig. 17d was due to necrosis caused by the parasite, not apoptosis (which is seen in Figs. 18c and 19d in uninfected areas of a monolayer).
Fig. 17a, b: Scanning electron micrographs of monolayers of HCT-8 cells, 48 hrs after infection with *C. parvum* excysted oocysts. *C. parvum* trophozoites are indicated by arrows. Note their rounder, more extracellular appearance and increased size. An excysted oocyst is indicated by an arrowhead.
Fig. 17c, d: Scanning electron micrographs of monolayers of HCT-8 cells, 48 hrs after infection with *C. parvum* excysted oocysts. The arrowhead in image c) indicates a growing HCT-8 cell infected with the parasite (arrow). The arrow in image d) indicates a necrotic HCT-8 cell in an infected area.

After 72 hrs *C. parvum* trophozoites appeared strongly engulfed by the host cell membrane (Fig. 18a, central arrow where two trophozoites formed next to their excysted oocyst). The membrane engulfing parasite stages still appeared smooth. The shape of the parasites nevertheless, did not appear round or oval any more, as the host cell membrane had encapsulated the invader further. Excysted oocyst shells were still seen adherent to the host cell surface (Arrowheads in centre and top left corner of Fig. 18a).
Fig. 18a: Scanning electron micrograph of a monolayer of HCT-8 cells, 72 hrs after infection with *C. parvum* excysted oocysts. Arrows indicate *C. parvum* stages. Arrowheads indicate excysted oocysts. Note their further change in appearance.

At 72 hrs the monolayer looked less healthy in contrast to its negative control. While necrosis was the dominant form of cell death in infected regions of a monolayer, apoptosis became abundant in non-infected areas and was more frequently seen than in negative control samples. Still, the monolayer kept its integrity and parasite stages were detectable at the host cell surface.
Fig. 18b, c: Scanning electron micrographs of a monolayer of HCT-8 cells, 72 hrs after infection with *C. parvum* excysted oocysts. The monolayer looks less healthy than at previous days or its negative control. This uninfected area of a monolayer shows features of apoptosis. Arrows indicate apoptotic cells and apoptotic bubbles.

After 96 hrs it was more difficult to identify *C. parvum* stages, because monolayers started to disintegrate (Figs. 19a, b, c). The location of parasite stages appeared far more extracellular than on previous days, leading to the suggestion that further developed parasite stages were starting to sever host cell contact at this stage. The strong engulfment by the host cell membrane seen at day 3 was not seen anymore. As the host cells disintegrated, microvilli were once again expressed in an irregular fashion (Fig. 19a), as described earlier for the case of non-confluent areas of a monolayer (see Fig. 16c).
Fig. 19a, b: Scanning electron micrographs of a monolayer of HCT-8 cells, 96 hrs after infection with *C. parvum* excysted oocysts. *C. parvum* stages are indicated by arrows. Note their far more extracellular appearance. The infected monolayer starts disintegrating. Necrotic HCT-8 cells infected by *C. parvum* are indicated by arrowheads in b).
Deterioration of the monolayer was not only visible in infected areas but also in uninfected areas of a sample (Fig. 19c). Features of apoptosis and necrosis were frequently seen throughout the sample (Figs. 19b-e). Necrosis occurred in infected regions of a sample (Figs. 19b and d) whereas host cell apoptosis was mainly seen in uninfected areas (Fig. 19c), the reasons for which will be explained in the discussion. No host cell overgrowth was detected in infected samples in contrast to negative controls (compare to Figs. 13 and 14).

After 5 days, many stages of the parasite were seen to be in an extracellular location (Fig. 20b). They appeared to have broken host cell contact. Microvilli were still expressed in an irregular fashion and mainly seen accumulated around parasite stages, whereas the majority of host cells was depleted of microvilli (Fig. 20a). The condition of monolayers was similar to the condition at day 4. Host cell apoptosis and necrotic cells were seen all through the sample.

Figs. 20c and d show necrosis of cells that are directly infected with the parasite. Thus, *C. parvum* directly causes necrosis in cells it infects. Still, no overgrowth was seen in infected samples after 5 days, in contrast to uninfected samples.
Fig. 19c, d, e: Scanning electron micrographs of a monolayer of HCT-8 cells, 96 hrs after infection with *C. parvum* excysted oocysts. Arrows labelled ‘t’ indicate parasite stages, whereas ‘a’ indicates an apoptotic host cell. In d) a necrotic host cell is labelled with an arrowhead.
Fig. 20a, b: Scanning electron micrographs of a monolayer of HCT-8 cells, 5 days after infection with *C. parvum* excysted oocysts. Parasite stages are indicated by arrows. Note the extracellular appearance of most *C. parvum* stages.
Fig. 20c, d: Scanning electron micrographs of monolayers of HCT-8 cells, 5 days after infection with *C. parvum* excysted oocysts. Necrotic HCT-8 cells infected by *C. parvum* are indicated with arrowheads. *C. parvum* stages are indicated with arrows.
4.5. Discussion

4.5.1 Uninfected HCT-8 Cells

HCT-8 is a eukaryotic cell line. Thus, cells multiply by mitosis like any other cells of eukaryotic organisms. As HCT-8 cells grow as a flat monolayer on a substrate, they may need to detach from the monolayer for the process of mitosis. For newly formed cell to attach to a substrate, microvillous material might be used, explaining the observation in Fig. 10c which shows elongated microvilli protruding from host cells and attaching to substrate. It was observed that cells in the course of mitosis are covered with microvilli (Fig. 15c). This suggests that the expression of microvilli on an HCT-8 cell does not depend on dense cell contact and a confluent monolayer.

Cellular growth and apoptosis occurs in all eukaryotic organisms continuously, to maintain homeostasis of cell numbers and replace old cells with fresh ones. The formation of membrane blebs that occurs in eukaryotic cells in the course of apoptosis was observed in the HCT-8 cell line (Fig. 15a). Host cell debris described in Figs. 10c and 11c was identified as the so called apoptotic vesicles (see Introduction) resulting from membrane blebbing in the course of host cell apoptosis (see Fig. 15a). The observation and description of apoptotic vesicles is essential to further studies examining *C. parvum* in the HCT-8 cell line as apoptotic host cell debris could be mistaken for *C. parvum* stages, if they had not been characterized earlier. Immunogold labeling of parasite stages may have been helpful at this point to differentiate between such apoptotic features of HCT-8 cells and parasite stages. On the other hand, debris in the form of small round balls that was commonly seen associated with host cell growth, did not appear attached to the monolayer, but floating away from the growing cell.
Thus, it can be distinguished from parasite stages, which are expected to be attached to host cells. In some cases the sizes of HCT-8 cells and/or cell line features can be misleading (for example Fig. 10c) but in other cases they can be used to distinguish between host cell line features and the parasite (Figs. 12b and 13b).

However, not all apoptotic debris had a round shape. Some host cell debris was spindle shaped (Fig. 11c) and might represent remnants of the disintegrated host cell membrane. When apoptosis is completed, dead cells detach from the monolayer (Fig. 13a). Dead cells are round but smaller than newly formed cells and no longer covered with microvilli. This makes it harder to differentiate them from parasite stages. On the other hand, parasite stages are known to be attached to the monolayer with dense contact (Huang et al., 2004; Valigurova et al., 2007) and thus are distinguishable from dead HCT-8 cells. Again, immunogold labeling of parasite stages might be a useful tool in some cases where the nature of a structure in culture is unclear.

An interesting observation in this study was that a regular microvilli brush border did not establish before a monolayer had reached confluence (Fig. 12). Before confluence was reached (Figs. 10 and 11), microvilli occurred in a variety of lengths and shapes and were distributed in an uneven fashion on cells. Therefore, the establishment of a confluent monolayer with dense contact between single cells allows for the expression of a regular microvilli brush border.

The observation made in this study that post confluent HCT-8 cells appeared highly heterogeneous with an irregular microvilli brush border, whereas
confluent HCT-8 cells established a homogenous monolayer expressing a regular microvilli brush border, is complimented by earlier findings of Barbat et al., (1998) and Rosenthal et al., (1977). They described the heterogeneity and homogeneity of post- and confluent cells, respectively.

Overgrowth in six day-old monolayers makes it difficult to culture *C. parvum* for longer than 5 days. It also appeared that the parasite failed to reinfect cells (see Chapter 3). As already mentioned in the previous chapter, exchanging media would eliminate extracellular parasite stages. On the other hand, inoculating old media containing parasite stages from 5 days of culture to a fresh monolayer would exclude all intracellular stages and thus also not enable monitoring of the complete life cycle. However, it might allow the maintenance of *C. parvum* in culture for longer. Attempts have been made (Hijjawi et al., 2001), but no immortal *C. parvum in vitro* culture has been achieved to date. Therefore, currently the best option is to observe the parasite in a monolayer incubated under oxygen depletion. Irradiation of HCT-8 monolayers to further slow down host cell growth has also proven a useful tool in previous studies by Hijjawi et al., (2001).

4.5.2. Infected HCT-8 Cells

Most of the previous research on *Cryptosporidium* has concentrated on host cell apoptosis to the exclusion of necrosis (Chen et al., 1999; Ojcius et al., 1999; Mc Cole et al., 2000; Widmer et al., 2000; Chen et al., 2001; Ponnuraj et al., 2002; Sasahara et al., 2003; Mele et al., 2004; O’Hara et al., 2007; Liu et al., 2007 and 2008). Only one paper explains host cell necrosis (Elliott and Clark 2003).
The most important finding of the present study was that *Cryptosporidium* causes necrosis, rather than apoptosis, to its host tissue. Apoptotic cells were detected in uninfected and infected monolayers; but necrotic cells were only seen in infected monolayers. Apoptosis was higher in uninfected cells in this study than infected cell monolayers. *Cryptosporidium* had been shown to suppress apoptosis (Chen *et al.*, 2001 and Liu *et al.*, 2008), explaining this observation. Necrosis has exclusively been monitored in infected samples, never in uninfected samples. This demonstrates that necrosis is caused by *C. parvum* infection. Necrosis was only seen in areas with visible infection. Thus, *Cryptosporidium* probably does not have an indirect necrotic effect on surrounding uninfected cells. Figs. 20c and d shows host cell lysis (necrosis) of infected cells. Thus, *Cryptosporidium* trophozoites can cause direct necrosis to the cells they infect. It is known that necrosis can be caused by infections (Peretti *et al.*, 2000), toxins (Voth *et al.*, 2005), trauma and inflammation (Fraser *et al.*, 1967; Aronsky *et al.*, 1997). Release of toxins from *C. parvum* stages has not been monitored. However, as the parasite is known to invade the cell in a feeding dependent fashion, it is apparent that *C. parvum* causes trauma to its host cell, which might be the cause for host cell necrosis. Harmful chemicals that can be released by necrotic cells (Ezepchuck *et al.*, 1996) might contribute to the pathogenesis of clinical cryptosporidiosis.

That apoptosis was mainly monitored in uninfected areas, supports the idea that *C. parvum* suppresses apoptosis rather than promotes it. This has already been suggested in studies by Chen *et al.*, (2001).
Little overgrowth was monitored in infected samples at 96 and 120 hrs in contrast to negative controls; most likely due to the fact that *C. parvum* causes cell death via necrosis in addition to the apoptosis that occurs in both infected and uninfected samples. Therefore, less dense HCT-8 monolayers were seen in infected samples on any day compared to uninfected ones.

Deterioration of the host cell monolayer was monitored at and after 4 days, only in infected samples. This observation again supports the finding that *C. parvum* causes host cell necrosis. Natural apoptosis and overgrowth of uninfected cells did not cause deterioration of the cell line. HCT-8 cell overgrowth was not monitored in infected samples in contrast to uninfected ones. Still the infected cell line deteriorated over time.

Membrane blebs were exclusive to the HCT-8 cell line during HCT-8 apoptosis, and were not found in parasites. Parasite stages opened for the release of endogenous stages, sometimes with their outer membrane becoming perforated to release their infective zoites or gametes.

For further studies, the addition of nutrients to the old media without exchanging the culture media would determine if the culture of *C. parvum* in HCT-8 cells can be extended beyond 5 days. On the other hand, host cell necrosis and deterioration of monolayers might be one reason that *C. parvum* can not live in culture for longer than 5 days due to the lack of uninfected cells. At day 5, necrosis of host cells is high. Furthermore, when cells undergo necrosis instead of apoptosis they release toxic chemicals that may contribute to the disappearance of *C. parvum* from the culture (Ezepchuck *et al.*, 1996).
4.5.3. Conclusions

When confluence is reached, HCT-8 cells form a homogenous monolayer with a regular microvilli brush border, suitable for an infection with *C. parvum*.

Through the findings of this study, that *C. parvum* infections cause a necrotic cell death, it can be concluded that it might not be possible to establish an immortal *in vitro* culture of this parasite. Thus, the propagation of *C. parvum in vivo* as described by Meloni and Thompson (1996) remains an essential tool to *Cryptosporidium* research.
5. Results

“The Hundred Faces of C. parvum”

5.1. Introduction

In this study, the life cycle of the parasite Cryptosporidium parvum was visualised from the excystation of oocysts through to the formation of new oocysts in vitro. Previously unknown life cycle stages as well as accepted life cycle stages of C. parvum and their interaction with host cells were morphologically examined. Electron microscopy was the method of choice for this purpose, as it resolves morphological features and thus enables identification of the parasite better than other microscopic methods. In this study, high resolution SEM enabled clear images of all parasite stages to be visualised with up to 800,000 times magnification. Additionally, surface receptors of C. parvum stages in cell culture were labeled with antibodies conjugated with immunogold, to confirm parasite stages and help in identifying parasite stages as well as morphological features of single stages that were difficult to interpret. Unfortunately, immunogold labeling of surface receptors compromised the structural stability of samples so that initial morphological examinations had to be undertaken without the aid of surface labeling. TEM was employed in this study to examine the parasite’s interaction with host cells and gain a better understanding of the parasite’s internal structures. Unfortunately, TEM is time consuming with a low hit to miss ratio. Even if every single cell in a certain area of an infected monolayer was infected with one or more C. parvum stages, there was still a higher chance to miss than to hit a parasite with a 100 nm wide TEM cross-section. Therefore, matching TEM
images could not be acquired for all parasite stages imaged with SEM in this study.

To perform this study, *C. parvum* was cultured in the HCT-8 cell line. Infected monolayers were examined with SEM and TEM over a 5 day period to monitor the entire life cycle. For this purpose, pure oocyst stock was also examined with SEM, after the trigger of excystation had been given. Previous experiments had shown that cultures from day 5 were best suited for examination, as all parasite stages (apart from sporozoites) were present at the same time. Thus, a 5 day old culture was the choice on which to perform immunogold labeling.

### 5.2. Aims

The hypothesis leading to this study was that the life cycle of *C. parvum* had been incompletely understood and that the morphology of many life cycle stages had not been correctly described. The same applies to the behaviours and host cell interactions of the parasite’s life cycle stages. This study aimed to visualise the life cycle of *C. parvum* and determine the morphology, behaviour and host cell interactions of all parasite stages. Topics of special interest were the establishment of parasite-host cell contact, the host cell invasion process and the involvement of microvilli material in the invasion process. Further investigations aimed to determine the location of various parasite stages within the host tissue and their attachment to host cells, including the morphology of the apical membrane.
Labeling of *C. parvum* surface receptors with immunogold conjugated antibodies aimed to confirm findings of the overall study and aid the detection of morphological features of *C. parvum*.

**5.3. Methods**

Cells were infected and cultured as explained in sections 2.1.4 to 2.1.7 of the Materials and Methods chapter. Electron microscopy was performed as stated in sections 2.2.1 to 2.2.8 of the Materials and Methods chapter. In addition to examining infected HCT-8 monolayers with SEM and TEM, excysted oocyst stocks were concentrated onto coverslips (as explained in section 2.2.1) and examined with SEM.

Sample preparation and imaging of parasites labeled with immunogold particles was performed as stated in section 2.3 of the Materials and Methods chapter. Unless otherwise stated, SEM images were acquired with in lens detection to achieve a maximal resolution.

**5.4. Results and Discussions**

The life cycle of *C. parvum* was visualised with SEM. Further morphological information about the parasite was acquired with the help of TEM analysis. Immunogold labeling of parasite surface receptors was performed to verify some results of this study; and help clarify the morphological findings made. The results in this chapter are presented in their developmental order as they are expected to occur *in vivo*; from inoculated oocyst stock through to the development of gamonts and oocysts.
It should be noted that not all inoculated oocysts excysted at once, but oocyst excystation occurred throughout the first 2 days after inoculation (Fig. 21b and c). Thus, the timing of appearance of the same stages of parasite development varied.

“Infected zoite” and “invading zoites” are terms used in this thesis. Infective zoites are free merozoites or sporozoites, whereas invading zoites are defined as merozoites and sporozoites in close host cell contact at the point of host cell invasion.

**5.4.1. Oocysts Excystation and Sporozoites**

Oocyst stock was treated for excystation and then inoculated into HCT-8 cell monolayers. Many of the treated oocysts were still intact 3 hrs after the trigger for excystation had been applied and incubated into culture as intact oocysts (Fig. 21a). Oocysts were oval shaped, measuring approximately 6 x 8 μm and possessing a suture for sporozoite release (Fig. 21a) in their otherwise smooth oocyst wall. The excystation of some oocysts occurred at a later time point up to 48 hrs post inoculation (Fig. 21c). However, some oocysts excysted directly after the trigger of excystation was applied, so that free sporozoites were incubated into the culture as well (Fig. 21b). Free sporozoites before inoculation, and as such before host cell contact, measured approximately 4.2 x 0.7 μm and possessed a blunt posterior end and a well defined, pointed anterior region (Fig. 21b).

The ultrastructure of oocysts had been examined with SEM before by Reduker *et al.*, (1985). According to the results of the present thesis, the oocysts
examined by Reduker were thin-walled oocysts as their size was reported to be 3.5 x 4 μm. The description of a suture for sporozoite release at one pole of the oocyst was first made by Reduker et al., (1985). The present study identified the same suture as previously described for thin-walled oocysts, on thick-walled oocysts (Fig. 21a). However, the size determined for sporozoites in studies by Reduker et al., (1985) slightly varied from the results of the present study. Some sporozoites identified in the present study measured approximately 8 x 1 μm (Figs. 21c and d). These sporozoites had excysted from thick-walled oocysts. As thin-walled oocysts measured in this study (see section 5.4.10) were as small as 2 x 3 μm, it can be proposed that there might be two slightly different types of sporozoites as a small thin-walled oocyst would encounter problems trying to accommodate 4 sporozoites of the same size as those which excyst from the larger thick-walled oocyst. Thus, the sporozoite described in Fig. 21b has most likely excysted from a thin-walled oocyst as described by Reduker, whereas the sporozoites described in Figs. 21c and d have excysted from thick-walled oocysts.

Excystation of intact oocysts continued throughout the first 48 hrs of culture (Fig. 21c). As our experiment created an artificial in vitro situation, it is possible that in in vivo, oocysts excyst simultaneously. However, the parasite appears to gain advantage by distributing oocyst excystation over a time frame of two days. This behaviour would ensure that at any time point a variety of different parasite stages are present, which have slightly different requirements of the host. Extracellular stages for example have less feeding dependency and do less damage to host cells than intracellular stages. Thus, the spread of oocyst
excystation over two days would enable the parasite to occupy the maximal niche within a host and thus replicate to its maximum possible number. Oocysts are known to contain four infective sporozoites which hatch from the suture during the excystation process (O’Donoghue, 1995). In Fig. 21c, the last of four sporozoites is hatching leaving a residual body behind in a peripheral location. Residual bodies have already been described in studies by Hijjawi et al., (2002) and presumably serve as a nutrient supply for the developing sporozoites until they have hatched to establish a feeding connection with host tissue. The apical region of this sporozoite hatching in host cell culture was elongated in contrast to sporozoites observed before the time point of inoculation into host cell culture. The same phenomenon of an elongated apical region was observed for sporozoites which were detected along the host cell surface (Figs. 21d and 22a). Whenever the apical region of a sporozoite was elongated, the main body towards the posterior region became rounder (Fig. 21c and d). Sporozoite apical region elongation has not been observed before and is first described in this study.

This elongation of sporozoites might be a result of the sample preparation procedure; however, apical end elongation appears to coincide with the time-point of expression of apical organelle structures. Apical organelle discharge occurs for the host cell invasion process of apicomplexan parasites (Blackman and Bannister, 2001). Therefore, it is possible that in the course of apical organelle discharge the apical region of sporozoites elongates. This again supports that the apical organelles of a sporozoite play the main part in sporozoite host cell attachment and invasion, as suggested by studies of Huang et al., (2004) and Valigurova et al., (2007).
The posterior body of hatched sporozoites was rounder in contrast to their slender apical region (Figs. 21c and d). This is probably a rudimentary effect of the apical area elongation but might already be a preparation for the expected host cell invasion and trophozoite formation.

Immunogold labeling of *C. parvum* surface receptors (see Figs. 22b and c) provided confirmation of the apical region elongation seen in sporozoites as a morphological feature within the parasite’s life cycle. The surface of free sporozoites appeared rough (Fig. 21d), unlike the surface of oocysts or the trophozoite stages of *C. parvum* that were ubiquitously seen and are described in Chapter 4. Free sporozoites were difficult to detect on the host cell surface as they invade cells very quickly upon oocyst excystation (Fayer *et al.*, 1997). They could only be detected if they were not washed away by the sample preparation procedure, thus attached to host cells but not yet invaded.

Immunogold labeling also served to enable the differentiation of *C. parvum* rudiments (such as the residual body described in Fig. 21c) from life *C. parvum* stages.
Fig. 21: Scanning electron micrographs of infective oocysts and sporozoites. a) Intact oocyst isolated from treated and partially excysted oocyst stock prior to inoculation. The arrow indicates the suture along which the oocyst opens for sporozoite excystation. b) Sporozoite isolated from partially excysted oocyst stock before inoculation. The apical end of the sporozoite is located towards the top left corner of the picture. c) Sporozoite excystation from an oocyst adherent to host cell, 48 hrs post inoculation. The apical end of the sporozoite points right. The arrow marks the rudimentary body in the oocyst. d) Sporozoite 7 hrs after inoculation. Note the elongated apical ends of sporozoites when apposed to the host cell.
Fig. 22: Scanning electron micrographs of apical end elongation of sporozoites as confirmed by immunogold labeling specific for *C. parvum* stages. The arrowhead in a) marks the main body, whereas the arrow indicates the apical end in contact with another *C. parvum* stage. Image a) was acquired with in-lens detection. Images b) and c) are acquired with backscattered electron detection (BSD) to reveal immunogold label which identifies the stages as *C. parvum*. Examples of immunogold particles are indicated by arrows in b) and c).
5.4.2. Host Cell Invasion

To monitor invasion of host cells by sporozoites, to the exclusion of merozoites which occur later in culture (Figs. 24a and b), samples were fixed as early as 6 and 7 hrs post inoculation. At this early stage, no mature trophozoites were seen. As mentioned above, sporozoite excystation occurred within 48hrs post inoculation. Thus, images taken 24 hrs post inoculation (a time point at which merozoites type I can be expected, as this study will show) showing sporozoite host cell invasion were included (Figs. 23a, b and 24c). The sporozoites seen in images presented here could be identified and distinguished from merozoites by their shape.

Fig. 23 shows two sporozoites at the early stage of host cell contact. Both sporozoites have already established a dense connection to their host cell and a host cell response has been initiated. When a sporozoite makes host cell contact, an intracellular cascade (in the host cell) is triggered (see General Introduction) that allows the parasite to establish itself in its niche, in feeding and protection dependency from the host cell. The establishment in the parasite niche includes the formation of a feeding connection to the host cell, known as the feeder organelle (Figs. 25a and b), as well as the formation of a protective membrane surrounding the parasite. Note how in Fig. 25b the engulfment of the invading parasite by the host cell membrane is not complete, although the establishment of a feeder organelle attachment has already begun. The feeder organelle is thought to be a tunnel-like feeding connection (Huang et al., 2004), connecting the parasite to the host cell in the middle of the attachment zone/electron dense plate. A similar tunnel-like feeding connection as the one observed with TEM for C. parvum by Huang et al., (2004) had been seen in
TEM studies of *C. muris* by Valigurova *et al.*, (2007), demonstrating the similarity between parasite species. In TEM cross sections (Fig. 30a) a developed feeder organelle had a granular appearance. The accumulation of small vacuoles on top of the invading parasite in Fig. 25b had been observed previously by Huang *et al.*, (2004) and is known to be a prerequisite for the parasitophorous vacuole (PV) formation. Like the studies by Huang *et al.*, (2004) further showed, small vacuoles were detected in the area where the parasite established a feeding connection (the feeder organelle) into the host cell (Fig. 25). Consequently, both processes, feeder organelle formation and the formation of the PV, may happen simultaneously.

The protective membrane is thought to be formed from host cell material. Studies by Valigurova *et al.*, (2007 and 2008) have identified *C. parvum* stages to be surrounded by 2-3 membranes. The parasite is thought to induce the host cell to engulf it with its apical membrane (see General Introduction) (Figs. 23b and 24a). Studies by Chen *et al.*, (2004a) and Huang *et al.*, (2004) suggested that the host cell membrane engulfment of the sporozoites occurs as a protrusion from the base of the host cell. However, the membranes surrounding the sporozoite in Fig. 23b did not appear to originate from a protrusion of the host cell. Fig. 23a shows how upon host cell contact sporozoites are embraced by elongated host cell microvilli. The sporozoite in Fig. 23b is already nearly fully engulfed by a thin membrane. Arrows ‘m’ indicate where the membrane engulfment is not yet complete. These gaps in the membrane initially enabled the identification of the thin membrane engulfing the invader. In comparison with the findings from Valigurova’s studies (of 2-3 membranes surrounding *C.
parvum stages), the thin membrane visible in Fig. 23b most likely represents the innermost membrane marking the boundary to the parasitophorous space. Note the smoother looking surface of the engulfed sporozoite in Fig. 23b in contrast to the non-engulfed patches left (as indicated by arrows in Fig. 23b). Elongated host cell microvilli were again found attached to the outside of the engulfed sporozoite. The microvilli-parasite contact seems to be a prerequisite for the host cell membrane to protrude and engulf the invading sporozoite (as seen in Figs. 23b and 24a) until it finally closes to wall off the invader from the outside (Figs. 24b and c). Fig. 23b suggests that the host cell membrane engulfment does not “only” occur as a protrusion from the base of the host cell, but that at least the inner membrane surrounding the invading parasite originates from the elongated microvilli that initially embrace the parasite. Note how the membrane engulfing the sporozoite in Fig. 23b is completely closed at the upper (posterior) end of the invader but still shows gaps at the anterior end and middle of it, as indicated by arrows. Thus, the final apical membrane surrounding parasites is not necessarily “only” a protrusion of the host cell, but may also be produced from elongated microvilli encapsulating the parasite similar to as it is seen in Fig. 23b. These findings are supported by the studies of Bonnin et al., (1999), which had previously detected microvilli components in the parasitophorous membranes (inner membranes) surrounding Cryptosporidium stages.

As the apical membrane engulfing the sporozoite is joined with the host cell, the parasite has now successfully invaded the cell. Within this protected niche, fuelled by nutrient supply through the feeder organelle, the sporozoite then transforms into a trophozoite stage and finally develops into a meront I. As Fig.
25a of a mature trophozoite shows, the feeder organelle remained during the life of the parasite to maintain host cell contact and ensure nutrient supply from the host cell to the parasite as long as it is needed.

Fig. 23: Scanning electron micrographs of sporozoite attachment to HCT-8 host cells 24hrs post inoculation. a) Sporozoite attached to host cell via apical end (a) and embraced by microvilli (mv). b) Host cell membrane engulfment (m) of sporozoite. The arrows point to where the membrane has not fully engulfed the sporozoite.
Fig. 24: Scanning electron micrographs of sporozoite host cell invasion. a) Sporozoite invasion of host cell 7 hrs post inoculation. The arrow indicates the development of an apical membrane to engulf the parasite. b) Sporozoite invasion of host cell 7 hrs post inoculation. The arrow indicates the junctions at which the apical membrane engulfing the parasite merges with the host cell. c) Sporozoite invasion 24 hrs post inoculation. The arrow indicates the growth and development of the apical membrane.
Fig. 25: Development of an intracellular feeding connection. Both images were taken 3 days after oocyst inoculation. a) Scanning electron micrograph of a feeder organellar attachment zone of a trophozoite. Arrows ‘f’ indicate the outline of the feeder organelle. The arrow ‘m’ marks the apical membrane engulfing the parasite before breakage due to host cell separation. b) Transmission electron micrograph of the formation of a feeder organelle by an invading parasite. The apical membrane engulfing the invader is not yet complete (arrow ‘m’).
5.4.3. Trophozoite Development

Once the invading parasite is fully engulfed by the host cell membrane, the transformation into the trophozoite stage begins. “Trophozoite” is a general term used to describe a Cryptosporidium stage that is engulfed by the apical membrane and too early in its development to be identified as a specific stage. Trophozoites were visible as rounded structures on the surface of host cells from 6 hrs post inoculation onwards (Fig. 27b). Their surface was smooth as trophozoites are encapsulated by the host cell apical membrane. Initially, trophozoites measured approximately 1 μm in diameter (Figs. 26 and 27a), finally reaching a size of approximately 3 μm for most trophozoites observed along the host cell surface (Fig. 31a). At very early stages trophozoites appeared hood-like in shape (Figs. 27a, b and c) on the host cell surface. A ring-like structure (Figs. 27a and 29c and d, labeled with arrows) was visible around developing trophozoites at this early stage, marking the boundary at which the apical membrane encapsulating the parasite maintains contact with the host cell (Figs. 30 a and d, arrow ‘j’). This area is also known as the host-parasite interface. Note how at this early stage the host cell membrane clearly engulfed the invading zoite without protruding membrane folds (Fig. 27a). As the parasite grows underneath the membrane, excessive amounts of membrane can be produced, resulting in folded membrane structures (Figs. 26 ‘mf’ and 27 c and d arrow). When the parasite was fully established in its niche, these excessive membranes disappeared (Fig. 31 a). Microvillous material also becomes incorporated into the membrane (Figs. 27b and 29a, b) which assists the parasite in forming its protective niche. Finally the parasite resides in its niche underneath the host cell membrane in a parasitophorous vacuole (PV)
(Fig. 31; and see Valigurova et al., (2008)). Studies by Valigurova et al., (2007 and 2008) identified two membranes forming the PV, with the inner membrane being connected to the parasite plasma membrane at the annular ring, whereas the outer membrane was continuous with the plasma membrane of the host cell. The outer membrane observed by Valigurova et al., (2007 and 2008) corresponds to the apical membrane merging with the dense band indicated by an arrow labeled ‘j’ in Fig. 30d. The inner membrane unfortunately was not observed in TEM sections acquired in this study. As Valigurova et al., (2007) describes the inner PV membrane to be of host cell origin as well as the outer membrane it is possible that the membrane, observed to initially form around invading sporozoites in Fig. 23b, represents this inner PV membrane, closely surrounding the parasite membrane so that it could not be clearly distinguished in TEM sections presented in this study.

Invaded parasites are separated from the host cell by an electron dense band (Fig. 30, Huang et al., 04). They do, however, remain connected to the host cell in a nutrient dependent fashion via a feeder organelle. The nucleus of a cell connects via the endoplasmic reticulum (ER) and golgi complex to the cell wall via the formation of transport vesicles. The host cell's nucleus produces amino acids, some of which are essential to C. parvum as it lacks certain de-novo synthesis pathways (Thompson et al., 2005). Ribosomes assemble amino acids into proteins at the rough ER. The smooth ER however, synthesises fatty acids some of which are essential to C. parvum. The Golgi is responsible for the modification and packaging of assembled proteins. Packaged proteins and fatty acids are packed in transport vesicles and migrate to the periphery of the cell.
The parasite seen in Fig. 30d appears to be located above these structures, potentially using them to obtain nutrient supply from the cell. This observation is a novel association, not reported previously for *C. parvum*. It supports the hypothesis, made in the general introduction, that *C. parvum* re-functions integral pathway of the host cell for its own purposes. However these observations remain highly speculative and require further examinations with higher magnification TEM. Yet, a similar mechanism of nutrient uptake had been observed in the related apicomplexan parasite *T. gondii*, as reviewed by Plattner and Soldati-Favre (2008): “The plausible way for scavenging host cell lipids could be via the association of the host cell ER and mitochondrion with the parasite’s PVM”.

That *C. parvum* does not ‘just’ reside at the surface of host cells and passively awaits nutrient supply, but actively establishes a feeding connection into the interior of the host cell has long been recognised (O’Donoghue, 1995). Fig. 25a demonstrates that the parasite forms a feeding connection into the interior of host cell. An ATP-binding protein, commonly associated with the transport of substances (CpABC) was localised at the host-parasite boundary (Perkins et al., 1999). It is thought that the CpABC forms the feeder organelle and serves to uptake nutrients from the host cells. However, at which point the parasite interferes with the host cell’s ‘machinery’ and what method it applies to obtain nutrients from the host cell are not understood. Studies by Zhu (2004a) identified that *C. parvum* is capable of elongating long-chain fatty acids, whereas earlier studies by Milne *et al.*, (2001) found a fatty acid acyl-CoA binding protein (ACBP) responsible for supplying myristoyl-CoA (a coenzyme involved in fatty acid oxidation) to the fatty acid remodelling machinery in related
parasites. Indeed a protein CpACBP1 had been found and located with the feeder organ of *C. parvum* at the host-parasite interface (Zeng *et al.*, 2006). This suggests that CpABP1 may function as a fatty acid scavenger to facilitate fatty acid uptake by *C. parvum*. As fatty acids are essential to all organisms, fatty acid metabolism had been considered a promising target for drug development against cryptosporidiosis (Zhu, 2004b). Similar studies would be essential to examine the amino acid/protein uptake of *C. parvum* from the host cell. As an external supply of amino acids is essential to *C. parvum*, protein synthesis may also represent a promising drug target. Further investigations of the parasite’s relationship with the host cell utilising TEM would aid in identifying at which stage the parasite interferes with the host cells ‘machinery’ and possibly narrow the drug target search.

Protrusions of the apical membrane encapsulating the trophozoite, as well as host cell microvilli, are involved in anchoring the parasite to the host cell surface (Fig. 31a). Even when two parasites co-invaded a cell, as seen in Figs. 26-29, each parasite developed its own niche with its own PV and feeding connection, isolated from each other by separate apical membranes as TEM images revealed (Figs. 28a and b). Results of this study presented in Figs. 42d, 49 and 50c, reveal that free zoites of *C. parvum* randomly adhered to each other via *C. parvum-C. parvum* surface receptor interactions. If two such stages make host cell contact in dense contact with each other, co-invasion of a host cell can be seen (Figs. 26-29).
The shape and appearance of a parasite is determined by several factors. As already seen earlier, the course of a parasite’s development determines its size and shape. Thus, for example, a trophozoite developing into a macrogamont will look completely different in its early stages than at the time point of maturation. Findings in this thesis show that the same parasite stage can have different morphological forms (see for example Figs. 39a-c and Figs. 41a-d). The trophozoite in Fig. 29c for example was round in shape with merozoites visible within, thus was developing into a meront. On the other hand the trophozoite in Fig. 29a appeared to be forming an oval stage with a peripheral nucleus and without internal merozoites, possibly developing into a macrogamont, thereby supporting the observations made by Hijjawi et al., 2001. Other factors like the expression or absence of host cell microvilli (Figs. 29a and b) appeared to influence the form and shape of trophozoites as well. As the state of a host cell seemed to influence the development of a parasite, it may be possible that parasite stages presented in this thesis appear slightly different in form compared to parasite stages in vivo, as all studies were performed in an in vitro model of cancer cells.

TEM cross-sections (Figs. 30b and c, arrows ‘m’) and SEM images both (Figs. 29a, b) showed spike-like membrane folds at the outer boundary of the parasite membrane, which were only seen for some trophozoites. The regularity of these spikes on the respective parasite suggests that they fulfil a defined function, the nature of which requires further investigation. Similar structures have been identified in gregarines (Butaeva et al., 2006). They were described as longitudinal ridges, serving structural support, strengthened through
microtubules running through every ridge. The examination of such ridges in further TEM studies could verify their importance and possibly suggest their role in the life cycle of *C. parvum*.

Further, TEM cross sections revealed folded membrane structures referred to here as “parasite membrane” (PM) located within the developing trophozoite (Figs. 30a and b). The presence of the PM is a new finding and the origin, nature and functions of these membrane structures have yet to be determined. The “PM” was given its name as it appeared to be of parasite origin rather than of host cell nature. This conclusion was made as the PM exclusively occurred in the interior of parasites and was thicker than the typical apical membrane surrounding trophozoites (for example, see Fig. 31 as a comparison). Oocysts that are passed in faeces form a thick cell wall and perhaps the PM described here plays a role in oocyst cell wall formation. The morphology of the developing parasite in Fig. 30a supports this suggestion. Not all stages were found to contain a PM. The stage in Fig. 30a however which did contain a PM appeared to be a macrogamont, identified by its oval shape and peripheral nucleus – developing into an oocyst after fertilisation. The peripheral location of nuclei in developing oocysts/macrogamonts has previously been seen in studied by Hijjawi *et al.*, (2001). The stage in Fig. 30b on the other hand, which also contains a PM, may very well develop into a different macrogamont-like stage, which will be described later.

Again the here described structures require further examination for their verification. Shrinkage and changes in cellular structures, which have occurred in standard sample preparation procedures here used, must be minimised to obtain more reliable results.
Fig. 26: Transmission electron micrograph of host cell invasion and trophozoite development by *C. parvum* invasive stages 3 days post inoculation. The membrane engulfing the invading zoite on the left is not completely closed (arrow ‘m’). Note how the host cell membrane gets folded in the course of the invasion process (‘mf’), utilising microvillus material. m=membrane, mf=membrane folds, mv=microvilli.
Fig. 27: Scanning electron micrographs of trophozoite development in HCT-8 cell monolayers 6 - 55 hrs post inoculation. a) 6 hrs post inoculation, encapsulated parasites appeared hood-like in shape on the host cell surface. The arrow marks the host-parasite interface. b) Incorporation of microvilli into the apical membrane surrounding the parasite at 6hrs of culture as indicated by an arrow, c) Different stages of trophozoite development at 55 hrs with an arrow marking membrane formation, d) Co-invasion of *C. parvum* stages at 24 hrs with the arrow indicating membrane formation and folding in the course of trophozoite development.
Fig. 28: Transmission electron micrographs of co-invading *C. parvum* stages. a) 3 days post inoculation several parasite stages have invaded two host cells in such close proximity that the apical membrane surrounding the single parasite stages are merged (arrow), b) 2 days post inoculation two parasites have invaded one host cell in direct proximity to each other. The arrow indicates the apical membranes surrounding and separating the parasites.
Fig. 29: Scanning electron micrographs of early trophozoites on HCT-8 cell monolayers. Trophozoites develop in different forms and shapes. a, b) Advanced stages of trophozoite development 24 hrs post inoculation. Microvilli are incorporated into the apical membrane encapsulating the parasites and anchor them to the host cell surface as indicated by arrows. c, d) Early stages of trophozoite development at 6 hrs post inoculation. The membrane and with that the niche is not fully established yet. The borders of the host-parasite interface are visible as ring-like zones around developing trophozoites as indicated by arrows. Note how the appearance of trophozoites in a) and b) depends on the expression of host cell microvilli, as compared to c) and d).
Fig. 30: Transmission electron micrographs of *C. parvum* stages in HCT-8 cell monolayers 3 days post inoculation. a) Development of a macrogamont from a trophozoite stage. Note the peripheral location of the nucleus, the oval shape of the parasite and the absence of infective zoites. Note the granular appearance of the feeder organelle structure. A membrane referred to as the ‘parasite membrane’ is visible within the developing structure of the parasite. b) *C. parvum* stage containing folded internal parasite membrane. Note the folds in the membrane ‘m’ surrounding the parasite as indicated with an arrow. c) Meront I containing internal merozoites type I. Note the folding of the membrane ‘m’ as indicated by an arrow. c) Magnification of the host parasite interface. Note the immediate location of the parasite above the host cell’s nucleus. 

*db=dense band, ER=endoplasmic reticulum, f=feeder organelle, j=junction of membrane to dense band, m=membrane, mv=microvillus, n=nucleus, pm=parasite membrane, pv=parasitophorous vacuole, z=zoite.*
Fig. 31: Example of a trophozoite. a) Scanning electron micrograph of a trophozoite on a HCT-8 cell monolayer 48 hrs post inoculation. The parasite is anchored to the host cell surface by the apical membrane ‘m’ surrounding the invader, and host cell microvilli ‘mv’ which are incorporated into this apical membrane. b) Transmission electron micrograph of a trophozoite on a HCT-8 cell monolayer 3 days post inoculation. *C. parvum* resides in its niche underneath the apical membrane ‘m’, in a parasitophorous vacuole ‘pv’, separated from the host cell by a dense band, with feeding connection via the feeder organelle ‘f’. Internal zoites ‘z’ are formed within the cytoplasm.
5.4.4. Trophozoite Characteristics

Invasive stages of *C. parvum* have the capability to co-invade cells. Clusters of two or more trophozoites were observed ubiquitously on the host cell surface after 48 hrs of culture (Figs. 32a-d).

Infective zoites must be in close proximity before and during host cell invasion to co-invade cells as seen in Figs. 32a-d. To maintain close proximity between infective zoites, the parasite must utilise some form of adherence surface molecules, which enable infective zoites to adhere to each other and maintain contact to co-invade host cells. Adherence of infective zoites has been observed in this study and is shown in Figs. 42, 44 and 50 of merozoites types I. It appeared as if the apical membranes engulfing two adjacent parasites had developed following the zoite invasion and PV formation, as small gaps were visible (as seen in Figs. 32a, c and d and indicated with an arrow in Fig. 32d). This observation complements the findings from Figs. 28a and b, which showed that each co-invaded parasite forms its own niche and PV.

The finding of a cluster of four trophozoites in Fig. 32a suggests that they resulted from sporozoite host cell invasion, as one oocyst always contains four sporozoites. It is likely that the four sporozoites excysted simultaneously, remained in dense contact via surface receptors and co-invaded a host cell as seen in Fig. 32a. Note how all four trophozoites are of the same size. The finding of a cluster of six trophozoites in Fig. 32b suggests that the present trophozoites resulted from merozoite type I host cell invasion, as one meront I is thought to contain six to eight merozoites type I (Figs. 41a-d; and see Hijjawi *et al.*, 2001). Note how all six trophozoites are of the same size. Nevertheless in many cases only two invasive zoites appeared to be released simultaneously,
adhere to each other and co-invade cells resulting in trophozoite pairs as seen in Figs. 32c and d. More trophozoite pairs were observed throughout the entire monolayer than single trophozoites or clusters of 4 or more (Figs. 33a and b). At first glance, it appeared as if the parasite would not gain advantage through this behaviour as it does not distribute the infection over a wide area. The longer the period post-inoculation, the more accumulations of trophozoites were seen as the monolayer became densely populated. As more pairings of trophozoites were seen than single trophozoites, it can be assumed that C. parvum does gain advantage by adhering to each other, possibly by the opportunity of parasite-parasite interactions. Thus, surface receptors that enable C. parvum-C. parvum interactions might be expressed specifically to serve this purpose. By co-invading cells, adjacent trophozoites have dense contact to each other as already discussed earlier. It is possible, that following invasion and establishment of their own niche, the invading parasites eventually establish a connection to each other, enabling parasite-parasite interactions similar to the ones described later in Fig. 37.

Apart from the apical membrane that is initially formed to engulf the invading parasite, host cell membrane was observed to grow over parasite stages and partially engulf them, as seen in Fig. 32d, growing over a trophozoite from the right hand side of the image; also Fig. 71b and Figs. 72a and b later on in this study. The formation of multiple membranes of host cell origin has already been observed in previous in vivo studies by Valigurova et al., (2007 and 2008) for C. muris. The additional outer membrane however observed in Fig. 32d seems to
be a third structure, possibly providing the parasite with extra anchorman to its host.

Fig. 32: Scanning electron micrographs of clusters of 4, 6 and 2 trophozoites on the host cell surface, 48 hrs post inoculation. Note how the apical membranes engulfing the parasites have merged between two adjacent stages (arrow).
Fig. 33: Scanning electron micrographs of trophozoite formation on HCT-8 cells by sporozoite directly next to oocyst, a) 3 days post inoculation. b) Trophozoites along the host cell membrane without evidence of their origin at 48 hrs of culture.
One important observation of this study was the presence of “gliding trails” along the host cell surface (Figs. 34a-c). Cryptosporidium gliding trails through the microvilli brush border of host cells have not been observed before in vitro culture or in vivo and were first described in this study. Gliding trails have been described previously in other parasites including Plasmodium and Toxoplasma (Soldati et al., 2008). Gliding motility is known to be a prerequisite for host cell invasion by apicomplexan parasites (Soldati et al., 2004; Wetzel et al., 2005). Gliding motility is actin based (Baum et al., 2006) and substrate dependent (Keeley and Soldati, 2004) causing interactions with host cell material. In in vitro, sporozoites move through the apical brush border of the intestinal epithelial lining and invade close to their origin, leaving trails of extended microvilli behind (Fig. 34c). A gliding trail is a visible trail in the microvillous brush border of host cells, consisting of abnormal microvilli (Figs. 34a and b). In contrast to the microvilli on the rest of the host cell, microvilli within the gliding trail were longer and denser (Fig. 34c). It appeared as if parasite contact had stimulated the extension of microvilli on the host cell membrane resulting in the gliding trails seen in this study. Figs. 34a and b show gliding trails between oocysts and trophozoites. It seems clear that the respective trophozoites had developed from sporozoites that excysted from these oocysts. After excystation from the oocyst, sporozoites gliding along the host cell surface resulted in the gliding trail prior to host cell invasion. In Fig. 34d all four sporozoites have invaded the host cell within the gliding trail region and are visible as trophozoites of which one is indicated as an example by an arrow. In Fig. 34a not all sporozoites seem to have invaded the host cell within the gliding trail region as only two trophozoites are visible at the end of the trail. This suggests
that not all sporozoites excysting from the respective oocyst were gliding along
the host cell membrane, but some must have travelled in the supernatant above
cells and invaded the monolayer elsewhere. This would be the case for most
infected zoites being released from its parent stage as gliding trails were not
ubiquitously observed throughout infected monolayers.
In other cases, sporozoites invade directly adjacent to the oocyst from which
they hatched and formed trophozoites there (Fig. 33a). However, the majority of
sporozoites seem to leave the parent stage and swim free in media, without
causing gliding trails, to invade elsewhere (Fig. 33b).
Many *C. parvum* receptors have been shown to be shed in trails by gliding
sporozoites onto substrate (Tilley and Upton, 1994 (CP15 and CP25); Cevallos
*et al.*, 2000a (Gp900); Okhuysen *et al.*, 2004 (Trap-C1); Tossini *et al.*, 2004
(Cpa135)). However, the observation of the so called “gliding trails” in microvilli
brush borders is new to this study. The present study has added to our
understanding of gliding motility, through the identification of gliding trails,
identifying host cell microvilli as the primary source of interaction of sporozoites
and other infective *C. parvum* zoites.
Fig. 34: Scanning electron micrographs of C. parvum gliding trails on HCT-8 cell monolayers. Expression of microvilli is enhanced through C. parvum contact. a) 3 days post inoculation, b) 24 hrs post inoculation, c) magnification of a). m= microvillus, o= oocyst, r= rudimentary body of oocyst, t= trophozoite.
Not only were infective zoites stages seen to affect the expression and nature of microvilli by gliding motion along the host cell membrane; but also meshworks of elongated microvilli were observed around parasite infections (Figs. 35a-c). The observation of microvilli meshworks is new to this study, introducing the term “meshwork”. The expression of microvilli around parasites appeared to have increased abnormally compared to non-infected areas of the same sample, and microvilli were much denser and longer than at non-infected areas of the same monolayer and even the same host cell (Figs. 35a-c).

Microvilli abnormality associated with meshwork formation was commonly observed in areas of *C. parvum* infection. Areas of increased microvilli expression around parasites in Figs. 35a-c clearly demonstrate that the parasite stimulates the host cell to express microvilli to utilise microvilli material for its own advantage to establish its protective niche. Many microvilli in such areas were elongated to more than double their normal length. This phenomenon has been discussed above in the context of sporozoite invasion (see Fig. 23). The expression and elongation of microvilli might not be alike in *vivo* but will probably occur, because negative controls or uninfected areas of the same monolayer did not show such areas of elongated microvilli and enhanced microvilli expression. Thus, it can be suggested that the parasite managed to stimulate the host cell to express microvilli for its own purposes, as the parasite utilises microvilli material for the establishment of its protective niche. On the other hand, it is likely that infective zoites preferably invade at regions where the expression of microvilli is high. As we have seen through negative controls at days 1 and 2 of culture before the monolayer was fully established, microvilli are not expressed in a ubiquitous manner but rather in an irregular fashion,
especially before the monolayer is fully established. This and the fact that cancer cells were used for this culture may also contribute to the expression of microvilli meshworks around parasite stages in this *in vitro* experiment.

Fig. 35: Scanning electron micrographs showing the stimulation of host cell microvilli expression by *C. parvum*. a) The expression of microvilli of a heavily infected host cell is enhanced and microvilli are expressed in a confused fashion 48 hrs post inoculation. b) A meshwork of elongated microvilli has formed around a cluster of a trophozoite pair by 55 hrs of culture. c) 6 hrs post inoculation a small trophozoite has formed in a nest of host cell microvilli.
Cryptosporidium is known to employ sexual as well as asexual reproduction in its life cycle to reproduce and amplify within the same host animal. To date one asexual cycle and one sexual cycle have been identified in the life-cycle of C. parvum (Fayer et al., 1997). The asexual cycle employs asexual reproduction via merogony resulting in the release of merozoites type I from meronts I that invade cells to form further meronts I and thus continue the asexual cycle. The sexual cycle however employs sexual reproduction via gametogony with fertilisation and zygote formation, followed by asexual multiplication (sporogony) to form sporozoites within oocysts.

Images seen during this study lead to the suggestion that C. parvum might employ other types of asexual reproduction which have not been described in its life cycle to date. Images as the one seen in Fig. 36a suggest that the parasite employs binary fission of one parent stage into two identical daughter stages. This is apparent as both stages are the same life cycle stage (microgamonts – identifiable by their internal microgametocytes as will be explained later) and are engulfed by the same apical membrane, not being separated by discs in contrast to stages in syzygy, which will be explained below. Binary fission is a type of asexual reproduction commonly employed by bacteria (Angert, 2005). If C. parvum follows the same rules, in the course of C. parvum binary fission, mitotic division must occur as well as the separation of the membrane surrounding the original parent stages to engulf the resulting two identical daughter cells. Unless a parasite stage is in the course of mitotic division it would be difficult to tell whether two identical stages have resulted from mitotic division of a parent stage or from two separate infective zoites. Nevertheless, it is likely that after mitotic division, one of the two daughter
stages does not possess host cell attachment as Fig. 36a suggests. Thus, stages originating from binary fission could be identified by their extracellular location. This new observation opens the possibility that some stages of *C. parvum* observed along the host cell surface without apparent host cell contact may have resulted from binary fission of a parent stage. The observation of another asexual replication process in the life cycle of *Cryptosporidium* in addition to merogony, which forms the meront I cycle, requires further investigation for its verification and acceptance into the life cycle. Similar observations have already been made by Hijjawi *et al.*, (2002) in Fig. 4. Hijjawi’s study shows light microscopic images of double nucleated stages (Figs. 4a-c) (containing one nucleus at each pole) of *C. andersoni* isolated from mice. Although Hijjawi described these double nucleated stages to be two *Cryptosporidium* stages in syzygy, they more closely resemble a stage in binary fission similar to the one described in Fig. 36a, as the stage(s) do not contain discs that have been identified to separate stages in syzygy (see Fig. 37) as well as in another image presented by Hijjawi *et al.*, (2002) in Fig. 4d.

Binary fission must be differentiated from the separation of parasites as seen in Fig. 36b. When host cells grow, the membrane surrounding adjacent trophozoites has to divide as they are pulled apart (Fig. 36b).
Fig. 36: Scanning electron micrographs of *C. parvum* binary fission in a HCT-8 cell monolayer. Binary fission follows mitotic division from one parent stage into two daughter stages as a means of asexual replication. a) Parasite stage (microgamont) in the course of mitotic division 4 days post inoculation. b) Separation of two adjacent *C. parvum* stages through host cell growth at 48 hrs in contrast to binary fission.
From other observations made in this study the question arises whether C. *parvum* is capable of syzygy like the related gregarines. In the phenomenon known as syzygy, parasite stages adhere to each other as described for gregarines by Lacombe *et al.*, (2002). It is hypothesised that in the course of syzygy, parasites exchange genetic material to gain advantage through genetic diversity. Syzygy has already been described for *C. andersoni* in *in vivo* studies by Hijjawi *et al.*, (2002). Parasite stages that showed morphological features of syzygy have also been observed in this study on *C. parvum* (Figs. 37 and 38). Evidence for this hypothesis is seen in Fig. 37 which shows connective discs between adjacent parasites as indicated by arrows. Such a disc has also been visualised in a cross-section in Fig. 38 separating and connecting two parasites as indicated by an arrow labeled 'm'. Discs connecting parasite to parasite have already been described by Toso and Omoto (2007) for gregarines and have been observed in this study in Fig. 37a-c. The studies by Toso and Omoto (2007) explain how gregarines start the sexual life cycle via the pairing of two gamonts, forming a syzygy junction which is similar to the connective discs seen in Fig. 37 of this study.

Further, Fig. 37b shows clearly how one parasite established a connection to another parasite instead of to a host cell. The same phenomenon was seen in TEM cross-section (Fig. 38) which shows one parasite attached to another one below via its feeder organelle. The same phenomenon is seen in Fig. 37a where one stage is open revealing its internal feeder organellar structure (the rudimentary body as also shown in Fig. 41d) which attaches to the adjacent parasite. This behaviour raises the question if one parasite feeds through other parasite, as it otherwise has no contact to the host cell but depends on external
nutrient supply during its developmental phase. This is one possible explanation for the behaviour seen, as the parasites which were seen attached to other parasites instead of to host cells, were found to be well developed (Fig. 38) to the point where they completed their life time and function (Fig. 37b). On the other hand, the adherence of two parasite stages may serve the exchange of genetic material via the feeder organelle. Findings of exclusive extracellular stages (meronts and trophozoites) in Chapter 3 suggest it is possible that stages of *C. parvum*, without apparent host cell contact, can obtain nutrients from the intestinal lumen through their cell wall. Stages seen attached to each other as described here appear to be microgamonts, most of which developed by day 4 of the parasite’s life cycle. Microgamonts will be described in detail in subsequent chapters. The observation that stages found in syzygy-like contact in this study are microgamonts supports the hypothesis that *C. parvum* employs syzygy. Studies on related parasites have revealed that syzygy is employed by stages of the sexual life cycle (Toso and Omoto, 2007).

If stages of *Cryptosporidium* employ syzygy to exchange genetic material similar to gregarines as Fig. 37b suggests, or if stages attach to each other in a feeding dependent fashion as Figs. 37a, c and 38 suggest, requires further investigation. The finding of three *C. parvum* stages in a row, similar to what had been described for gregarines by Lacombe *et al.*, (2002) and Toso and Omoto (2007), supports the hypothesis that *Cryptosporidium* employs syzygy in its life cycle. However, more intensive TEM studies would be needed to determine conclusively whether *C. parvum* stages are capable of syzygy.
Fig. 37: Scanning electron micrographs of *C. parvum* syzygy. Images a) and b) were taken 5 days post inoculation; Image c) 3 days post inoculation. Arrows indicate connective discs between parasite stages. Image a) shows most clearly how one microgamont attaches to another microgamont via the basal disc. The rudimentary body ‘b’ of the left open microgamont is located above the attachment area where the left microgamont attaches to the right intact microgamont.
Fig. 38: Transmission electron micrograph of *C. parvum* syzygy. *C. parvum* above connects with a feeder organelle to the parasite below 5 days post inoculation. f=feeder organelle, m=membrane, z=zoites.
5.4.5. Meronts I and Merozoites type I

Trophozoites resulting from initial sporozoite host cell invasion develop into meronts I (O’Donoghue, 1995) which were seen in Figs. 39a-c and 40 after 24 hrs of culture. Meronts I were identified through the formation of six or eight internal merozoites type I (Hijjawi *et al.*, 2001), which were aligned in parallel under the smooth apical surface of meronts I (Figs. 39b-c).

In general, meronts I were the same size as trophozoites detected at day 1-2 of culture with a diameter of approximately 3 μm. At the corresponding developmental stage (here observed after 24 hours of culture) merozoites type I were seen underneath the smooth apical membrane. Their shape resembled that of sporozoites, were smaller in size than sporozoites yet larger in number with at least six merozoites contained in one meront I (Fig. 40). Soon after merozoites type I became visible under the apical membrane, merozoites type I were released into culture (see Figs. 42a-d which shows merozoites type I at day 1 of culture). Previous light microscopic studies by Hijjawi *et al.*, (2001) proposed the development of meronts I after 48hrs of culture. In contrast to Hijjawi’s observations with the help of light microscopy, the present study clearly identified mature meronts I after only 24 hrs of culture with merozoites type I visible below their smooth surface membrane (Fig. 39) with free merozoites type I being present in culture as well (Fig. 42). SEM and TEM used in this study enabled the more precise determination of meront I and merozoite type I morphology than it had been possible to achieve in the past. Due to their small nature, merozoites type I could be easily overlooked with light microscopy if they appeared in small numbers as often the case after only one day of culture.
Fig. 39: Scanning electron micrographs of meronts I, 24 hrs post inoculation on HCT-8 cell monolayers. a) Four meronts I of equal size and developmental stage. b) Cleft like opening of apical membrane indicated by an arrow revealing internal merozoites type I which are parallel aligned. c) Enlargement of a).
Fig 40: Scanning electron micrograph of a Meront I at the time-point of merozoite release, 24hrs post inoculation. As an example one hatching merozoite is indicated by an arrow.

Meronts I appeared in different sizes and forms, especially after 5 days of culture (Figs. 41a-d). The size of meronts I appeared to mainly depend on the developmental stage of the individual meront I, as it has been described for trophozoites in the previous section. A comparison of both meronts I seen in Fig. 41a suggests that the final size of a meront I depends on its location and relationship with(in) a host cell. The meront I seen in the lower left corner of Fig. 41a was rounder and smaller than the larger meront visible in the top right corner of the same image. The round developing trophozoite seen in Fig. 29c probably developed into a meront I similar to the small round meront I seen in Fig. 41a. These meronts I were smaller in size and possessed less host cell
attachment than other meronts I (for example Figs. 39, 40 and 41) seen along the host cell surface.

Figs. 41b-d show typical meronts I at the time point of merozoite release. A rudimentary body ‘b’ (for example arrow Fig. 41b) was visible in each of the three parent stages. This rudimentary body was located centrally in the parent stage and seemed to be connected to the host cell. Its margins appeared the same as those marked with ‘j’ in Figs. 30a and d in cross-sections through parasite stages. Merozoites type I appeared to be formed by budding of this rudimentary body which is clearly visible in Figs. 41c and d labeled with arrows ‘mz’. The rudimentary body remains behind once the parent stage has released all its endogenous stages. Thus, the rudimentary body and the left behind feeder organelle may be one and the same parasite structure.

In Fig. 41b six mature merozoites type I are aligned in parallel on the rudimentary body, whereas in Fig. 41c eight merozoites were counted within the apical membrane of their parent stage. On the one hand, two merozoites might have left one of the meronts already, but on the other hand, this finding supports the suggestion made earlier that there are different morphological options for meronts I.

The host cell membrane appeared to have ruptured at this time point to allow merozoites to leave the parent stage. Fig. 39b suggests that the apical membrane initially opens along a cleft (arrow) before it breaks away further. Visible in Fig. 41c is a residual body ‘r’ that might be enclosed by parasite membranes. The residual body possibly serves as nutrient supply before the parent stage parasite established host cell contact itself. A similar residual body has already been described for oocysts.
Figs. 41b-d show that sample preparation had damaged the apical membrane surrounding meronts I, as not all merozoites had completed budding of the rudimentary body yet. In other cases the apical membrane might rupture as well as open along a suture as seen in other cases (Fig. 39) to enable merozoites type I to leave the meront once the first merozoites have completed budding off the rudimentary body. This way, not all merozoites type I leave at once and co-invade cells, but they invade cells with a higher distribution to cover a larger area and contribute to the success of the parasite infection.

Merozoites type I are released to infect cells of the same organism (O'Donoghue, 1995). Trophozoites that originate from merozoite type I invasion can either continue in the asexual life cycle by developing new meronts I, or initiate the sexual life cycle by forming meronts type II (O'Donoghue, 1995).

Fig. 42a shows a merozoite type I apparently ‘swimming’ freely through the apical brush border of its host cells. Merozoites type I measured approximately 2 μm in length and 0.5 μm in diameter with a pointed apical tip and a blunt posterior region (Figs. 41a-c). Free merozoites type I appeared to make host cell contact not only with the apical tip but alongside the whole parasite surface (Figs. 42b-d and Fig. 43). As the TEM cross section in Fig. 43 shows, it is most likely that merozoites type I establish host cell contact via their entire surface. Consequent reorientation of apical organelles within the parasite is thought to occur to enable host cell invasion (Huang et al., 2004). The short and stout shape of the merozoite type I observed in Fig. 42d might be a result of the apical organelle reorientation, leading to host cell invasion as seen in Fig. 42d. Once a merozoite type I has established host cell contact, host-parasite
interactions take place leading to parasite encapsulation and trophozoite formation (Fig. 42d).

Fig. 41: Scanning electron micrographs of meronts I 5 days post inoculation. a) Two meronts I of different form. b) Classical meront I with 6 mature merozoites type I. c) Meront I with 8 merozoites type 1 and a residual body (r). d) Immature meront I with merozoites ‘mz’ still budding off the rudimentary body ‘b’. b=rudimentary body, mz=merozoite, r=residual body.
Fig. 42: Scanning electron micrographs of merozoites type I at day 1 of culture.

a) Merozoite ‘swimming’ freely through the microvilli brush border. b) Merozoite on the host cell surface. c) Merozoites in host cell contact. d) Merozoites in the course of host cell invasion. An arrow labeled ‘m’ in image d) indicates the junction between the apical membrane surrounding the invader and the host cell. Another arrow indicates the contact region between two co-invading merozoites.
Fig. 43 shows a cross-section through a merozoite that had established host cell contact. Structures in the contact region between the parasite and the host cell may have resulted from receptor interactions between the parasite and its host, mediating parasite-host contact. Note how the apical end ‘a’ of the merozoite points towards the host cell, suggesting that reorientation of apical organelles had just started to take place. Visible within the merozoite are internal organelles as described in sporozoites by Tetley et al., (1998). The most abundant ones are labeled ‘r’ for residual bodies and ‘rhp’ for rhoptries.

Fig. 43: Transmission electron micrograph of a merozoite type I 5 days post inoculation in dense host cell contact ‘cn’. a=apical end, cn=contact region, r=residual body, rhp=rhoptry.
Fig. 44: Scanning electron micrographs of merozoites type I with lateral protrusion (arrows) on the host cell surface at day one post inoculation.

In contrast to the images of merozoite type I seen in Figs. 42a-d, many merozoites possessed a lateral protrusion as seen in Figs. 44a-d. Immunogold labeling of *C. parvum* surface receptors confirmed this lateral bulge as an important feature in the life cycle of *C. parvum* (Figs. 45-48), with its main function in the host cell contact and invasion process. Fig. 45b shows that receptors were ubiquitously distributed over the entire merozoite type I surface apart from one area: the lateral protrusion. The lateral protrusion showed the highest density of surface receptors (Fig. 45b) leading to the suggestion that it plays a mayor role in the establishment of host cell contact. Indeed, the establishment of host cell contact via this lateral protrusion was observed with immunogold labeling in Figs. 46 and 47. The finding of this lateral bulge
suggests the possibility that apical organelle contact to host tissue might not occur to mediate initial host cell contact of merozoites type I. Apical organelle reorientation probably occurs during the invasion and developmental processes of the invading parasite, as it is an essential prerequisite of host cell invasion of apicomplexan parasites (Blackman et al., 2001) and brings internal structures into the right location for trophozoite development. Not all merozoites type I observed at any one time possessed the lateral protrusion, suggesting that it is produced at a certain time point after merozoite emergence from the meront. This is supported by findings shown in Figs. 44a-d, with the merozoite in Fig. 44a just starting to form the lateral protrusion, whereas for the merozoite in Fig. 44d the protrusion is well developed. These observations demonstrate that the protrusion develops internally within the merozoite type I.

The observation that C. parvum surface receptors may be left behind on the host cell next to the attaching merozoite in Fig. 47b, supports the hypothesis that merozoites type I initially contact the host cell via their entire surface as suggested by the TEM image in Fig. 43.

Merozoites type I can not only adhere to host tissue, but also to each other (Figs. 42c and d and Fig. 48). This is most likely to occur via parasite-parasite surface receptor interactions as the high density of surface receptors in Fig. 45 suggests. Whether two adhering merozoites utilise the lateral organelles described above for this purpose remains speculative as the establishment of host cell contact may depend on receptors present in this area. Adherence of infective zoites to each other leads to co-invasion of host cells as observed, for example, in Fig. 32.
Fig. 45: Scanning electron micrographs of a merozoite type I confirmed by immunogold labeling of surface receptors, measuring approximately 0.5 x 1.6 μm. The apical end of the merozoite points towards the upper right corner of the images. Images a) and b) are images of the same merozoite acquired with SE2 detection (a) and BSD (b) respectively. Note the high density of receptors at the merozoite’s lateral protrusion (arrow in b).
Fig. 46: Scanning electron micrographs of a merozoite type I attaching to host tissue via its lateral organelle (arrow in b). The apical end of the merozoite points towards the lower right corner of the images. Images a) and b) are images of the same merozoite acquired with SE2 detection (a) and BSD mixed with in lens detection (b) respectively.

Fig. 47: Scanning electron micrographs of a merozoite type I attaching to host tissue via its lateral organelle. The apical region of the merozoite points towards the bottom of the images. The large arrow in b) indicates the host cell attachment area, whereas the slim arrow indicates an area where the parasite had contacted the host cell as well, leaving surface receptors behind. Images a) and b) are images of the same merozoite acquired with SE2 detection (a) and BSD mixed with in lens detection (b) respectively.
Fig. 48: Scanning electron micrographs of two merozoites type I. Merozoites type I are adhering to each other via parasite-parasite surface receptor interactions. Images a) and b) are images of the same merozoite acquired with SE2 detection (a) and BSD mixed with in lens detection b) respectively. Surface labeling of *C. parvum* receptors (made visible with BSD in b)) on these two difficult to identify parasite stages, demonstrates how immunogold labeling can help in the identification of parasite stages.

Merozoites, unlike sporozoites, did not seem to attach to host cells via their apical end but along their whole body length (Fig. 43) with a defined attachment organelle at their lateral side (Figs. 44). Studies by Tetley *et al.*, (1998) had identified two crystalline bodies in the interior of sporozoites. Two such bodies were also detected in this merozoite (Fig. 43). The lateral protrusion of merozoites type I might result from one of the crystalline bodies seen in Fig. 43 as it must somehow develop from the parasite. However, this aspect remains very speculative and requires further investigations. Labeling of surface receptors was needed to demonstrate that this lateral protrusion seen on some, but not all merozoites type I, was of *C. parvum* origin.
(Fig. 45). The lateral protrusion indeed showed the highest density of surface receptors (Fig. 45b) leading to the suggestion that it plays a major role in the establishment of host cell contact (Figs. 46-47). Whether it also plays a major role in the initiation of the invasion process remains doubtful, as the apical organelles are known to be the invasion ‘machinery’ (Soldati-Favre, 2008). Merozoites can be expected to share at least some receptors with sporozoites that are involved in the host cell attachment and invasion process. Most studies however have been performed on sporozoites as they are easier to study than merozoites. Nevertheless, a *C. parvum* sporozoite ligand (CSL) to an intestinal epithelial cell receptor has also been localised in the apical complex of merozoites (Langer *et al.*, 2001). An orchestra of other proteins already described in sporozoites might play a similar role in merozoite adhesion and invasion. However, further studies utilising monoclonal antibodies against these receptors in a similarly designed immunogold SEM study would be essential to test this hypothesis. Thus far, it can be concluded that host cell contact is established via the lateral protrusion identified in Fig. 44, followed by apical organelle reorientation (see Chapter 1), followed by host cell invasion initiated through apical organelle discharge (Huang *et al.*, 2004).

Merozoites type I probably adhere to each other via their entire surface rather than the lateral bulge (Figs. 42c and 48), as the receptors in the lateral organelle are needed to establish host cell contact.

It is possible that different types of meronts I release different types of merozoites type I to either form meronts I or meronts II. Different types of merozoites type I might not be distinguishable by their morphology. On the
other hand it is possible that variation and genetic differentiation may occur during the trophozoite development process which determines whether a trophozoite develops into a meront I or II. However, the presence of more than one subtype of merozoites would explain the stouter shape of the merozoite type I in Fig. 44a compared to the slender shape of the merozoite type I in Fig. 44d. According to the morphology of the many merozoites type I observed during the overall experiments, different subtypes of merozoites type I might exist. Genetic diversity amongst merozoites type I may lead to the development of different subtypes of meronts II which were observed in this study and are presented below.

5.4.6. Meronts II and Merozoites type II

Merozoites type I invade host tissue to form meronts I and continue in the asexual cycle but can also invade host tissue to form meronts II and initiate the sexual cycle. In stark contrast to merozoites type I, merozoites type II were round and measured approximately 0.8 μm in diameter. Yet a wide range of variation was seen in the size of merozoites type II, ranging from not much more than the size of a microgametocyte, 0.55 μm to 1 μm (Fig. 49 and Figs. 52-58).

Fig. 49 shows one possible meront II of approximately 3 x 4 μm. In contrast to meronts I, the cell wall of this meront II is thicker, contributing to its larger size. The open meront in this image still contained two merozoites of approximately 1 μm in diameter. The arrow indicating the merozoite points to a ring-like structure that separates a rough region of the merozoite (bottom right) from a smoother, slightly pointed region (top left). The smoother slightly pointed region of a
merozoite type II may be identical to the apical tip of sporozoites or merozoites type I, probably housing the invasion ‘machinery’. The exact number of merozoites type II contained in a meront could not be determined in this study, as only open meronts II which still housed some merozoites could be identified as such. It is most likely that meronts II house approximately eight merozoites type II (Fig. 59a).

Fig. 49: Scanning electron micrograph of a meront II with two internal merozoites type II (arrow) 5 days post inoculation.

Once merozoites type II are released, they attach to host cells and initiate the invasion process similar to merozoites type I and sporozoites (images presented in Fig. 50). Figs. 50a to c suggest that parasite encapsulation by the host cell membrane takes place in a fashion described for sporozoites.
previously. Fig. 50a suggests that microvilli are involved in host cell membrane protrusion visible in that image. Fig. 50c shows how microvilli are incorporated into excess membrane formations. In Fig. 50b an arrow indicates an incompletely closed apical membrane of an invading merozoite type II which is embraced by elongated host cell microvilli.

Like merozoites type I or sporozoites, merozoites type II can adhere to each other to co-invade a host cell as seen in Fig. 50c. Adherence of parasite stages is likely to have occurred already at the parental stage as Fig. 49 suggests. Note how the two remaining merozoites type II are connected to each other in their parental stage.

Without immunogold labeling, *C. parvum* merozoites type II were difficult to distinguish from apoptotic host cell debris (see Figs. 10 and 11 from Chapter 4). Thus, to identify merozoites type II as being of *C. parvum* origin, surface receptor labeling was essential. Fig. 51a shows a large *C. parvum* stage of approximately 5 μm in diameter, which appears to be at the time-point of infective zoite release, similar to the one seen in Fig. 49. Immunogold labeling in Fig. 51b confirmed the stage to be of *C. parvum* origin. The release of the three rounded stages seen in window b) lead to the suggestion that this large stage is most likely to be a meront II rather than a macrogamont (the only other stage of comparable size). Even if unlikely, the impact of sample preparation might have caused sample damage in such a way that the here described stages might be *C. parvum* artefacts.
Fig. 50: Scanning electron micrographs of merozoite type II host cell invasion.

a) Host cell contact. Host cell membrane protrusion initiated by microvilli is indicated with an arrow.

b) Host cell membrane engulfment. Note the incomplete closure of the apical membrane engulfing the invader as indicated by an arrow.

c) Co-invasion of host cell by two merozoites. Arrows indicate the outer membrane surrounding the invaded stages and connecting them to the host cell.
Fig. 51: Scanning electron micrographs of *C. parvum* stages on HCT-8 host cells. a-b) Meront II releasing three merozoites type II. c) Two trophozoites forming next to their parent stage. Arrows indicate surface receptor labeling. Image a) was acquired with SE2 detection and images b) and c) were acquired with BSD combined with in lens detection.

Small round stages detected on the host cell were identified as being of *C. parvum* origin with the help of surface receptor labeling. Such stages were most commonly identified when they were located in close proximity to merozoites type I, which are unmistakable (Fig. 52). They were thought to be a *C. parvum* stage as their surface morphology resembled that of merozoites type I (see Fig. 53). Similar to sporozoites and merozoites type I, merozoites type II became embraced and overgrown by host cell microvilli (Figs. 54a and c and Figs. 55a and b). Merozoites which measured approximately 0.55 μm in diameter showed
surprisingly little surface labeling (Figs. 52b, 54c, 55b and 56b). In comparison, merozoites type II that measured close to 1 μm in diameter showed a large amount of surface labeling. This might be accounted for by the subtype of merozoite or alternatively, the expression of surface receptors might increase during parasite development.

Fig. 52: Scanning electron micrographs of a merozoite type II in comparison to a merozoite type I. Identified with immunogold labeling. Images a) and b) are images of the same merozoites acquired with SE2 detection (a) and BSD mixed with in lens detection b) respectively. These images show a merozoite type I (bottom left) and II (top right). The merozoite type I measured approximately 1.5 x 0.5 μm and the merozoite type II 0.6 x 0.6 μm. The arrow in a) points onto the lateral protrusion commonly found on merozoites type I whereas the arrow in b) indicates surface labeling identifying the merozoite type II as a C. parvum stage.
Fig. 53: Non-immunogold comparison picture of a merozoite type I and II.

Fig. 54: Scanning electron micrographs of merozoites type I and II surrounded by host cell microvilli. Image a) was acquired with SE2 detection. Images b) and c) are magnifications of a) acquired with BSD mixed with in lens detection. Image b) shows a merozoite type I. The merozoite type II seen in c) measures only 0.55 μm in diameter. The arrow in c) indicates immunogold labeling.
Fig. 55: Scanning electron micrographs of merozoites type I and II embraced by host cell microvilli. Image a) was acquired with SE2 detection. b) and c) are magnifications of a) acquired with BSD mixed with in lens detection. The merozoite type II in b) measures 0.6 μm in diameter. The double arrow in b) indicates immunogold labeling on the merozoite type II. The single arrows in b) and c) point to host cell microvilli that have overgrown merozoites type I.
Fig. 56: Scanning electron micrographs of two small merozoite type II (0.55 x 0.55 μm) on the host cell surface. Images a) and b) are images of the same merozoites acquired with SE2 detection (a) and BSD (b) respectively. Two small merozoite type II (0.55 x 0.55 μm) on the host cell surface. The merozoites adhere to each other and to the host cell. Groups of merozoite type II surface receptors are indicated with arrows in b).
Fig. 57: Scanning electron micrograph of a larger merozoite type II on the host cell surface. BSD mixed with in lens image of a merozoite type II measuring approximately 0.75 x 0.75 $\mu$m in diameter. Note the higher amount of surface labeling and thus receptor expression as compared to smaller merozoites type II.

Fig. 58: Scanning electron micrographs of a large merozoite type II on the host cell surface. Images a) and b) are images of the same merozoite acquired with in lens detection (a) and BSD mixed with in lens (b) respectively. This merozoite type II measuring 0.95 x 0.95 $\mu$m. Note the even higher amount of receptor expression.
Considering the variety of stages that merozoites type II can possibly develop into, it is likely that merozoites type II of different sizes represent different subtypes of this developmental stage. They develop into a wide range of gamont-like stages which will be presented in subsequent sections of the present Chapter 5. However, the different sizes of merozoites type II may be caused by the sample preparation procedure. This shrank stages a little more than without immunogold although it only accounts for minimal differences of 1.05 μm in Fig. 49 in contrast to a size of 0.95 μm with immunogold labeling. As described for merozoites type I, different subtypes of merozoites type II might exist, accounting for the variation in size of 0.55 to 1 μm that has been observed in merozoites type II. One subtype of merozoites type II may exist which invades host cells to form microgamonts, whereas another subtype may invade cells to form macrogamonts. Further, these two different subtypes might be subdividable into different subtypes of micro- and macrogamonts, which will be explained later. As speculated for merozoites type I earlier, it may also be possible that differentiation occurs within the trophozoite following host cell invasion by merozoites type II that are undifferentiated. This possibility however would not explain the different sizes observed amongst merozoites type II. Therefore different types of merozoites type II are thought to be present.

If there are different subtypes of merozoites type II this poses the question whether different subtypes of meronts II exist which vary in their morphology. The occurrence of two different types of meronts (meront I and meront II) in the life cycle of *C. parvum* is a well documented phenomenon (O'Donoghue, 1995). Diversity within the same life cycle stage as reported in the present study for *C. parvum*, has not been reported for other apicomplexan species. However, for a
different species of Cryptosporidium, C. baileyi, three meront generations have been described (Current et al., 1986) whereas for some other species (for example Cryptosporidium sp. from lizards) only one meront generation had been reported (Ostrovska and Paperna, 1990). These observations support the suggestion that more than two different types of meronts can exist and contribute to the life cycle of C. parvum.

It must always be kept in mind that this study was performed in vitro, including many different parasite cultures. Therefore future in vivo experiments should be undertaken to support these observations.

5.4.7. Novel Meront-Like Stages

Until the present study a meront II was thought to resemble the stage in Fig. 49. The results of this study suggest that a number of different forms of meronts II may exist. Each distinct subtype of meront II is thought to release merozoites type II that, dependent on their subtype, form one of the many different gamont stages that have been discovered in the present study and will be presented in the following section.

Fig. 59 shows a different type of meront II that was discovered in cell culture after 4 and 5 days, which has not been described previously. This novel meront-like stage contains at least eight merozoites (Fig. 59a, one of which is indicated with an arrow labeled ‘z’) which develop into a trophozoite stage of yet unknown future, without resolving contact with the rudimentary body of the parent stage and thus without leaving the parent meront. This meront was very large, reaching a diameter of approximately 8 μm with an overall round shape (Fig. 59b). The parent stage had a stalk-like connection to the host cell as indicated
with arrows labeled ‘s’ in Figs. 59a and b. This stalk-like connection probably serves as a feeding connection for the parent stage, and later on for the developing daughter stages. Note how every single daughter stage within the novel meront is engulfed by a smooth membrane, resembling the apical membrane of trophozoites. Fig. 59a possibly shows an earlier stage as the membranes of the single daughter stages still have openings and were relatively small, approximately 1.3 μm in diameter. New growth of host cell membrane might be initiated to engulf daughter stages. Fig. 59b shows a more mature stage with every daughter stage having reached the size of a trophozoite, approximately 2.5 μm in diameter and with a complete apical membrane. Note the highly perforated remnants of the apical membrane, which had once been the parent stages’ outer membrane, in between the single daughter stages. It is possible that the parasite also utilises material of the parent stage’s apical membrane to engulf the daughter stages. However, the incomplete closure of the membranes engulfing each single zoite in Fig. 59a suggests that more material is needed and new host cell material is employed to engulf each daughter stage. Thus, the membrane engulfing each single daughter stage may consist of remnants of the old parent cell membrane as well as newly formed membranes using fresh host cell material.

The development of this meront II might lead to the clusters/ accumulations of microgamonts and other gamont-like stages that have been observed frequently along the host cell surface from 5 days onwards. That the formation of this different meront was only observed at the initiation of the sexual life cycle, supports its nature and place in C. parvum’s life cycle.
Fig. 59: Scanning electron micrographs of novel meronts II. a) 5 days post inoculation. b) 4 days post inoculation. Note how both parent meront II stages are connected to host tissue via a stalk-like attachment organelle labeled ‘s’. Each parent stage houses more than eight daughter stages labeled ‘z’, each of which is engulfed by its own apical membrane. s=stalk, z=zoite
5.4.8. Gamonts

Merozoites type II develop into a range of gamonts. All gamonts start as a trophozoite and develop into a different size, shape and morphology dependent on their destiny. Gamonts are divided into two major categories: macrogamonts ♀ and microgamonts ♂. Macrogamonts are fertilised by microgametocytes released from microgamonts. Upon fertilisation some macrogamonts develop thick-walled oocysts that are passed in the faeces to spread from host to host. Other macrogamonts develop into thin-walled oocysts or different auto-infective gamont-like stages which are described in this section. Diversity has not only been observed amongst macrogamonts but also amongst microgamonts. Yet, if diversity amongst microgamonts has any impact is speculative, as their part in C. parvum’s life cycle consists of releasing gametocytes for the fertilisation of macrogamonts.

The final extracellular location of some parasite stages appeared to occur through the breakage of host cell contact (as seen in Figs. 72-73 and 76) rather than through cell-free development as suggested by Hijjawi et al., (2004). Some micro- (Figs. 62c and d and Fig. 76) and macrogamont-like stages (Figs. 70-73) broke host cell contact before completion of the life cycle as they still contained their endogenous stages. It is most likely that gamonts exhibit the same behaviour in vivo. Gamonts gain advantage through the breakage of host cell contact. Breaking of host cell contact enables gamonts to travel through the lumen of the small intestine, fertilise each other and infect the host in a different area of the small intestine, thus spreading the infection within the same host.

However, not all gamonts observed in this study broke host cell contact. At least half of all gamonts observed retained a dense connection to their host cell upon
their time point of maturation and gametocyte or infective zoite release, leaving their emptied structures behind on host cells (see Figs. 61a, 65a and 70).

### 5.4.8.1. Microgamonts and Microgametocytes

Microgamonts were seen on the surface of host cells as early as 2 days of culture (Fig. 60b). Their early occurrence in culture could be explained if the inoculant consisted of other stages than the expected thin-walled and thick-walled oocysts. The discovery of large free gamonts, see section 5.4.8.2., opens the possibility that the inoculated oocyst stock may have contained other stages apart from the proposed oocysts. Stages like the large gamont were possibly isolated together with oocysts. Such stages inoculated into culture could have infected cells to form microgamonts directly, without prior meront I and meront II formation. However, other species of *Cryptosporidium* are known to complete their life cycle in as little as 2 days (O’Donoghue, 1995). Whether this is possible for some isolates of *C. parvum* remains speculative and will be discussed in more detail in the General Discussion. The microgamont seen in Fig. 60b was an isolated finding and apart from that, microgamonts occurred after 4 days of culture with their highest prevalence after 5 days. They appeared on the host cell surface similar to trophozoites that have been described earlier, for example in Fig. 31. Microgamonts were round in shape reaching approximately 3.5 μm in diameter at the time point of maturation and microgametocyte release (see for example Fig. 61b). Yet, not all microgamonts reached the same final size. Variations between 2 μm (Fig. 61d) and 4 μm (Fig. 62c) were observed. As already mentioned above, two different types of microgamonts were observed: attached and free microgamonts. Both types of
microgamonts produced stalk-like attachment organelles (compare Figs. 61a of an attached microgamont to 62d of a free microgamont). Variations in size amongst microgamonts and microgametocytes were observed in this study. The different sizes for microgamonts were seen in context with two different morphologies that will be explained below.

Like meronts, microgamonts were found to contain a rudimentary body at the host-parasite interface from which microgametocytes are formed and released via budding off the body (Fig. 61a). In some cases the rudimentary body is located above a stalk-like structure, here referred to as “stalk”, which lifts the microgamont off the host cell (Figs. 61a and b). Similar stalk-like structures have also been described by Valigurova et al., (2008). The stalk might enable the whole microgamont to break host cell contact. This phenomenon was observed in Fig. 61d which shows an accumulation of microgamonts, with their stalks pointing upwards after breaking contact with the host cell. Fig. 61c shows how such a stalk forms during the early developmental process of the parasite stage. However, not only microgamonts with the long stalk seen in Figs. 61b and d break host cell contact. Figs. 62c and 62d reveal how entire microgamonts can become released from their attachment site to swim free in the lumen of the gastrointestinal tract (here culture media). These microgamonts show a different morphology than the ones with the long stalks. The microgamont seen in Fig. 62c was round with a suture-like opening for gametocyte release. The microgamont in Fig. 62d on the other hand resembled the one described in Fig. 61a, having taken the entire stalk-like structure with it after breaking off. Nevertheless, not all microgamonts break contact with the host cell. Figs. 60a and b demonstrate how parent stages remain attached and
release fertile microgametocytes into the lumen of the intestine (here culture media). Their thick membrane initially opened along a suture for gametocyte release (Figs. 60a and b, 61b and 62c). Figs. 60a, b and Fig. 61b show how each microgamont is packed with a large number of microgametocytes at a high density. The microgamonts of *C. parvum* observed in this study appeared to contain approximately 50 fertile gametocytes, according to the images presented in this chapter. Microgamonts were seen to accumulate in culture and even adhere to each other because of non-specific surface receptor interactions (Figs. 62a, b and 63). As microgametocytes rely on chance to contact a macrogamont, as they lack any flagella, it comes as no surprise that *Cryptosporidium* produces such a large quantity of microgamonts. According to the results of this study a microgamont contains approximately 50 microgametocytes, in contrast to the proposed 12 microgametocytes in studies by Hijjawi *et al.*, (2001). Perhaps a meront was mistaken for a microgamont in previous light microscopic studies and a microgamont like the ones described here had not been visualised before.

Microgametocytes were shaped like red blood cells measuring approximately 0.2 x 0.4 μm. However, their exact size was difficult to determine due to their small nature. Microgametocytes appeared to adhere randomly to any *C. parvum* stages (Fig. 62a) as well as to host tissue (Fig. 62b and Fig. 63). Fig. 63 is a magnification of Fig. 62b, revealing a ring-like structure on each microgametocyte as already suggested by close examination of Figs. 60a and 61b. As microgametocytes initially showed a red blood cell-like shape, the ring-like structure appeared to develop subsequently. This ring-like structure
probably enables the microgametocytes to adhere to and fertilise macrogamonts.

Fig. 60: Scanning electron micrographs of microgamonts at the stage of microgametocyte release on the host cell surface after a) 69 hrs and b) 48 hrs. Notice how the microgamonts open up along a suture-like opening (arrows) to release their fertile gametocytes ‘gz’. gz=gametocyte.
Fig. 61: Scanning electron micrographs of microgamonts with a stalk attached to the host cell after a, b and d) 5 days and c) 48 hrs. a) Microgametocytes are initially formed by budding of a rudimentary body ‘b’. Images b)-d) Observe how the stalk ‘s’ enables the microgamonts to break host cell contact. b=rudimentary body, s=stalk.
Fig. 62: Scanning electron micrographs of microgamonts and microgametocytes after 5 days of culture. a) Microgametocytes (arrows) randomly attaching to merozoites type I. b) Microgametocytes (arrows) randomly attaching to host tissue. c) Large microgamont without visible host cell contact. d) Microgamont after breakage of host cell contact. s=stalk.
Fig. 63: Magnification of Fig. 62b) showing microgametocytes on host cell. This high magnification image of microgametocytes randomly adhered to host tissue reveals a ring-like structure at one end of each microgametocyte (arrows).

Microgametocytes were seen to be budding off a rudimentary body as it has been described for merozoites. However, unlike the smooth rudimentary body of meronts I, the rudimentary body of microgamonts resembled the feeder organelle attachment area of the macrogamont see in Fig. 70. Granular structures of feederorganelle lamellae had already been observed by Valigurova et al., (2008). However the present study proposes that this so-called feeder organelle actually resembles the rudimentary body that zoites or gametocytes are formed from and that the feeder organelle locates below within the host cell (see summary, Fig. 88). A cross-section through a couple of microgamonts in Fig. 64 shows how the feeder organelle - that has been described in for example a standard trophozoite in Fig. 31 or for a developing macrogamont in Fig. 30a - and the stalk that has been described for
microgamonts in this chapter, are one and the same structure. These findings suggest that the granular structure actually resembles the rudimentary body of parasite stages, whereas the feeder organelle locates underneath these structures within the host cell, probably densely connected to the host membrane to enable nutrient uptake by the parasite from the host cell.

Not only does the stalk-like structure of microgamonts (Fig. 64) resemble those of macrogamonts (Fig. 30a and Fig. 70), but it also resembles the intracellular arrangement of organelles shows similarities. A peripheral location of nuclei in macrogamonts had been described earlier by Hijjawi et al., (2001) and was also seen in Fig. 30a. The cross-section of a microgamont in Fig. 64 reveals that also in a microgamont the nucleus (n) resides in a peripheral location, rather than in the centre. The nucleus seems to be encapsulated by a parasite membrane similar to the one described in Fig. 30, which separates the nucleus from the rest of the cytoplasm containing the fertile microgametocytes (gz). The cross-section through the larger of the two microgamonts in Fig. 64 shows two microgametocytes (labeled with arrows) located in vesicle-like vacuoles, just beneath the apical surface of the parent stage, probably just prior to their emergence from the microgamont. This would enable the microgamont to release its gametocytes one by one, rather than all at once, an ability through which the parasite would gain advantage as movement of intestinal fluids would distribute the microgametocytes to a larger area.
Fig. 64: Transmission electron micrograph of two developing microgamonts, 3 days post inoculation. Both gamonts connect to the host cell via their feeder organelle ‘f’ which appears as a stalk-like structure ‘s’. Gametocytes ‘gz’ are formed in the cytoplasm, possibly encapsulated in vesicles for their release. Note the peripheral location of the nucleus ‘n’ of the larger of the two gamonts. 

f=feeder organelle, gz=gametocyte, n=nucleus, s=stalk.
Since the morphology of microgamonts revealed in this study has never been described in previous studies, immunogold labeling of specific *C. parvum* surface receptors was applied to demonstrate that the microgamonts and gametocytes are *C. parvum* stages. Fig. 65 shows an open microgamont with a small amount of surface labeling. There was no internal labeling detected which is to be expected as the antibodies used in this study were directed against surface epitopes.

![Scanning electron micrographs of an open microgamont. Arrows in b) point onto specific *C. parvum* receptors labeled with immunogold particles. Image a) was acquired with SE2 detection. Image b) is a magnification of a) acquired with BSD.](image)

On the other hand, free microgamonts showed a considerable amount of surface labeling (Fig. 66). Due to their small nature, free microgametocytes were virtually impossible to identify in culture without the use of surface labeling.
Fig. 66: Scanning electron micrographs of a microgametocyte on host cell surface measuring approximately 0.3 x 0.3 μm. Image a) was acquired with in-lens detection. Image b) shows the same stage as a) acquired with BSD.

Once microgametocytes are released they swim free in the lumen of the intestine (here culture medium) in the hope to make contact with a macrogamont. Figs. 67-69 show microgametocyte attachment to large *C. parvum* stages which may very well be macrogamonts. Note how the free microgametocytes in Figs. 67-69 display a higher number of surface receptors than the large *C. parvum* stage they are attached to. The density of surface receptors was especially high at the area of attachment (Fig. 68b) which correlates with the finding of the ring-like attachment structure from Fig. 63.

Microgametocytes depend on their surface receptors to adhere to a macrogamont, whereas macrogamonts have a larger surface area to offer. The large stages seen in (Figs. 68) showed a considerable amount of surface receptor expression which suggests that *C. parvum* macrogamonts express surface receptors on the apical membrane to facilitate microgametocyte adherence.
Fig. 67: Scanning electron micrograph of large *C. parvum* stages (see box ‘a’) with microgametocytes adhering (see boxes ‘b’ and ‘c’).
Fig. 68: Magnifications of Fig. 67 acquired with BSD. a) Surface receptor expression on the large *C. parvum* stage as indicated by arrows. b, c) Microgametocytes adhered to the large *C. parvum* stages. Note the concentration of receptors at the attachment area in b).
Fig. 69: a) Scanning electron micrographs of a microgametocyte (enlarged in b)) adherent to a large *C. parvum* stage (a). The microgametocyte could only be distinguished via immunogold labeling using BSD in image b).
5.4.8.2. Macrogamonts and other Gamonts

Macrogamonts were given their name because of their size. They are the largest stage formed in the life cycle of Cryptosporidium. Presently the complex nature and variety amongst macrogamonts remains incompletely understood, however, novel information about microgamonts is provided in this section.

In general, a macrogamont is the stage that becomes fertilised by a microgametocyte and, upon fertilisation, forms internal zoites. It is thought that a fertilised macrogamont develops sporozoites within its body (Marshall et al., 1997; Hijjawi et al., 2001), either developing into a thin-walled, auto-infective oocyst or into a thick-walled oocyst for release in faeces. Nevertheless, results from this study have shown that there are several morphological variations amongst macrogamont with a variable developmental potential.

Fig. 70 shows a macrogamont at the time point of maturation and zoite release. Note the extracellular location and granular structure of the feeder organelle (f) which is similar to those of microgamonts. The granular nature of the macrogamont feeder organelle had already been observed and described by Valigurova et al., (2008) in a different Cryptosporidium species, which confirms the results presented here. The macrogamont in Fig. 70 measured nearly 6 x 7 μm a size consistent with earlier literature (O'Donoghue, 1995) and more recent studies (Hijjawi et al., 2001; Valigurova et al., 2008). Its apical membrane had become perforated, remaining as a dead remnant which is not required anymore. The area of apical membrane closest to the feeder organelle suggests that it once had been smooth. The zoite that was released measured more than 2 μm in diameter with an oval shape. Macrogamonts like the ones seen in Figs. 70-73 probably do not develop into an oocyst, and are not passed
in the faeces but reinfect the same host. Their wall appeared even thinner than that of thin-walled oocysts (Fig. 81) as it became perforated (died off) before infective zoites were released (Figs. 70 and 79c and d), instead of possessing a suture. Internal zoites that were released into culture (Figs. 70 and 79c and d) had either transformed from a sporozoite to a trophozoite internally or were never a sporozoite. The results from this study support the proposal that zoite development in macrogamonts originated from sporozoite-like precursors, but which were different in morphology (see. Fig. 70); probably closer to the morphology of merozoites, yet larger in size.
Fig. 70: Scanning electron micrograph of a matured macrogamont type 1, 4 days post inoculation. Note the extracellular location and granular morphology of the feeder organelle ‘f’, the perforation of the outer membrane and the shape and size of the released zoite ‘z’. f=feeder organelle, z=zoite.
Many macrogamonts were hard to identify as most looked like the one seen in Fig. 71a rather than that in Fig. 70. The results of this study suggest that a macrogamont may develop into more than the two possibilities known and accepted today. As strong variations in the morphology of macrogamonts was detected, the existence of different subtypes has been suggested above. Apart from the formation of thin-walled (Fig. 80) and thick-walled oocysts (Fig. 76c), the possible formation of extracellular large gamont-like stages (see i.e. Fig. 73) and macrogamonts that did not develop into an oocyst before the release of endogenous stages (see Figs. 70 and 71) was observed. (As such stages have not been described previously, it must be considered that they could be artefacts due to the sample preparation procedure – C. parvum stages to denatured to be indentified.) These possible large free gamonts and macrogamonts that did not form oocysts occurred more frequently in this study than newly formed oocysts. The reason for this may be that the culture was terminated after 5 days of observation. On the other hand, in vitro culture systems may favour the development of different stages compared to their occurrence in vivo.

The macrogamont in Fig. 71a was identified by its attachment to the host cell as indicated with an arrow. In stark contrast to the macrogamont seen in Fig. 70, this one left a large attachment area behind with a width similar to that of the entire macrogamont, rather than possessing a small and precise feeder organelle attachment structure. The size of the macrogamont in Fig. 71a was the same as the one in Fig. 70 with approximately 6 x 7 μm, supporting the hypothesis that this C. parvum stage is another subtype of macrogamonts. Like the macrogamont in Fig. 70, the one shown in Fig. 71a had a perforated outer
membrane. Other macrogamont-like stages resembling the one in Fig. 71a were seen in culture after 5 days (Figs. 71b, c and 72b). All stages had in common that their outer membranes were highly perforated. Perforation of oocyst membrane had already been described by Hijjawi et al., (2001) and appeared to occur during maturation to facilitate the release of endogenous stages. Further, all stages described in this study showed host cell attachment, and were thus identified as C. parvum stages. The macrogamont in Fig. 71b appeared slightly larger than the one in Fig. 71a. It was found in dense host cell contact next to a developing trophozoite of yet unknown future, which was clearly engulfed by, and anchored into the host cell membrane. Another such trophozoite-macrogamont pair is shown in Fig. 71c. Unlike the two types of macrogamonts described so far, the macrogamont in Fig. 71c was not oval to round in shape but measured approximately 10 μm in length and only 4 μm in width. It is of course possible that the macrogamonts seen in Figs. 71a and b would have still developed into such a stage, but as they had already resolved host cell contact, the finding of the large macrogamont may be another (third) subtype of macrogamont. This subtype is referred to here as the large gamont-like stage. Similar gamont-like stages have already been observed in the course of this study with light microscopy of cell-free culture (see chapter 3, Fig. 9). Previous studies by Hijjawi et al., (2002) had also observed “novel” gamonts with light microscopy for the species C. andersoni isolated from the intestine of mice. The similarity of this large gamont-like stage to a developing trophozoite as seen in Figs. 72a and b demonstrates its affinity with C. parvum. Fig. 72a shows how two trophozoites become overgrown by the host cell apical
membrane. In Fig. 72b the same phenomenon of host cell membrane overgrowth is shown for a large gamont-like stage.

Fig. 71: Scanning electron micrographs of macrogamonts/large gamonts, 5 days post inoculation. a) Macrogamont subtype 2 breaking of attachment zone. b) Macrogamont paired with a trophozoite. c) Large gamont-like stage paired with a trophozoite. Note the host cell attachment and perforated membranes of the three stages described here. G=gamont, M=macrogamont, T=trophozoite.
Fig. 72: Scanning electron micrographs of different stages of *C. parvum* origin engulfed by a tertiary host cell membrane. a) Two trophozoites engulfed by host cell membrane. b) Large novel gamont still attached to host cell membrane. G=gamont, m=membrane (host cell), T=trophozoite.

The pairing of the possible large gamont-like stages with trophozoites was a commonly observed phenomenon as Figs. 73a-c further show. The large gamont-like stages observed in Fig. 73 seemed to be mature stages as they were close to breaking host cell contact (Figs. 73a and 73b) or had broken it already (Fig. 73c). As described earlier, a mature stage measured 4 x 10 μm and its outer membrane was completely perforated. Each large gamont possessed a more pointed and a blunter end (Figs. 73a and 73c), similar to the shape of merozoites type I. This stage seems to be produced to break host cell contact and travel in the lumen of the intestine (in contrast to macrogamonts type1), as the stages found still attached to host cells in this study, did not release zoites while still attached.
The possible macrogamonts and extracellular large gamonts as described in Figs. 70-73 occur in a multitude of different shapes, of which some were seen to release endogenous stages in culture, whereas others broke host cell contact prior to maturation. An extracellular location of the feeder organelle like the one seen in Fig. 70 enables macrogamonts to break host cell contact. However, other possible macrogamonts were observed to sever host cell contact differently, without possessing any stalk-like organelle as seen in Fig. 71a. Their breakage of host cell contact presumably results in free swimming macrogamonts like the ones seen in Figs 79c and d that only show an attachment ring on their surface.

With the help of immunogold labeling of *C. parvum* surface receptors, a large *C. parvum* stage of the same size as the large gamont-like stages described above was identified (Fig. 74a). This stage had an attachment zone located in the right-hand corner of Fig. 74c, which was rich in receptors. Nevertheless, it looked as if the entire stage was attached to the host cell along its entire body length. The receptor labeling on the rest of the parasite was less than in the attachment area (Fig. 74b) and there was no labeling detected immediately surrounding the parasite, suggesting that the structure in the right-hand corner of Fig. 74c is the parasite's main host cell attachment area.
Fig. 73: Scanning electron micrographs of large gamont-like stages ‘G’. Arrows indicate where gamonts have contact to *C. parvum* trophozoites.
Fig. 74: Scanning electron micrographs of a) large gamont-like stage identified with immunogold labeling of surface receptors. b) Label was detected over the entire surface indicated by arrows. c) The density of *C. parvum* receptors was particularly high in the parasite attachment area as indicated by the lower arrow in c). Images b) and c) are higher magnification of a) acquired with a mixture of BSD and in lens detection.
Since macrogamonts release their endogenous stages into culture without transforming into an oocyst and large gamonts break host cell contact to travel free in the lumen of the small intestine, stages other than thick-walled or thin-walled oocysts exist that break host cell contact, travel free in the lumen of the intestine and thus possibly become isolated together with thin-walled oocysts, as proposed earlier.

What accounts for the, at least, five different morphological forms of macrogamont-like stages (thick-walled oocyst, thin-walled oocyst, free large gamont, free macrogamont, attached macrogamont) and two different morphological forms of microgamonts (attached and free) that have been observed in this study still remains speculative. The possibility of diversity amongst merozoites type II has already been mentioned above. On the other hand, it is possible that upon merozoite type II invasion, genetic regulation determines which final stage a macro- or microgamont develops into.

5.4.8.3. Attachment Zone

When gamonts break host cell contact, feeder organelles, stalks, rudimentary bodies and residues of the parasites’ apical membranes are left behind, and are referred to here as attachment zones (Figs. 75-77). Some stages of *C. parvum* did not initially release infective zoites or fertile gametocytes into culture, but first broke attachment to their host cell altogether. Such stages include microgamonts (see Figs. 62c and 62d) and the different types of macrogamonts and newly formed oocysts (for example see Figs. 70, 77a and 77c). The same phenomenon has never been observed for *C. parvum* meronts. In contrast to gamonts, meronts I and II were observed to retain dense host cell contact
during their entire life-span. Observations from this study demonstrated that the further a *C. parvum* stage was progressed in the *C. parvum* life cycle, the less host cell contact it showed at the time point of maturation. This eventually enables gamonts to sever host cell contact completely in order to spread the infection within the same host or to other host.

When gamonts of *C. parvum* broke host cell contact, in many places attachment zones were left behind, which consisted of remnants of the apical membranes that once engulfed the parasite and feeder organelle structures (Fig. 75). In studies by Valigurova *et al.*, (2008) similar attachment zones have been described. The four attachment zones in this image all showed a slightly different morphology, suggesting that they were associated with morphologically different parasite stages. In the top right attachment zone of Fig. 75, a small round feeder organellar attachment area is visible in the centre (f). The attachment zone in the top left corner in contrast shows a broader attachment area (f) at the base of the attachment zone. Unlike the top right and the top left attachment zone, the membranes of the fourth attachment zone were not highly folded (mf). The fourth attachment zone neither showed folded membranes nor any distinct attachment organelle at its base. The lower attachment zone might have accommodated a round internal stage as seen in Figs. 76b and c, and the one at top right most likely a macrogamont like that seen in Fig. 70 or Figs. 77a and c. In contrast, the top left attachment zones were probably associated with microgamonts possessing defined stalks similar to those seen in Fig. 62d. However, the morphology of an attachment zone alone is not sufficient to determine the nature of the *C. parvum* stage. As the results presented in Figs. 60-62 as well as Figs. 70-73 have demonstrated, micro and macrogamonts
exhibit at least three different behaviours: retain host cell contact during their entire life-span, break host cell contact but leave their attachment organelle behind or break host cell contact but retain their attachment organelle. Each of these different behaviours would result in a different morphology of the attachment zone.

Fig. 75: Scanning electron micrograph of gamont attachment zones 5 days post inoculation. mf=membrane folds, commonly associated with parasite membrane attachment to host cell, f=feeder organellar attachment area.

The parasites leaving the top three attachment zones in Fig. 75 presumably severed host cell contact by employing a mechanism similar to controlled-apoptosis along a ring-like structure marking the boundaries of the attachment zones seen in Fig. 75. Figs. 76a and b show two microgamonts in the process
of leaving attachment zones similar to those seen in Fig. 75. Fig. 76a shows how the microgamont took some apical membrane with it following detachment whereas the remnants stayed behind on the host cell. In Fig. 76b the ring-like structures, are indicated by arrows. The left arrow indicates the ring-like structure where the apical membrane surrounding the parasite was cut; the arrow on the right marks the boundary where the contact to the host cell along the host-parasite interface was severed.

However, there appears to be another way for *C. parvum* stages to leave their protective niche. The attachment zone at the bottom of Fig. 75 looked different to the other three. It appeared as if the stage had opened its membrane to release endogenous stages. Indeed, the release of endogenous stages from a similar site has been observed. Figs. 76c and d shows attachment zones similar to the one at the bottom of Fig. 75, still containing the endogenous stages as indicated with arrows. Surprisingly, the attachment zones in Fig. 76c and d appeared to contain more than one endogenous stage. The TEM cross-section of a *C. parvum* trophozoite in Fig. 30b suggests that more than one *C. parvum* stage can develop below the same apical membrane and in this case the entire membrane would need to open up for the release of endogenous stages. From this new finding the question arises; why entire stages are released rather than continuing the life cycle where they are. As already explained earlier, the parasite gains advantage if gamonts travel in the lumen and fertilise and infect a host at a different location. However, the morphology of the stages observed in Figs. 76c and d suggests that these stages, which are released into culture, might not be microgamonts (as it is thought to be the case for Figs. 76a and b) but undescribed stages, possibly infective to host cells.
Fig. 76: Scanning electron micrographs of different extracellular *C. parvum* stages. Release of entire *C. parvum* endogenous stages from their attachment zone. Images a) and b) show single stages resolving host cell contact by severing contact along ring-like structures as indicated with arrows in image b). In images c) and d) one parasite membrane opens up to release entire *C. parvum* stages that have formed internally. More than one endogenous parasite stage was encapsulated by the same apical membrane as indicated by arrows.
Not only attachment zones similar to those described in Fig. 75 were left behind on the host cell surface, but also stalk-like organelles containing the feeder organelle and the *C. parvum* rudimentary body were found along the host cell surface (Figs. 77d-f). Discarded stalks with the same morphology have already been described by Valigurova *et al.*, (2008) for *C. muris*.

The discarded stalk-like structure indicated ‘s’ in Fig. 77d showed membrane folds similar to those described for the attachment zones in Fig. 75. Two stalks in Figs. 77e and f showed similar membrane folds which embrace a rudimentary body. The rudimentary body could be identified as such, as in Fig. 77e two microgametocytes were seen attached to the discarded rudimentary body on top of its stalk-like feeder organelle structure. Such residual stalks which still contained the rudimentary body as part of their structure, demonstrate that a stage had not broken host cell contact but released endogenous stages at its original location. As already mentioned earlier, some microgamonts broke host cell contact before the time point of gametocyte release whereas others released gametocytes at their original location.

The same applies to macrogamonts. Fig. 77a shows a macrogamont measuring approximately 6 x 7 μm, which severed host cell contact between the feeder organelle and the host cell, taking its feeder organelle with it as indicated by an arrow. Fig. 77c shows a newly formed thick-walled oocyst still containing an attachment organelle like the macrogamont in Fig. 77a. The stage seen in Fig. 77c was identified as a newly formed thick-walled oocyst as it possessed the same morphology as thick-walled oocysts from the inoculant (Fig. 21a) with a suture for sporozoite release. However, the newly formed thick-walled oocyst in Fig. 77c still possessed a stalk. Studies by Valigurova *et al.*, (2008) showed that
oocysts are initially produced with a stalk. It is possible that some oocysts take their stalk with them when breaking host cell attachment and that detachment from the stalk occurs at a later time point. The loss or retention of the stalk in *C. parvum* gamont-like stages probably depends on whether the contact is broken first with the host (Figs. 77a and c) or with the stalk (Fig. 77d). Observations made in this study suggest this is a random process.

The process of a gamont-like stage breaking host cell contact but retaining its feeder organelle is thought to leave a hole-like attachment area behind in a host cell, similar to the one seen in a dead host cell in Fig. 77b. The occurrence of holes in the host cell surface was initially overlooked in this study. Fortunately, research by Huang *et al.*, (2004) identified a tunnel-like feeding connection between a parasite and its host cell. These holes seen in this study might be similar to the tunnel-like feeding connection described by Huang *et al.*, (2004), if they are not due to cell damage in the course of sample preparation. Thus, Huang’s findings help to explain the holes seen in the host cell membranes in this study. On two occasions, holes were observed in the host cell monolayer (Figs. 77c, 85c). The hole seen in Fig. 77c was located next to the newly formed thick-walled oocyst that had retained its stalk. The hole in Fig. 85c was seen next to a large round parasite stage which showed a hole of equal size at its base. The large parasite stage in Fig. 85c had probably been attached to the host cell via this tunnel-like connection. The finding of three smaller holes in the host cell surface around the hole in Fig. 85c, indicated by an arrow, resembles the attachment zones of three smaller parasite stages. These three smaller stages might be daughter stages which have broken host cell contact and left
their attachment site. Fig. 85d shows a parasite, which as it severed contact from its attachment zone ‘m’, showed a hole-like gap in its outer membrane, as indicated by an arrow.

These observations thus support those of Huang et al., (2004). However, either the formation of a tunnel-like connection is uncommon or most host cells are still alive after the breakage of parasite-host contact and the holes close again rapidly.
Fig. 77: Scanning electron micrographs of different *C. parvum* stages and stalk-like host cell connections. a) Extracellular macrogamont with stalk, 5 days post inoculation. b) Attachment zone left behind in a dead host cell after 3 days. c) Thick-walled oocyst still containing a stalk after only 2 days of culture. d, e) Discarded stalks of a microgamont after 5 days. f) Discarded stalk and rudimentary body after 2 days.
5.4.9. Zoite Development from within Parent Stage

A phenomenon that had already been described in studies by Hijjawi et al., (2004) and Boxell (personal communication, Fig. 78) has also been observed in this study: trophozoite development from within a parent stage.

Findings by Boxell with light microscopy (personal communication, see. Fig. 78) did not conclusively answer the question as to whether trophozoites can develop directly within oocysts. As the studies of Hijjawi et al., (2004) and Boxell were based on light-microscopy it is possible that cross sections through internal sporozoites were mistaken for trophozoites as they appear round. Images presented to support the hypothesis (see Fig. 78) lead to this assumption since the internal sporozoites that, according to Boxell, had internally transformed into trophozoites, still possessed their longitudinal body.

Employing light-microscopy, the cross section through the body of internal sporozoites was mistaken for trophozoites as the cross section through sporozoites is round.

Fig. 78: Light micrograph by Boxell. Light-micrographs of oocysts from water with internal sporozoites. Some sporozoites are indicated by arrows. In the left image all sporozoites remain internal whereas in the right image some sporozoites appear to be excysting.
On the other hand, observations of the present study in Fig. 79c and d show that trophozoite development can sometimes occur directly within the parent stage, without prior contact between the newly forming trophozoite and host cell. Fig. 79a shows an excysted large *C. parvum* stage, possibly an oocyst (arrow labeled ‘O’). Trophozoites were seen to develop directly next to the oocyst, one of which was still engulfed by the membrane of the oocyst (arrow labeled ‘m’). This observation opens the possibility that the sporozoites had started to develop into a trophozoite stage when still being in the oocyst. In Fig. 79a and b however, it appears that zoites develop more commonly once they emerge and make host cell contact, with the newly formed trophozoite still engulfed by the parent cell membrane.

Fig. 79b shows a similar finding. An arrow labeled ‘m’ indicates the membrane of the original parent stages. Three internal zoites have developed into a trophozoite stage without leaving the parent stage. It is possible that they established a feeding connection to the host cell, from within the parent stage. In contrast, the two parent stages seen in Figs. 79c and d had broken host cell contact already as a disc-like attachment structure, indicated with an arrow labeled ‘f’ in Fig. 79c. Both stages presumably resemble oocyst-like stages as their perforated outer membrane is similar to macrogamonts described in Figs. 70 and 71. Both oocyst-like stages had round zoites labeled ‘z’ emerging from their membranes. Again, a similar behaviour has been described for the macrogamont in Fig. 70. It can be assumed that whatever zoite the oocyst-like stage originally contained, developed into a round trophozoite-like stage from within the parent stage prior to the point of zoite emergence from the parent stage.
It must always be kept in mind that the sample preparation procedure causes cellular damage and might lead to rupturing of parasite stages to reveal their endogenous contents.

Fig. 79: Scanning electron micrographs of trophozoite development from within parent stages. a) Trophozoites ‘z’ have developed within the oocyst membrane ‘m’ 48 hrs post inoculation. b) Internal zoites ‘z’ have established host cell contact and developed into a trophozoite stage without leaving the parent stage membrane ‘m’ at day 5. c, d) Zoite ‘z’ development into trophozoites from within the oocyst developed after 5 days of culture. f=feeder organelle attachment, m=membrane, O=oocyst, z=zoite.
5.4.10. What formed the Oocyst?

As already demonstrated in Figs. 77a and c and Figs. 79c and d, *C. parvum* had completed its life cycle after 5 days of culture with the formation of thick-walled oocysts and other macrogamont-like stages. Fig. 80 shows a TEM cross-section of a zygote detected after 5 days of culturing with four internal sporozoites and a shape distinctive for oocysts. However, the zygote measured only 3 x 4 μm and did not show a thick cell wall. Thus, the zygote in Fig. 80 most likely resembles a thin-walled auto-infective oocyst as described by Reduker *et al.*, (1985). Indeed, not only thick-walled oocysts were produced in culture after 5 days (Fig. 77c), but also the development of thin-walled oocysts had begun (Fig. 81). Thin-walled oocysts were identified by comparison to thin-walled oocysts from the inoculant (Fig. 82), which were identified with the help of immunogold labeling and the application of a higher electron accelerating voltage (see Fig. 91 in the next chapter).
Fig. 80: TEM cross-section through a zygote, after 5 days of culture measuring 3 x 3.5 μm. The four structures located in the centre of the oocyst represent four newly formed sporozoites. The arrow indicates the place where sporozoite release might possibly occur.

In contrast to macrogamonts, thin-walled oocysts developed flat on the host cell surface and showed smooth membranes that appeared thin, as the folding of the outer membrane in Fig. 81b suggests. Thus, the developing structures are most likely to be developing thin-walled oocysts, finally developing into a structure similar to the one seen in Fig. 82a. Thin-walled oocysts like the ones described in this section have also been observed in previous studies by Valigurova et al., (2008) in vivo. In contrast to the macrogamont-like stages and thick-walled oocysts described earlier which develop at day 5, the thin-walled oocysts did not seem to possess any stalk-like attachment organelle.
The thin-walled oocysts measured only approximately 2 x 3 μm in their mature form (Figs. 82a-d). The size of thin-walled oocysts measured in this study contrasts to findings made in light microscopic studies by Hijjawi et al., (2001) who reported round structures with a diameter of 5 x 5 μm to be thin-walled oocysts. In the present study however, thin-walled oocysts with a consistent size of approximately 2 x 3 μm were detected 6 hrs post inoculation of the oocyst stock into cell culture (Fig. 82) as well as 5 days post inoculation. As they were shown to be consistent in size, these dimensions are more likely to reflect the correct size and shape of the small thin-walled oocysts as they were acquired with higher magnifications.

The arrow in Fig. 82a indicates a suture for sporozoite release similar to the one seen on thick-walled oocysts in Figs. 21a and 77c. Note the different shapes of all four thin-walled oocysts in Fig. 82. That each of the shapes is in fact a thin-walled oocyst was confirmed with immunogold labeling and a higher acceleration voltage of the SEM electron beam as detailed in the next Chapter (6).

Since numerous thin-walled oocysts were observed at 6 hrs post inoculation it can be concluded that they formed a large proportion of the original oocysts stock. As already mentioned earlier, this is due to harvesting of oocysts directly from the mouse intestines for the harvest of oocysts rather than collecting faeces which would mainly contain thick-walled oocysts.

Thick-walled and thin-walled oocysts might contain two different types of sporozoites as already mentioned earlier.
Thin walled oocysts do not seem to develop via the intermediate formation of macrogamonts as assumed previously by for example Hijjawi et al., (2001). Rather, macrogamonts similar to the ones described earlier in section 5.4.8.2 appear to be independent stages, fulfilling their function in *C. parvum*’s life cycle without further development into oocysts. Precursors developing into either thin or thick-walled oocysts should be referred to as zygotes and not as macrogamonts (which are a life cycle stage on their own).

Fig. 81: Scanning electron micrographs of thin-walled oocysts newly formed 5 days post inoculation. a) A thin-walled oocyst develops flat on the host cell. b) The thin membrane of an oocyst folds.
Fig. 82: Scanning electron micrographs of thin-walled oocysts 6 hrs after inoculation of *C. parvum* oocyst stock measuring approximately 2 x 3 μm. The arrow in image a) indicates a suture for sporozoite release. Note the different shapes of all four oocysts.

5.4.11. Host Cell Parasitism

The TEM cross-sections in Fig. 83b lead to the suggestion that *C. parvum* might interact with the nucleus of infected cells. Fig. 83a demonstrates that the parasite does grow a connection into the host cell as outlined by arrows labeled ‘f’. Fig. 83b reveals a host cell structure located directly underneath the parasite. The structure of this organelle suggests that it is the host cell nucleus, containing the central dense nucleolus surrounded by the ER (as indicated with an arrow) and golgi (not visible). The cellular transport mechanisms of the ER
and golgi are probably used by the parasite to obtain essential amino acids and fatty acids that are synthesised in the nucleus, ER and golgi and packaged and transported by the golgi and ER to the periphery of the host cell. As previously noted, *C. parvum* lacks certain *de novo* synthesis pathways. Thus it is dependent on the host cell for nutrient supply. How the parasite satisfies its feeding needs still remains unclear. Results from this study suggest that the parasite might utilise the host cell organelles including nucleus, ER and Golgi to obtain essential nutrients as explained in section 5.4.3. This ‘feeding’ on the host cell is the likely cause for the premature cell death of infected host cell as explained in Chapter 3.

In Figs. 84a and b the interior of infected HCT-8 cells has disintegrated in a fashion similar to apoptosis. It is possible that the dying HCT-8 cells undergo a natural course of apoptosis. On the other hand, the feeding dependency of *Cryptosporidium* might cause premature host cell death as described above. Host cell death induced by *C. parvum* infections has already been discussed in detail previously (see Chapter 4).
Fig. 83: Scanning (a) and transmission (b) electron micrographs of *C. parvum*’s feeding connection. a) Intracellular anchorman and presumably feeding connection of a trophozoite into the host cell. b) Location of a *C. parvum* stage directly above the host cell nucleus. *db*=dense band, *f*=feeder organelle, *m*=membrane, *n*=nucleus, *no*=nucleolus.

Fig. 84: TEM cross-sections through *C. parvum*-infected HCT-8 cells 3 days post inoculation. Images a) and b) show dying cells following infection by the parasite ‘P’. The host cell nucleus ‘n’ shows signs of disintegration proceeding host cell death. *n*=host cell nucleus, *P*=parasite.
As stated earlier (see Chapter 5.4.3), the location of the parasite in relation to host cells and parasite interactions with host cells remain highly speculative and require further investigation with higher magnification TEM.

### 5.4.12. Atypical Features of *C. parvum* which require Future Investigation

Several features that were seen in culture throughout the 5 day examination period could not be readily classified as being of either parasite or host cell origin. After more detailed observations, these atypical features were considered to be of *C. parvum* origin as they had never been observed in negative controls or showed features which resembled those of known *C. parvum* stages. Fig. 85a shows a dead host cell with two parasite rudimentary bodies-stalk combinations still attached to it. By the time this image was acquired the parasites had fulfilled its function and the host cell had died. Such atypical formations were seen commonly after 5 days of culture as many stages had released their endogenous stages and or severed host cell contact by breaking from or with their stalk. Fig. 85b shows another cluster of dead HCT-8 cells and parasite remnants as indicated with arrows. The left arrow indicates an empty microgamont still attached to a necrotic HCT-8 cell.

The stage in Fig. 85f was nearly mistaken for debris if it was not for the connection to the host cell as indicated by an arrow. This connection together with its morphology suggests that this stage might resemble another possible form of the large gamont-like stage described previously.
Fig. 85: Scanning electron micrographs of atypical parasite stages in 5 day old cultures. a, b) Clusters of dead cells and *C. parvum* remnants. c, d) Tunnel-like feeding connections leaving holes in host cell membranes. e) Yet unexplained *C. parvum* stage. f) Large novel gamont.
Initially the stage in Fig. 85e was considered too atypical to be a life cycle stage of *C. parvum*. It was thus unexpected, when during the examination of monolayers with immunogold labeling of *C. parvum* surface epitopes, a similar stage was confirmed to be *Cryptosporidium*. Still, the nature and function of this stage can not be explained. Further studies examining parasite stages beyond day 5 of culture, might help to reveal the nature, subsequent development and function of these atypical stages seen in Figs. 85e and 86. Comparison of results between researchers will aid to identify parasite stages that have been dismissed and clarify their role in *C. parvum*’s life cycle.

Finally, a long, worm-like stage was observed on the host cell surface which appeared to be of parasite nature rather than of host cell origin due to its surface morphology (Fig. 87a), which was confirmed by immunogold labeling (Figs. 87b and c). The slender worm-like stage, measuring 7-8 μm in length with an even width of approximately 0.8 μm resembling the size of a sporozoite, was associated with a round structure next to its centre. Surprisingly it showed host cell attachment at both ends as well as at its central round bulge. Stages like the ones seen in Figs. 86 and 87 may represent gamont-like stages different to the ones previously described. However, the further development of these stages would need to be monitored to be able to answer the question about their nature. It is possible that the stage seen in Fig. 87a resembles the multinucleated stages described with light microscopy by Hijjawi *et al.*, (2002) for *C. andersoni* isolated from mouse intestines. Longitudinal TEM cross-sections of isolated parasites would be helpful to identify internal structures.
Fig. 86: Scanning electron micrographs of a) *C. parvum* stage similar to the one shown in Fig. 85e. b) Immunogold labels detected with BSD (arrows) identify the stage to be of *C. parvum* origin.
Fig. 87: a) Scanning electron micrographs of a long *C. parvum* stage attached to the host cell after 5 days of culture. Immunogold labeling in images b) and c) detected with BSD confirmed the stage to be part of the *C. parvum* life cycle.
5.4.13. Summaries

5.4.13.1. Scheme of Standard Trophozoite
Fig. 88: Schematic drawing of a typical trophozoite, here developing into a meront I. The trophozoite resides above the host cell surface, encapsulated by the parasitophorous vacuole membrane (PVM) (inner membrane) and host cell apical membrane (outer membrane), and separated from the host cell cytoplasm by an electron dense band. The trophozoite establishes a connection into the host cell cytoplasm (the feeder organelle ‘f’) to obtain essential nutrients. This study hypothesis that the parasite utilises the host cell’s protein and fatty acid synthesis machinery, consisting out of the nucleus (‘n’), endoplasmatic reticulum (‘ER’) and golgi (‘G’), for its own feeding purposes. Asexual replication occurs within the trophozoite via merogony - in the case of meronts resulting in the formation of merozoites (‘z’) or sporogony – in the case of oocysts resulting in the formation of sporozoites. Zoites are formed via budding of the rudimentary body (‘b’) which is located at the base of the trophozoite.
5.4.13.2. The Host-Parasite Interface

The attachment zone between Cryptosporidium and its host cell has been given many names. Scientists often speak of the host-parasite interface (Chen et al., 2005) when they refer to the region at which Cryptosporidium merges and makes contact with its host cell. This area includes many structures. With TEM, an electron dense band had been identified at this host parasite interface (Huang et al., 2004) separating the parasite from the host cell; apart from a region where the parasite establishes a feeding connection into the host cell. Feeder organelle is the most common name for this feeding connection (Valigurova et al., 2008). Studies by Huang et al., (2004) have described a tunnel-like feeding connection into the host cell. This tunnel-like connection was observed in the present study (see Fig. 85c), as well as the feeder organelle (see Fig. 25) and both structures most likely represent two ‘faces’ of the same functional organelle. Studies by Valigurova et al., (2008) described the feeder organelle lamellae’s granular structure. The present study identified this granular structure for microgamonts and macrogamonts (Figs. 89a and c). However, as the feeder organelle is known to be a feeding connection into the host cell, the granular structure is most likely the residual body of which zoites and gametes are formed, located directly above the feeder organelle (see Fig. 88). Comparing Figs. 89c to 89a reveals how this granular structure is possibly one and the same structure as the rudimentary body which has been extensively described in section 5.4.5. It can be assumed that initially this rudimentary body is all that the trophozoite is composed of. As zoites or gametocytes are starting to be formed from this rudimentary body, it gradually
reduces giving its substance to the newly forming daughter cells or gametocytes (see Figs. 89a and c).

The parasite’s structure composed of feeder organelle merged with the rudimentary body above, shows different morphological forms dependent on the parasite stage. In some cases, stages of *C. parvum* develop stalk-like structures (see Fig. 89d) which allow them to break off and sever host cell contact altogether. Figs. 89e and 89f show how this stalk-like structure develops from the rudimentary body. The development of the rudimentary body determines whether a stage develops a stalk and is able to sever host cell contact, or whether a stage stays in dense host cell contact until its function is fulfilled.

The granular feeder organelle structure of a macrogamont (see Fig. 89b) is the same functional structure as the feeder organelle-stalk structure seen in microgamonts (see Fig. 89c), which is the same structure as the rudimentary body seen in for example meronts I (see Fig. 89a). Consequently, the granular ‘feeder organelle’-structure is the rudimentary body of the parasite, of which zoites or gametocytes are formed, and not the feeder organelle. In the case of macrogamonts, this granular rudimentary body functions as a stalk (Fig. 89b). The feeder organelle is located underneath this structure, forming a feeding connection into the host cell. Previous findings of this study support the use of this organelle by *C. parvum* to obtain nutrients from the host.
Fig. 89: The host-parasite Interface. Scanning (a-d) and transmission (e,f) electron micrographs of different C. parvum stages. a) Rudimentary body ‘b’ of a meront. b) Rudimentary-body ‘f’, functioning as a stalk of a macrogamont. c) Rudimentary body ‘b’ and stalk ‘s’ of a microgamont. d) Stalk ‘s’ of a microgamont. e, f) Development of the rudimentary body ‘f’ determines the formation of stalks. b=rudimentary body, f=previously feeder organelle, s=stalk.
One achievement of this study was new information on Cryptosporidium parvum’s life cycle. When a thick-walled oocysts measuring 6 x 8 μm becomes ingested, 4 sporozoites measuring 0.8 x 5 μm hatch in the small intestine (Fig. 21). Immediately after their emergence from the oocyst, they attach to a host cell via their apical end which elongates upon host cell presence to start the invasion process (Figs. 21, 22 and 23). Initially sporozoites become embraced by host cell microvilli, which elongate and grow along the sporozoite surface.
Emerging from the host cell surface and from the microvilli embracing the sporozoite, protective membranes are formed that engulf the sporozoites in their protective niche (Figs. 23 and 24). Before growth and transformation into a trophozoite stage can begin, the invader needs to form a feeding connection to the host cell, the feeder organelle (Fig. 25). The feeder organelle aids the parasite to obtain essential amino and fatty acids from the host cell. The rest of the parasite is separated from the host cell via an electron dense band marking the parasite-host interface (Fig. 31). Now the transformation into a trophozoite is complete and further development into a meront I can begin. Initially the cytoplasm of the young trophozoite is the original cytoplasm of the invaded sporozoite, containing the sporozoite’s nucleus (Fig. 25b). The cytoplasm reduces to a rudimentary body of which the merozoites type I are formed in a process known as merogony. The whole process resembles merozoites type I budding off the residual body, which keeps reducing as merozoites type I are produced and released (Fig. 41). A residual body remains behind (Fig. 41c) which may have served as nutrient supply for the parent stage before host cell contact via the feeder organelle had been established. Meronts type I reach a size of 3 μm. Eventually the meront’s membrane opens up to release merozoites type I into the lumen of the intestine. Initially merozoites type I resemble stout sporozoites measuring between 0.5 x 1.5 and 0.5 x 2 μm (Fig. 42). Unlike sporozoites, merozoites type I do not establish host cell contact via their apical end but laterally, using their entire body surface (Fig. 43). To create an initial secure connection with the host cell, merozoites type I develop a lateral protrusion (Fig. 44) with which they adhere to their host (Fig. 46). Merozoites type I either invade cells to form meronts I and continue in the
asexual life cycle, or they invade cells to form meronts II and initiate the sexual reproductive cycle.

Meronts II are larger than meronts I measuring approximately 3 x 4 μm with a thicker cell wall than meronts I (Fig. 49). Each meront houses several merozoites type II which measure 0.55-1 μm (Figs. 49-58), are round in shape but also possess a well defined, lightly pointed apical end for invasion (Fig. 49). Another type of meront that was observed measured more than 8 μm, with each single merozoite type II developing into a trophozoite without leaving the parent meront (Fig. 59). The function of this different meront in the parasite's life cycle still requires clarification.

Merozoite type II host cell invasion leads to the formation of gamonts. Gamonts are divided into two major categories: macrogamonts ♀ and microgamonts ♂. Macrogamonts are fertilised by microgametocytes released from microgamonts. Two types of microgamonts have been observed in this study ranging from 2-4 μm in diameter. Each microgamont is densely packed with at least 50 microgametocytes, which are as small as 0.2 x 0.4 μm and possess a ring-like structure for adherence to macrogamonts (Fig. 63). The first type of microgamont is roundish, measuring approximately 2 μm in diameter, and appears on the host cell surface similar to meronts. This type of microgamont opens its membrane along a suture for the release of fertile microgametocytes (Fig. 60). The second type of microgamont can measure up to 4 μm in diameter and breaks contact to the host cell before the release of its internal microgametocytes, to travel free in the lumen of the small intestine (Figs. 62c and d). Likewise there are different types of macrogamonts. Some macrogamonts, upon fertilisation, remain in host cell contact and release zoites
into culture (Fig 70). These macrogamonts are oval in shape, measuring approximately 6 x 7 μm and have a perforated outer membrane (Fig. 70). Another type of macrogamont, the “large gamont”, shows a similar perforated membrane but measures up to 2-3 x 10 μm with a merozoite type I-like shape (Figs. 71-73). These large gamonts break host cell contact to travel free in the lumen of the intestine. Other fertilised zygotes transform into oocysts. There are two types of oocysts. One is small measuring 2 x 3 - 3 x 4 μm with a thin wall (Fig. 82) and the other larger, measuring 6 - 8 μm with a thick outer cell wall (Fig. 21a). The large oocyst initially possesses a stalk (Fig. 77c), but breaks host cell contact before being passed in the faeces. Thin-walled oocysts show a cell wall similar to thick-walled oocysts with a suture for sporozoite release, but because of their thin non-protective cell wall their purpose is to reinfect the same host and initiate C. parvum’s life cycle once again.
### 5.4.13.4. Summary of C. parvum Stages

<table>
<thead>
<tr>
<th>Time</th>
<th>Stage</th>
<th>Size</th>
<th>Morphological Feature</th>
<th>Fig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0hrs</td>
<td>Thick-walled oocyst</td>
<td>6x8 μm</td>
<td>Ovular, rough surface with cleft for sporozoite release, initially produced with stalk like macro- and microgamonts I</td>
<td>21</td>
</tr>
<tr>
<td>0 hrs</td>
<td>Thin-walled oocyst</td>
<td>2x3–3x3.5 μm</td>
<td>Ovular, smooth surface with cleft for sporozoite release, released from attachment zone like macro- and microgamonts II</td>
<td>80, 81, 82</td>
</tr>
<tr>
<td>&gt;24hrs</td>
<td>Excysted thick-walled oocyst</td>
<td>6x8 μm</td>
<td>Perforated surface</td>
<td>21</td>
</tr>
<tr>
<td>&gt;0hrs</td>
<td>Excysted thin-walled oocyst</td>
<td>3X3.5 μm</td>
<td>Smooth surface, opened along the cleft</td>
<td>93</td>
</tr>
<tr>
<td>&gt;3hrs</td>
<td>Sporozoite from thick-walled oocyst</td>
<td>0.2-1x8 μm</td>
<td>Rough surface, pointed apical region (elongated when in proximity to host cells), rounded posterior region</td>
<td>21</td>
</tr>
<tr>
<td>&gt;0hrs</td>
<td>Sporozoite from thin-walled oocyst</td>
<td>4.2x0.7 μm</td>
<td>Slender body with pointed apical region</td>
<td>21</td>
</tr>
<tr>
<td>&gt;6hrs</td>
<td>Early trophozoite</td>
<td>1-2.5 μm</td>
<td>Smooth surface formed by the host cell apical membrane, hood like shape</td>
<td>24, 27, 29</td>
</tr>
<tr>
<td>&gt;24 hrs</td>
<td>Trophozoite</td>
<td>2.5-3.5 μm</td>
<td>Epicellular, smooth surface, electron dense band, feeder-organelle, PV, cytoplasmic granulation, hood like shape</td>
<td>24, 30</td>
</tr>
<tr>
<td>&gt;24 hrs</td>
<td>Meronts I</td>
<td>2.5-3.5 μm</td>
<td>Epicellular, smooth surface</td>
<td>39-41</td>
</tr>
<tr>
<td>&gt;24 hrs</td>
<td>Open meront</td>
<td>3.5 μm</td>
<td>6 or 8 Merozoites aligned within, residual body</td>
<td>40</td>
</tr>
<tr>
<td>&gt;24 hrs</td>
<td>Merozoites type I</td>
<td>0.5x2-1.2x1.7 μm</td>
<td>Rod like shape, pointed apical region, rough surface</td>
<td>42</td>
</tr>
<tr>
<td>&gt;72 hrs</td>
<td>Meronts II a</td>
<td>3x4 μm</td>
<td>Epicellular, smooth thick membrane</td>
<td>49</td>
</tr>
<tr>
<td>&gt;72 hrs</td>
<td>Merozoites type II a</td>
<td>0.55-1.2 μm</td>
<td>Rough surface, round shape with pointed smooth apical end</td>
<td>49, 52</td>
</tr>
<tr>
<td>&gt;96 hrs</td>
<td>Meronts II b</td>
<td>8-10 μm</td>
<td>Round grape-like cluster of merozoites II, residual parent meront membrane and host cell attachment organelle</td>
<td>59</td>
</tr>
<tr>
<td>&gt;96 hrs</td>
<td>Merozoites</td>
<td>1.7-2.5</td>
<td>Remain within parent meront,</td>
<td>59</td>
</tr>
</tbody>
</table>
Table 2: Summary of *C. parvum* stages including variation in size and morphological features of each stage.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Size</th>
<th>Description</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microgamont I</td>
<td>&gt;96 hrs</td>
<td>2-4 µm Residing on stalk, densely packed with ~50 microgametes, Binary fission, Syzygy</td>
<td>60, 61</td>
</tr>
<tr>
<td>Microgamont II</td>
<td>&gt;96 hrs</td>
<td>2-4 µm Extracellular, released from attachment zone to travel in lumen of intestine</td>
<td>62</td>
</tr>
<tr>
<td>Microgamete</td>
<td>&gt;96 hrs</td>
<td>0.2x04 µm Red blood cell shape with central attachment organelle</td>
<td>60, 62-63</td>
</tr>
<tr>
<td>Macrogamont I</td>
<td>&gt;96 hrs</td>
<td>6x7 µm Residing on stalk, Ovular, rough surface, releases endogenous stages without severing host cell contact</td>
<td>70</td>
</tr>
<tr>
<td>Macrogamont II</td>
<td>&gt;96 hrs</td>
<td>6x6 µm Extracellular, released from attachment zone, perforated surface</td>
<td>71</td>
</tr>
<tr>
<td>Large gamont</td>
<td>&gt;96 hrs</td>
<td>3x8-5x10 µm Extracellular, released from attachment zone, perforated surface</td>
<td>71-73</td>
</tr>
<tr>
<td>Oocysts</td>
<td>110 hrs</td>
<td>2x3 µm New formation in culture</td>
<td>81</td>
</tr>
</tbody>
</table>

5.4.14. Conclusions

Observations of oocyst stocks and infected monolayers demonstrated that large thick-walled oocysts (Fig. 21a) and small thin-walled oocysts (Fig. 82) had both been inoculated into culture. However, before high voltage SEM studies had been performed (see Chapter 6, Fig. 91) the thin-walled oocyst had not been identified as such due to their size which, according to the present study is much smaller than stated by previous light microscopic studies on *C. parvum* (Hijjawi *et al.*, 2001). Thus, thick-walled and thin-walled oocysts were isolated together using the protocol for oocyst isolation by Meloni and Thompson (1996). 6 hrs post inoculation, many thin-walled oocysts were seen along the host cell surface (Fig. 82); however thick-walled oocysts were not detected in culture.
before 24hrs. A reason for this observation might be that macromolecules on the surface of thick-walled oocysts hindered their attachment (Kuznar and Elimelech, 2006) whereas thin-walled oocysts were able to attach to their target immediately. This conclusion seems logical, as thin-walled oocysts are not excreted in faeces but never leave their target tissue and reinfect the same host. Therefore, they would gain advantage by immediately expressing host cell adhesive receptors. Alternatively, thick-walled oocysts are passed in faeces, and therefore gain advantage by not expressing adhesive receptors before specific triggers through ingestion and digestion are given.

*C. parvum* had completed its life cycle after 5 days of culture with the formation of thick and thin-walled oocysts and other macrogamont-like stages.

These findings stand in contrast to observations by Hijjawi *et al.*, (2001) who reported the life cycle of *C. parvum* to be complete after 7 days. Earlier research, however, reported the entire life cycle of distinct *Cryptosporidium* species to be completed in as little as two days dependent on the host (O’Donoghue, 1995). The possibility of a life-cycle shorter than 7 days has not been considered for *C. parvum*. The inoculation of stages other than thick walled oocysts or the unnatural conditions *in vitro* may account for the shortened life cycle as it occurred in this study. Nevertheless, the present study reported the occurrence of many life cycle stages earlier than reported to occur previously (Hijjawi *et al.*, 2001). In the present study, meronts I, for example, were observed to form after only 24 hours post inoculation. It is possible that the inoculation of parasite stages other than thick-walled oocysts accounts for the early occurrence of life cycle stages. However, in the case of meronts I it is most likely that previous light microscopic studies (Hijjawi *et al.*, 2001) which
observed *Cryptosporidium* over a period of more than 5 days, could not differentiate early meronts I from trophozoites. Thus, the observations of this study suggest that the life cycle of *C. parvum* might be completed earlier than previously thought.

Another observation of this study was that *C. parvum* stages in one area of an infected monolayer were found to be of the same kind, for example, one area was heavily infected with meronts type I whereas another area was heavily infected with microgamonts. An explanation for this observation has not yet been determined. It does not seem to give the parasite any advantage not to have all stages present in one area, since this, for example, facilitates the fertilisation of macrogamonts by microgamonts.

Further, this study revealed that *Cryptosporidium* stages are not confined to one morphological form. The same stages, for example, meronts I, can be well attached to host cells and open up for merozoite release, or meronts I can be completely round, showing hardly any host cell attachment. However, the existence of different morphological forms of certain life cycle stages requires verification through *in vivo* studies.

Observations made in this study demonstrated that the further a *C. parvum* stage is progressed in the life cycle, the less host cell contact it has at the time point of maturation. At the beginning of *C. parvum*’s life cycle, dense host cell contact and host cell membrane engulfment appears to be advantageous to the parasite’s survival, by supplying a feeding connection and a protective outer shield. Later on in the parasite’s life cycle, less host cell contact might be desired for auto infective stages to sever host cell contact and infect new areas
of the ileum and for oocysts to be passed in faeces and spread the infection to new hosts.

Unfortunately, this study was confined to standard sample preparation procedures utilising conventionally fixed material and critical point drying. High pressure freezing TEM or CryoSEM was not available to this study. Thus, shrinkage and changes in cellular structures were unavoidable, which must be considered in all observations.

5.5. Final Remarks

The main aim of this study was to visualise the life cycle of *C. parvum* and morphologically examine every life cycle stage detected in this study and its interactions with host cells. Gaining a better understanding of the parasite’s life cycle and interaction with its host is an essential prerequisite for the prevention and treatment of human and life stock infections. The life cycle of *C. parvum* remained incompletely understood until now and the morphology of many life cycle stages, for example microgamonts, remained unclarified. The present study adds onto the understanding of *C. parvum*’s life cycle, with the addition of previously undescribed life cycle stages and the morphological characterisation of accepted ones. The limited information obtained when examining *C. parvum* in culture with light microscopy at the start of this study has shown the necessity of EM methods to identify and morphologically characterise parasite stages. Thus, several parasite stages visualised in this study have not been morphologically characterised by earlier studies which relied on light microscopy only, or utilised EM techniques on limited aspects of the parasite’s
life cycle only. Therefore, several stages and/or their behaviours described in this study have not yet been included in the life cycle of the parasite *C. parvum* before. In this chapter the morphology of all known life cycle stages has been visualised, and host cell interactions have been described. In addition, new life cycle stages of *C. parvum* have been detected and begun to be morphologically characterised. For some new features detected in the life cycle of the parasite, their role still needs clarification.

An essential tool in the detection of new life cycle stages or previously unknown morphological features was immunogold labeling of *C. parvum* surface epitopes. Without the application of surface receptor labeling many features in the life cycle of *C. parvum* would have been dismissed as unidentifiable structures, on the basis of morphological data from SEM and TEM only.
6. Results

“The Expression of C. parvum Surface Receptors”

6.1. Introduction

Surface receptors play an essential role from the beginning of C. parvum’s life cycle. Macromolecules are expressed on the surface of ingested oocysts to hinder attachment to gastrointestinal epithelium until the target tissue is reached (Kuznar and Elimelech, 2006). After passage through the gastrointestinal tract and exposure to trypsin, these lectin receptors (macromolecules) are then altered in such a way as to enhance adherence to the ileal lining (Kuznar and Elimelech, 2006) and possibly enable the transduction of excystation signals through the oocyst wall to the endogenous sporozoites. Studies by Snelling et al., (2007) showed that the expression of a variety of sporozoite proteins (for example Cpa135) is increased during excystation. Such proteins are located in apical complex organelle structures of C. parvum, which demonstrates a possible role for C. parvum sporozoite surface molecules in the excystation process. After excystation mucin-like receptors on the surface of sporozoites facilitate the adherence to and penetration of intestinal mucous (Petersen et al., 1992a). These receptors include the GP900 and GP40 (Cevallos et al., 2000a and b). A prerequisite for host cell invasion by apicomplexan parasites is gliding motility (Wetzel et al., 2005). Gliding motility is mediated through parasite surface receptors. Antibody labeling revealed that the C. parvum GP900, CP40 and Cpa135 as well as GP15 and CPS-500 are shed during gliding motility of infective zoites (Riggs et al., 2002; Tossini et al., 2004; O’Connor et al., 2007a;
Wanyiri et al., 2007). *C. parvum* zoite surface receptors associated with the early host cell attachment and invasion process are Cpa135, TRAP-C1, CSL, CP47 and CP12 (Nesterenko et al., 1999; Tossini et al., 2004; Yao et al., 2007; Putignani et al., 2008). Other *C. parvum* surface receptors, for example the CP2 (Chen et al., 2004b), are thought to be associated with sexual development during the parasite’s life cycle. Further, *C. parvum* surface receptors can be thought to be essential later in the life cycle to mediate microgametocyte adherence to macrogamonts and facilitate re-attachment of thin-walled, auto-infective oocysts.

To visualise the location of such receptors with electron microscopy, immunogold labeling of respective antigens is needed. Immunogold labeling of antigens relies on the binding of a primary antibody to the antigen of interest. Most commonly, monoclonal antibodies are used, which are specific for one antigen (O’Hara et al., 2006; Biggs et al., 2008). To visualise the location of the receptor bound by the primary antibody, a secondary antibody labeled with gold particles has to be directed specifically against the primary antibody. Two antibodies are needed for immunogold labeling as one antibody can either bind to receptor or carry a gold particle, but not both. It is important to prevent non-specific interaction of antibodies with other receptors, therefore serum-based buffer solutions must be utilised to block free aldehyde groups.

Immunogold labeling of *C. parvum* stages for SEM has not been performed previously. In this study a protocol was established which enables the labeling of surface receptors of a parasite for SEM. For the experiment, *C. parvum* infected HCT-8 cultures as well as oocyst stock and cell-free cultures were labeled with *Cryptosporidium* gold conjugated antibodies. A primary polyclonal
antibody was directed against a wide range of C. parvum surface antigens. By using a polyclonal antibody, the protocol ensured that a large variety of C. parvum surface epitopes would be bound, thereby visualised at one time. A secondary antibody conjugated with gold particles was selected to be directed against the first antibody. The 10 nm immunogold particles were visualised on SEM micrographs using a higher electron acceleration voltage of 10kV (instead of 3kV which was used in Chapter 5). Coincidentally, C. parvum stages with a thin cell wall were transparent at a higher kV, allowing differentiation of some endogenous stages of cell-free culture or oocyst stock.

The visualisation of a large variety of C. parvum surface receptors within one study enabled the answering of several key questions: “Are there receptors expressed onto the membranes of thin-walled oocysts and cell-free stages? Are C. parvum receptors expressed on the apical membrane encapsulating invaded stages? How and where do receptors interact with host cell material? Are C. parvum receptors involved in microgametocyte-macrogamont binding” By selecting primary antibodies against a selection of C. parvum receptors elucidated to date, many receptors could have been missed and with that, not all of these questions answered.

6.2. Aims

This study examined whether C. parvum epitopes are expressed into the apical membranes surrounding intracellular parasite stages. Further, this study examined interactions between C. parvum surface antigens and host tissue. It aimed to identify if and how C. parvum interacts with the host cell and/or host cell microvilli. With the help of immunogold labeling, cultures were scanned for
parts of parasite origin, which otherwise would have been dismissed. As such, this study extended information on the host parasite relationship, building on the results presented in Chapter 5.

Additional goals of this study were to examine oocyst stocks and cell-free cultures for the expression of *C. parvum* receptors on the surface of respective stages. Labeling of oocyst stock was performed to examine the expression of surface receptors in the absence of host cells and in context with the occurrence of internal sporozoites. Cell-free culture was labeled to confirm stages that had been hypothesised to form in cell-free culture in Chapter 3 of the present study, as well as by Hijjawi *et al.*, (2004).

### 6.3. Methods

The experiments in this chapter were carried out according to section 2.3 of the Materials and Methods Chapter 2.

Ideas from protocols of studies using immunogold labeling together with TEM on *Cryptosporidium* (Yu and Chai, 1995; Yu and Lee, 1996; Yu, 1998; Matsubayashi *et al.*, 2008) or using immunogold labeling together with SEM on cell surfaces (Baharloo *et al.*, 2005; O'Hara *et al.*, 2006; Biggs *et al.*, 2008; Muscariello *et al.*, 2008) were used to establish the protocol for the present study. The protocol for sample fixation used for the experiments in Chapter 5 had to be altered to enable specific, but prevent unspecific, binding of primary and secondary antibodies in the course of the immunogold labeling procedure. The idea for the primary antibody was obtained from protocols of Boxell *et al.*, (2008) who utilised the same primary antibody for fluorescence labeling of *C. parvum* stages. The primary antibody had to be applied to viable cultures
without any prior fixation. Fixation before the application of the primary antibody had been shown to denature surface receptors on *C. parvum* and thus hinder antibody binding. Further, the blockade of free aldehyde groups on host cells using FCS in the washing steps, before the application of the two antibodies respectively, proved to be essential to exclude non-specific interactions between the antibodies and host cell receptors.

To test the specificity of the antibodies this experiment included two negative controls for cell culture. The first control consisted of uninfected HCT-8 cells incubated with both antibodies. The second control consisted of infected cultures incubated with the second antibody only. The aim of the first negative control was the exclusion of nonspecific antibody binding to HCT-8 cells. The second negative control was included to exclude nonspecific binding of the second antibody to *Cryptosporidium*.

The protocol, essential to perform immunogold labeling of *C. parvum* surface epitopes, compromised the structure of samples as paraformaldehyde was included, as well as glutaraldehyde, and the concentrations of fixatives had to be higher which lead to a higher degree of membrane shrinkage.

Secondary electrons (SE) do not allow visualisation of immunogold particles. Backscattered electrons visualise gold particles, as the intensity of their signal is proportional to atomic numbers, but less structural information is revealed. To obtain both structural and immunogold information, BSE and in lens (SE) signals were combined using the instrument software with approximately 0.3 SE and 0.7 BSE signals.
6.4. Results

6.4.1. Oocyst Stock

A sample of oocyst stock, obtained with the same methods as for the inoculation of the experiments in Chapters 3-5, was examined for the presence of surface receptors. After the trigger of excystation had been applied, surface receptors of oocysts were labeled with immunogold particles. Labeled oocysts were visualised with SEM using 10 kV to obtain immunogold information. Simultaneously the high kV enabled visualisation of some oocysts internal structures. Surprisingly, all oocysts identified in this experiment were thin-walled oocysts. Thus, the mouse intestines used for the production of the corresponding oocyst stock must have nearly exclusively contained thin-walled oocysts at the time point of harvest. These findings correlate with findings from Chapter 5 which identified a large number of thin-walled oocysts on the host cell surface 6 hrs post inoculation, whereas only a small number of thick-walled oocysts were detected.

Oocysts examined with immunogold labeling measured between 2.5 x 3.5 (Fig. 91) – 3.5 x 3.5 μm (Fig. 95c). This size correlated with the size of thin-walled oocysts detected on the host cell surface (Fig. 92). Oocysts were frequently observed to occur in accumulations in the oocyst stock (Fig. 91a). Accumulations of oocysts suggest the presence of surface receptors to enable adherence. Indeed, small dots were seen ubiquitously on the surface of oocysts even without the aid of immunogold detection (Fig. 91a). BS detection revealed the presence of immunogold labels, and with that the presence of *C. parvum* receptors ubiquitously distributed over the surface of the oocysts (Fig. 91c). Combining the SE image of Fig. 91a with the BSE information of Fig. 91c,
revealed that the location of the surface receptors identified in Fig. 91c overlapped with the small dots seen on the surface of oocysts in Fig. 91a (Fig. 91b). It is possible that these small dots seen represent the gold-conjugated antibodies and would not be visible without antibody labeling. However, as similar dots can be seen on oocysts which were not labeled with immunogold (Chapter 5, Fig. 82 and Fig. 92), it is most likely that they mark the position of macromolecules, which might play a role in oocyst adhesion.

Fig. 91: Receptors on oocyst surfaces. a) In lens scanning electron micrographs of three oocysts from oocyst stock. Image b) is a magnification of a) acquired with a combination of in lens and BSD detection revealing immunogold labeling (arrows) on the oocysts surface, whereas c) is acquired with BSD only. Note the better visibility of gold particles in image c) as compared to image b). Also note the structural information seen in image b) as compared to image c).
Fig. 92: Scanning electron micrograph of an oocyst from inoculant on the host cell surface at 24 hrs of culture. Standard sample preparation without immunogold labeling.

As well as intact oocysts, excysted oocysts were identified in the inoculant (Fig. 93a). Excysted oocysts measured 3.5 x 3.5 μm, due to their opened structure, and the thinness of their cell wall was evident. In contrast to unexcysted oocysts, no immunogold labeling was detected on the surface of fully excysted oocysts (Fig. 93b).

Oocysts shown in Fig. 94a were immature, measuring 2.5 x 3.5 μm with incompletely formed sporozoites visible in their interior. Oocysts seen in Fig. 94b had matured with sporozoites visible in their interior, measuring 2.5 x 2.8 μm. Note the rounder shape of the mature oocysts seen in Fig. 94b, as compared to the egg-like shape of the oocysts seen in Fig. 91a. Hence, two different basic forms of thin-walled oocysts exist, as already seen in Chapter 5: round and oval.
Increased magnifications of Fig. 94a and b reveal that immature oocysts, with sporozoites still forming in their interior (Fig. 94c), appeared to express fewer surface receptors than mature oocysts (Fig. 94d).

Fig. 93: Excysted oocyst. a) In lens scanning electron micrograph of an excysted oocyst. b) Magnification of a) acquired with BSD only. Excysted oocysts (a) showed no immunogold labeling, hence there was no expression of surface receptors (b).
Fig. 94: Immature and mature oocysts. 

a) In lens micrograph of two immature oocysts with sporozoites still forming. 
b) In lens micrograph of two mature oocysts with sporozoites aligned. 
c) Magnification of a) acquired with BSD only, showing few surface receptors (arrows). 
d) Magnification of b) acquired with BSD only, showing a large number of surface receptors (arrows).
Fig. 95: Immature and mature oocyst. a) In lens micrograph of an immature oocyst. White arrows indicate round structures other than sporozoites. b) Magnification of a) acquired with BSD only, revealing no surface labeling. c) In lens image of a mature oocyst with four internal sporozoites. A white arrow indicates round structures other than sporozoites. d) Magnification of c) acquired with a combination of in lens and BSD detection. Two examples of immunogold surface labeling are indicated with yellow arrows.

The size difference of the two oocysts seen in Figs. 95a and 95c, 3.5 x 3.5 μm compared to 2.5 x 3.5 μm respectively, may be caused by the sample preparation procedure causing membrane shrinkage of the oocyst in Fig. 95c. The more immature oocyst of both did not show any surface labeling (Fig. 95b), whereas the mature oocyst did (Fig. 95d).
Using a high kV, round structures measuring approximately 0.25 μm in diameter were seen in the interior of oocysts. The two dots visible in the interior of the oocyst in Fig. 95a were initially thought to be the apical tips of sporozoites, but the cluster of four of these dots in the oocyst in Fig. 95c clearly showed that these round structures were separate structures.

In addition to the oocysts described so far, round stages, smaller than 2 μm and with a thicker surface membrane, were detected in the oocyst stock (Fig. 97a), sometimes attached to an oocyst (Figs. 96a, b, c). Immunogold labeling of *C. parvum* surface receptors confirmed these stages to be *C. parvum* (Figs. 96d and 97b). With a diameter of approximately 1.8 x 1.8 μm (Fig. 96a), compared to oocysts which measure approximately 2.8 x 2.8 μm in diameter, these cyst-like stages can not be mature oocysts. However through the discovery of a cyst-like stage in the oocyst stock which measured 2.5 x 2.9 μm in diameter (Fig. 98a) and was confirmed by immunogold labeling (Fig. 98b), the hypothesis was made that the cyst-like stages represent a stage in transition into an oocyst. In contrast to mature oocysts, no interior sporozoites were visible (for example Fig. 98).
Fig. 96: Round cyst-like stages attached to oocysts. a, b) In lens micrographs of oocysts attached to round cyst-like stages (1.8 μm). Image c) is a magnification of image a) acquired with BSD only, to show the attachment between the oocyst and the cyst-like stage. Receptor labeling is indicated with arrows. Image d) is a magnification of the cyst-like stage in image b), acquired with BSD only. As an example for immunogold labeling detected on the surface of the *C. parvum* cyst-like stage, three groups of immunogold particles are indicated by arrows. O=oocyst, C=cyst-like stage.
Fig. 97: Single cyst-like stage. a) In lens micrograph of a cyst-like stage in oocyst stock measuring 1.8 μm. Image b) is a magnification of image a) acquired with BSD only, showing gold particles on the parasite’s surface. As an example of immunogold labeling detected on the surface of this *C. parvum* stage, one group of three immunogold particles is indicated by an arrow.

Fig. 98: Oocyst-like stage. a) In lens micrograph of an oocyst-like stage measuring 2.5 x 2.8 μm. b) Magnification of a) acquired with BSD. A couple of immunogold labels are indicated by arrows.
Apart from oocysts, an accumulation of different, much larger cyst-like structures was detected in the oocyst stock, with each structure measuring approximately 2 x 5 μm (Fig. 99). The size and shape of these cyst-like structures was similar to the large gamont-like stages that have already been described to develop in \textit{in vitro} culture in Chapter 5. That they were isolated together with oocysts supports the hypothesis made in Chapter 5 that large gamont-like stages may be isolated together with thin-walled oocysts, as they fulfil a similar function in the parasite’s life cycle, which is the re-infection of the same host. This hypothesis was further supported through findings by Hijjawi \textit{et al.}, (2002) which identified similar “extracellular” gamont-like stages in the lumen of the mouse intestine. However, as the large gamont-like stages presented in Fig. 99 showed no surface labeling (data not shown) their definitive nature remains speculative.

![In lens micrograph of possible large gamont-like stages in oocyst stock.](image)

No immunogold labeling was detected on the surface of these stages.
6.4.2. Cell-Free Culture

Samples of cell-free culture were processed at the same time points as cell culture samples (5 days) to ensure a valid comparison. The cell-free culture was carefully examined, however no other stages than the three examples presented (Fig. 100-102a) were observed. All stages detected in cell-free culture appeared as round cyst-like stages measuring between $2 \times 2 \, \mu m$ and $2.8 \times 2.8 \, \mu m$ in diameter. Thus, their size did not differ from oocysts and cyst-like stages shown in the previous chapter.

Fig. 100: Meront from cell free culture. a) In lens micrograph of a meront in cell-free culture measuring $2.8 \times 2.8 \, \mu m$. b) Magnification of a) acquired with a combination of BSD and in lens signal to reveal surface receptor labeling.
Fig. 101: Meront from cell free culture. a) In lens micrograph of a meront in cell-free culture measuring 2.5 x 2.5 μm with zoites aligned in the interior. b, c) Magnification of a) acquired with BSD only to reveal surface receptor labeling.
Fig. 102: In lens micrographs of oocyst-like stages in cell-free culture. a) Cyst measuring 2.6 x 2.6 μm. c) Cyst measuring 2.5 x 3 μm. b) Magnification of a) revealing immunogold labeling as indicated by an arrow. d) Magnification of c).

All stages observed in cell-free culture proved to be *C. parvum* via surface receptors labeling (Figs. 100b, 101b, c and 102b, d). Internal merozoites were identified in some stages (Fig. 100a, 101a), resembling meronts I of Chapter 5 (see Fig. 39). They were shorter and stouter than the sporozoites seen in the interior of oocysts detailed in the previous chapter. The cell-free stage in Fig. 101 appeared similar to the meront I in Fig. 39 of Chapter 5, possibly containing merozoites type I. The cyst-like stages in Fig. 102 however, still seemed to be developing. These stages appeared similar to thin-walled oocysts correlating with their diameter of approximately 3 μm. They may have been oocysts
remaining from the inoculation of the cell-free culture. However, after 5 days of culture, their definite nature remains unknown.

6.4.3. Cell Culture

For this study *C. parvum* was cultured in HCT-8 cell monolayers. After 5 days of culture the cells were fixed to obtain the maximum variety of *C. parvum* stages. The sample preparation procedure was optimised for immunogold labeling of *C. parvum* receptors as explained in section 6.3. As a result some structural preservation was compromised (Fig. 103 as compared to Chapter 4), as explained in section 6.2. The loss of the structural integrity of microvilli and fine surface features as a result (i.e. Fig. 105 a) could not be prevented.

Fig. 103: Overview of a 5-day infected monolayer acquired with scanning electron in lens detection, processed for immunogold labeling of *C. parvum* surface receptors.
6.4.3.1. Apical Membrane

The main reason for this study was to determine the composition of the apical membrane engulfing intracellular parasite stages: whether it consists purely of host cell material or if it contains parasite elements as well. As Fig. 23 of Chapter 5 demonstrated, the apical membrane engulfing parasite stages is formed from host cell material. However it is likely that the parasite expresses receptors into this membrane as *C. parvum* is known to play an active role in the apical membrane formation process, by stimulating a host cell response, which results in membrane formation and protrusion to encapsulate the invader (see Chapter 5). This experiment was performed to confirm the presence of these hypothesised parasite receptors in the apical membranes surrounding intracellular stages.

As already seen in Chapter 5, free merozoites type I possessed a large number of surface receptors. However, when merozoites type I established host cell contact and started the invasion process, forming an early trophozoite (labeled “T” in Fig. 104a), surface receptor expression was reduced (see Fig. 104 b) as the invading zoite became covered by the host cell apical membrane. Much less immunogold labeling was detected on the surface of an early trophozoite, than on a merozoite that had just attached to a host cell (compare stage “T” to stage “M” in Fig. 104b).

Following this tendency of reduced surface receptor expression with increased maturation, trophozoites that were slightly larger in size and thus further developed (Fig. 105a) expressed fewer surface receptors than very early
trophozoites (compare Fig. 105b to Fig. 104b). These findings suggests a role for *C. parvum* surface receptors in the invasion process.

Mature trophozoites (Fig. 106a) however expressed more receptors on the apical membrane than young trophozoites, or developing trophozoites, which indicates that over time more receptors were expressed in the apical membrane. Large groups of receptors were identified on the surface of mature trophozoites (Fig. 106b). Small receptor groups and single receptors were also identified (Fig. 106b). However the general tendency for apical membrane receptor expression was in groups of up to 50 receptors (Fig. 106b).

![Fig. 104: Surface receptor expression on invading merozoites. a) In lens micrograph of merozoite type I invasion and early trophozoite formation. b) Early trophozoites show much less surface receptor labeling than free merozoites type I as revealed with BSD only. M=merozoite, T=trophozoite.](image)
Fig. 105: Surface receptor expression on early trophozoites. a) In lens image of a young trophozoite and merozoite type I. b) Immunogold surface receptor labeling on the trophozoite revealed with BSD is indicated with arrows.
Fig. 106: Surface receptor expression on mature trophozoites. a) In lens image of a mature trophozoite expressing groups of receptors on its surface as seen in a magnification in b) acquired with BSD.

*C. parvum* trophozoites with adjacent small round stages were frequently seen in this study (Fig. 107a) and immunogold labeling of surface receptors on these stages demonstrated their affinity with *C. parvum* (Fig. 107b).

Immunogold labeling in Fig. 107a, identified *C. parvum* surface receptors elsewhere than only on parasite surfaces. *C. parvum* receptors were found in close proximity to a small trophozoite (see right arrow in Fig. 107b). The image suggests that these *C. parvum* surface receptors may locate on protrusions of the apical membrane encapsulating the parasite. Though, it is possible that the receptors locate on host cell microvilli material in close proximity to the small *C. parvum* stage. As negative controls presented in subsequent sections of this study will show, no such immunogold label was detected on host cell surfaces of non-infected samples or areas which were not infected by *C. parvum*. Therefore
the here described observation was a result of specific receptor binding, not artefact.

Fig. 107: Surface receptor expression on empty and newly formed *C. parvum* stages. a) In lens micrograph of a mature, possibly excysted *C. parvum* stage with small trophozoite forming to the right. b) BSD image of immunogold labeling of surface receptors confirmed both stages to be of *C. parvum* origin and identified *C. parvum* receptors on host cell material (see arrows).
Trophozoites appeared in all forms and shapes on the host cell surface. Some appeared so atypical that initially they could not be identified as *C. parvum* stages (see Fig. 108a). The atypical trophozoite in Fig. 108 was only identified as such because it occurred in a cluster of three more readily identifiable trophozoites. As presented in Chapter 5, trophozoites commonly occur in clusters of 4, originating from the 4 sporozoites that excysted from one oocyst. Thus, the atypical trophozoite in Fig. 108 might represent an earlier stage on its way of transformation into a trophozoite, still possessing the shape of a sporozoite. With the application of immunogold labeling, such morphologically atypical stages can now also be identified as *C. parvum* even if occurring as single stages. Immunogold labeling of *C. parvum* surface receptors revealed receptor groups on the surface of each of the four trophozoites (Fig. 108b-d).

Fig. 108: In lens image of four trophozoites on the host cell surface. The arrow indicates the former apical tip of the now invaded sporozoite.
Fig. 108: Magnified images of a) acquired with BSD. All four trophozoites identified as *C. parvum* by immunogold labeling as indicated for three examples by arrows.

A phenomenon that was hypothesised in Chapter 5 was also observed in the present study: Binary fission of a *C. parvum* stage resulting in two identical daughter stages (Fig. 109a). Immunogold labeling on the surface of the
corresponding stage in binary fission helped in identifying it as *C. parvum* (see Figs. 109b and d). The other *C. parvum* stages which occurred in clusters did not appear as typical trophozoites. However immunogold labeling confirmed these structures as *C. parvum*, as shown with an example in Fig. 109c.

**Fig. 109:** Surface receptor expression on atypical stages and stages in binary fission. a) In lens micrograph of binary fission of stage(s) identified as *C. parvum* and accumulation of atypical stages. A magnified image acquired with BSD, presented in b) revealed immunogold labeling on the surface of the stage(s) in binary fission.
Fig. 109: Magnifications of a) acquired with BSD, identifying stages as C. *parvum*. Arrows indicated groups of immunogold particles.

Meronts I containing 6-8 internal merozoites type I were observed in 5 day old samples in Chapter 5. A stage, possibly resembling a meront I (Fig. 110a), was examined in this study. The amount of surface receptor expression was lower than seen for other trophozoites earlier (Fig. 110b).
Fig. 110: Surface receptors on a meront. a) In lens micrograph of a trophozoite resembling a meront I. b) Magnification of a) acquired with BSD. Arrows indicate small amounts of surface receptor labeling.
6.4.3.2. Microvilli Interactions

As already seen in Fig. 47b of Chapter 5, invasive stages of *C. parvum* use surface receptors to establish host cell contact. In this study, *C. parvum* specific labeling was identified on the surface of host cells. Detection of *C. parvum* receptors in for example gliding trails (Fig. 113) lead to the hypothesis that gliding parasites shed receptors onto host cell material. To further examine this phenomenon, this part of the study examined areas of monolayers populated with excysted parent stages (see i.e. Fig. 111a).

The large *C. parvum* stage in Fig. 111a was identified as an empty meront I because merozoites type I were seen emerging from it (Fig. 111c), forming trophozoites in close proximity to the parent stage (Fig. 111b). Apart from a large amount of surface labeling on the free merozoite in Fig. 111c, *C. parvum* surface receptors were detected on the host cell surface directly next to the free merozoite and young trophozoite (Fig. 111b), possibly left behind during gliding motion. Figs. 111b and 111c show clearly that labeling of *C. parvum* receptors was always found on host cell microvilli, rather than on the host cell surface itself.
Fig. 111: *C. parvum* surface receptors on host tissue. a) In lens micrograph of an empty meront I with merozoite type I host cell invasion and trophozoite formation in close proximity to the parent stage. Images b) and c) are magnifications of a) acquired with BSD, revealing labeling of *C. parvum* surface receptors on host tissue.

Further investigations of the monolayer revealed that merozoites type I directly interact with microvilli (Fig. 112a) in a fashion similar to sporozoites (see Fig. 23 of Chapter 5). Fig. 112a shows elongated host cell microvilli (labeled with arrows “mv”) embracing a merozoite type I. In Fig. 112b immunogold labeling of *C. parvum* surface receptors can be identified on these two microvilli embracing
the merozoite. This supports earlier observations (Fig. 111) which suggested that *C. parvum* interacts with host cell microvilli via surface receptors which are shed onto substrate during gliding motility of the *C. parvum* zoite.

Gliding trails similar to the one seen in Fig. 113a were previously observed and explained in Chapter 5. However, *C. parvum* surface receptors within gliding trails have not yet been detected. Magnifications of Fig. 113a revealed groups of *C. parvum* surface receptors left behind on host cell microvilli (Figs. 113b and c). These receptors were probably shed by gliding merozoites (being released from the parent stage in Fig. 113a) as they made their way through the microvilli brush border to find a suitable place for host cell invasion. Again, receptor labeling on microvilli was not detected in any negative control, which demonstrates the validity of this finding, that *C. parvum* receptors were shed onto host cell microvilli.
Fig. 112: *C. parvum* surface receptors on host cell microvilli embracing a merozoite. a) In lens micrograph of a merozoite type I embraced by host cell microvilli. b) BSD image of a): Immunogold labeling was not only detected on the surface of the merozoite, but also on the host cell microvilli embracing the invading parasite, as indicated with arrows in b).
Fig. 113: *C. parvum* surface receptors in gliding trails on host cells. a) In lens micrograph of a gliding trail with developing trophozoites. b, c) Magnifications of a) acquired with a combination of BSD and in lens detection revealing immunogold labeling of *C. parvum* surface receptors on host cell microvilli.
6.4.3.3. *C. parvum* Stages only Identified via Immunogold Labeling

Some stages of *Cryptosporidium* were not identified without the application of *C. parvum* surface receptor labeling. These stages are described below.

Fig. 114a shows a gliding trail with merozoites type I tangled in meshworks of host cell microvilli (Figs. 114b and c). However, at the periphery of the gliding trail (Fig. 115a) small round stages, exhibiting *C. parvum* surface receptor labeling, were seen in contact with microvilli, measuring approximately 0.55 μm in diameter (Fig. 115b). Their size suggests they are small merozoites type II. However, other round structures discovered in this area (Fig. 115c) were even smaller in size, measuring approx 0.3 μm. Their size suggests they are microgametocytes. However, unlike microgametocytes seen in Fig. 115b, their structures appeared flat, similar to that of host cell microvilli which had been damaged by the sample preparation procedure.

The flat appearance of the round stage in Fig. 115c may be an illusion caused by light and dark patterns within the image. The round structure in Fig. 115c showed a large amount of *C. parvum* surface receptor labeling and was densely attached to host cell microvilli, which suggests it is a *C. parvum* stage. *C. parvum* receptors have also been detected on microvilli close by (see double arrow in Fig. 115c).
Fig. 114: Merozoites type I engulfed by host cell microvilli. a) In lens micrograph of a gliding trail around an empty *C. parvum* meront I. b, c) Magnifications of a) acquired with a combination of in lens detection and BSD. Merozoites type I get tangled in the microvilli meshwork of the gliding trail.
Fig. 115: Merozoites type II engulfed by host cell microvilli. Magnifications of Fig. 114a acquired with a combination of BSD and in lens detection. b) Merozoites type II tangled in a meshwork of host cell microvilli. c) Previously unidentified round structures with a large amount of *C. parvum* surface receptor labeling. Arrows indicate immunogold particles.

Similar stages to those described in Fig. 115c were seen in the proximity of many other *C. parvum* stages (Figs. 116a and 117a). The round structures seen in Fig. 116c and 117c are in close proximity to the parasites’ apical membrane.
Their large number suggests that they represent microgametocytes which have adhered to host cell tissue as explained in Chapter 5. Thus, the large *C. parvum* stages may be microgamonts, which are know to frequently occur at day five of culture.

Fig. 116: Surface receptors on microgametocytes. In lens micrograph of proposed microgamonts with microgametocytes (b, c, d) attached to the host cell surface in close proximity.
Fig. 116: Magnification images of a) acquired with BSD. Immunogold labeling revealed a large number of *C. parvum* surface receptors on the surface of the microgametocytes seen in Fig. 116a as indicated by arrows.
Fig. 117: Surface receptor labeling of a large *C. parvum* stage. a) In lens micrograph of an open *C. parvum* stage with small round structures attached to the host cell in close proximity (c). b) Magnification of a) acquired with BSD revealing labeling on the open *C. parvum* stage. c) Magnification of the small round structures acquired with BSD also showing *C. parvum* surface labeling.

### 6.4.4. Negative Controls

To confirm that no non-specific labeling occurred, negative controls were performed. A negative control consisted of infected monolayers which were incubated with only the second antibody, to demonstrate that the second antibody carrying the gold atoms did not bind non-specifically to
Cryptosporidium or host cell membranes. Further, uninfected HCT-8 cells were examined to see if any gold label was detected on the monolayer itself. A magnification of at least 80kx and BSD detection only was used to ensure that no gold particle was missed.

Fig. 118: Negative control, merozoite type I. a) In lens micrograph of a merozoite type I on the host cell surface. b) BSD image of the same merozoite: No immunogold labeling was detected on the merozoites surface without the presence of the primary antibody.
If only the secondary, gold-conjugated antibody was applied to the culture, no labeling of *C. parvum* stages occurred (Figs. 118b and 119b). Stages like merozoites type I (Fig. 118a) or trophozoites (Fig. 119a), which had proven to express surface receptors, did not show any gold labeling if the primary antibody was withheld.

**Fig. 119:** Negative control, trophozoite and microgametocytes. a) In lens micrograph of a trophozoite (T) and microgametocytes (arrows) on the host cell surface. b) Magnification acquired with BSD: No immunogold labeling was detected without the presence of the primary antibody.
Microvilli of non-infected areas of infected monolayers, incubated with both antibodies, did not show any signs of antibody labeling (Figs. 120a-d). Likewise, no immunogold labeling was seen on the host cell surface if the primary antibody was withheld (Figs. 121a-b).

Fig. 120: Negative control, host cell microvilli. a, c) In lens micrographs of host cell microvilli in a non-infected area of a monolayer labeled with both antibodies. b, d) With BSD no immunogold labeling was detected.
Fig. 121: Negative control, host cell surface. a) In lens image of the host cell surface of a monolayer labeled with the secondary antibody only. b) With BSD no immunogold labeling was detected.

6.5. Discussion

In many previous studies (Cevallos et al., 2000a; Strong et al., 2000; Tosini et al., 2004) monoclonal antibodies had been used to detect specific C. parvum receptors on substrate. Immunogold labeling of C. parvum receptors has been performed as well, but for a specific receptor group only (Tilley and Upton, 1994). Immunogold labeling of C. parvum receptors on the surface of C. parvum stages for SEM has not been undertaken previously. Although this approach does not provide specific information about the involvement of single receptors in parasite processes, it allows the simultaneous visualisation of a large variety of C. parvum surface receptors.
Critical point drying causes membrane shrinkage, which did not only have an impact on size, but also form; the ability to identify *C. parvum* stages and host cell features such as microvilli is consequently impeded. The sample preparation for immunolabeling was found to stronger compromise the structural preservation of samples, possibly due to higher concentrations of fixatives. Therefore, it was found essential to first acquire high quality structural information on *C. parvum* life cycle stages without immunogold labeling. Thus, the results from the non-immunogold studies presented in Chapter 5 were essentially needed to reveal the morphology of life cycle stages.

The visualisation of gold atoms requires the usage of a higher electron acceleration voltage which coincidentally allowed for the transparency of thin-walled stages in this study. A higher acceleration voltage causes the electrons to have more energy, which enables the electrons to penetrate more deeply. Consequently, the usage of a higher electron acceleration voltage, in combination with gold labeling, gave novel insight into the interior of some parasite stages in context with their surface receptor expression.

**Oocysts**

This study has shown that thin-walled oocysts express surface receptors which may either hinder or enable thin-walled oocysts to adhere to tissue (Fig. 91) similar to thick-walled oocysts (Kuznar and Elimelech, 2006; Yu *et al.*, 2007). Studies by Kuznar and Elimelech (2006) have described macromolecules on the surface of thick-walled oocysts and concluded they hinder attachment to epithelium before contact with digestive enzymes. After contact with digestive enzymes the attachment efficiency of thick-walled oocysts increases. It is likely
that during further passage through the intestine, receptors are expressed once again to mediate adherence to target tissue. A glycoprotein, Cp12, has been detected on the surface of oocysts in recent studies by Yao et al., (2007). As glycoproteins are known to be involved in host cell attachment, Cp12 is one candidate which might mediate adhesion of oocysts to host tissue. The disruption of the initial oocyst adherence to host tissue presents an additional drug target which could serve to prevent initial infection in cases where exposure is known to have occurred; for example in a laboratory accident or when contaminations of drinking water or other sources are detected immediately after consumption.

However, this study found that not all macrogamont-like extracellular stages do express receptors for the adherence to host cells. Some macrogamont-like stages release their internal zoites without reattaching to host tissue (Chapter 5). Similar to these macrogamont-like stages that travel free in the lumen of the intestine (see Chapter 5), thin-walled oocysts are not passed in faeces but excyst within the intestine of the same host. Therefore, the expression of surface receptors on thin-walled oocysts had not been expected and thus not realised before immunogold labeling was applied. Combining the three structural images of Fig. 91a with the immunogold information of Fig. 91c revealed that the location of the surface receptors identified on thin-walled oocysts in Fig. 91c overlapped with the small dots seen on the surface of these oocysts in Fig. 91a (Fig. 91b). Thus, receptors are present in the absence of host cells. The adherence of thin-walled oocysts to each other as seen in Fig. 21, however, may also be caused through sample centrifugation.
Immunogold labeling was only detected on the surface of intact oocysts containing developed sporozoites (Fig. 91) or developing sporozoites (Fig. 94a), but not on the surface of excysted oocysts (Fig. 93) or very immature oocysts (Fig. 95a). Once sporozoites have left the oocyst, it would appear that there is no longer a need for receptors in the oocyst wall, as the oocyst has fulfilled its function and there is no further requirement for adhesion to the host.

It is likely that thin-walled and thick-walled oocysts are not produced at exactly the same time point. Consequently there were mainly thin-walled oocysts contained in the oocyst stock. Only a few thick-walled oocysts seemed to be present in the oocyst stock, as experiments in Chapter 5 showed (see for example Fig. 21). It can be assumed that thick-walled oocysts might take longer to develop and mature as they have to form a much thicker cell wall than thin-walled oocysts, but otherwise produce the same complex structure.

Other structures, apart from internal sporozoites, were identified within thin-walled oocysts (Fig. 95). These round structures may have been cytoplasmic granules involved in the process of sporozoite formation, or residues left over after sporozoite formation. Their exact nature however could not be determined based on this experiment. Further intensive TEM studies, building upon the findings of the present studies would be essential to obtain more information on the nature and function of these various structures.

Oocysts detected in this study measured between 2.5 x 3.5 – 3.5 x 3.5 μm. Size variations could depend on the degree of membrane shrinkage due to the
sample preparation procedure, and indeed some oocysts seemed to be more affected (Fig. 95c) than others (Fig. 95a). In contrast, a similar oocyst size of 2.5 x 3.5 μm was observed in non-immunogold samples (Fig. 92), showing how minimal the impact of the sample preparation procedure on the size of most parasites had been.

The differences in size and shape of oocysts seen in Fig. 94, compared to oocysts seen in Fig. 91, are partially due to the sample preparation procedure and partially due to the fact that oocysts exhibit different shapes as already mentioned in Chapter 5. One type of thin-walled oocyst is oval (Fig. 91a) and the other one round (Fig. 94b).

With a size of approximately 1.8 x 1.8 μm in diameter the small cyst-like stages that were seen in the oocyst stock (i.e. Fig. 96a) do not conform to mature oocysts. One might presume that they represent a trophozoite-like stage in transition to an oocyst. If this was the case, one would expect them to be attached to host cells and not isolated together with oocysts. However, through the finding of a cyst-like stage measuring 2.5 x 2.8 μm in (Fig. 98a) the possibility exists that these cyst-like stages represent a transition stage, developing and growing to the size of an oocyst. It may be possible that such stages are isolated together with oocysts. A second possibility would be that the here described cyst-like stages represent trophozoites that have developed, during the storage of the oocyst stock, from within oocysts. TEM examinations of oocyst stock would be needed to determine the nature of the cyst-like stages. They might either be found to contain four endogenous stages and represent a stage developing into oocysts, or they might be found to contain 6-8 endogenous stages and represent trophozoites that formed from within oocysts.
According to their length, the large gamont-like stages seen in Fig. 99 could also be thick-walled oocysts which were expected to be contained in the inoculant but not discovered otherwise. It is possible that, during the sample preparation procedure, the diameter of thick-walled oocysts had shrunk to the dimensions here described. However, thick-walled oocysts are expected to express macromolecules onto their surface (Kuznar and Elimelech, 2006). Large gamont-like stages may not express receptors, as they represent exclusively extracellular stages (as Chapter 5 suggests) that never adhere to host tissue unlike oocysts. Still, it is possible that the macromolecules on thick-walled oocysts were not detected by the antibodies used in this study.

**Cell-free Culture**

Due to the transparency of thin-walled stages under a high kV, some stages detected in cell-free culture could be differentiated from oocyst and identified as different life cycle stages of *C. parvum*. However, no extracellular meront (accumulated from single merozoites) or novel stages, that had been observed by Hijjawi *et al.*, (2004) with light microscopy (see Chapter 3), were seen in this experiment. It is possible that life cycle stages of the cell free culture were missed in this study, as a multitude of cell-free stages had been detected with light microscopy in Chapter 3. Extracellular meronts, resulting from accumulations of merozoites, were observed without sample centrifugation in the supernatant above cells, and were subsequently identified as *C. parvum* stages by the cell walls surrounding them (see Chapter 3). Whether these so called extracellular meronts represent a life cycle stage that contributes to *C. parvum*’s propagation in a host, or merely represent a random accumulation of
C. parvum stages via non-specific C. parvum-C. parvum interactions, could not be clarified in this study and remains to be determined. Isolation of extracellular meronts from the supernatant above cells or from cell free culture would be essential. Re-infection of such isolated stages into fresh cell lines and fresh cell-free media would show whether they are capable to propagate and progress in C. parvum’s life cycle.

Even though different C. parvum life cycle stages were seen in cell-free cultures with light microscopy in Chapter 3, the completion of C. parvum’s life cycle could not be detected in the present study in a 5 day cell-free culture, in contrast to cell culture.

Yet it must be noted that the cyst-like stages in the cell-free culture resemble meronts type I seen on the host cell surface from Chapter 5. However, as no other stages were observed in this experiment, cell-free culture proved not to be suitable for observation with immunogold SEM. A reason for this may be that the sample preparation procedure, essential to process samples for SEM, damaged cell free stages to a degree that they could no longer be identified.

Indeed, a large amount of debris was detected in cell free samples prepared for SEM, in contrast to a small amount of debris in the oocyst stock prepared for SEM. It is likely that cell free cyst-like stages (like thin-walled oocysts) withstand the SEM sample preparation procedure, protected by their cyst wall, whereas other stages which lack this wall become damaged more easily. Combining light microscopy with antibody labeling of C. parvum surface receptors (using fluorescence markers as undertaken in studies by Boxell et al., 2008) could overcome this problem and offers another possibility in examining surface
receptor expressions on cell-free stages and extend the studies by Petersen et al., (1992b) which examined sporozoites antigens with a similar technique.

**Apical Membrane**

Results of this study have shown that *C. parvum* stages express receptors into the host apical membrane surrounding them. Less immunogold labeling was detected on the surface of early trophozoites than merozoites type I (Fig. 104). This indicates that fewer receptors were present on the surface of invasive stages once the host cell invasion process had been initiated, which again indicates that at this stage (Fig. 104) host cell membrane engulfment had already begun. That there were a few immunogold labels on the surface of early trophozoites nevertheless, suggests that *C. parvum* utilised receptors, which were then found in the apical membrane surrounding the invading parasite, in order to orchestrate its engulfment by the host cell apical membrane (Fig. 105).

Again there was more receptor labeling indentified on the surface of mature trophozoites (Fig. 106b), because the parasite had time to develop. Consequently the parasite must have expressed the receptors, seen in Fig. 106b, into the apical membrane during its development. Why surface receptors were mainly seen to occur in groups (Fig. 109c and d) of up to 50 receptors (Fig. 106b) is not clear. Comparing surface receptor expression on mature macrogamonts to other mature *C. parvum* stages like meronts would require intensive TEM studies, to identify stages based on their internal morphology. Such studies might determine if receptors, expressed in groups, are indeed found on the surfaces of macrogamonts, for example. The use of specific
primary antibodies could help to determine whether all receptors within one receptor group are the same, and are expressed as a group to offer sufficient adhesive surface area for the adherence of external stages (which in this case are microgametocytes).

C. parvum receptors identified on what appeared to be the host cell membrane, in close proximity to a young trophozoite (Fig. 107), probably represent receptors used before and/or during invasion, in the course of establishing host cell contact or initiating the host cell to start the apical membrane engulfment. At the end of these processes, receptors are discarded. It can be hypothesised that C. parvum invasive stages shed receptors to mediate host cell contact and to induce the host cell apical membrane engulfment of themselves. Receptor shedding had been observed in previous studies on Cryptosporidium, for example by Strong et al., (2000) who microscopically observed the shed antigen trails of a 15 kD protein (CP15) by gliding sporozoites. Other receptors associated with gliding motility include the Cpa135 (Tosini et al., 2004), CP25 (Tiley and Upton, 2004), GP40 and GP900 (Cevallos et al., 2000a) and CPS500 (Riggs et al., 1999) (see Chapter 1 for a full description of proteins associated with gliding motility). Further, studies by Nesterenko et al., (1999) demonstrated an association between a 47 kD protein found in the apical complex of sporozoites with receptor/ligand interactions between C. parvum and the host cell. It is more than likely that an orchestra of many proteins are involved in host cell attachment and invasion process (see Chapter 1). The TSP-related adhesive protein of Cryptosporidium-1 (TRAP-C1) has also been localised in the apical end of sporozoites (Spano et al., 1998; Okhuysen et al., 2004) and is
associated with host cell attachment and invasion, as shown by its structural similarities with proteins found in *Toxoplasma* (Carruthers and Sibley 1997), *Plasmodium* (Frevert *et al.*, 1993; Mueller *et al.*, 1993; Robson *et al.*, 1995) and *Eimeria* (Tomley *et al.*, 1991).

Meronts I (as seen in Fig. 110) may show less surface receptor labeling because they have nearly completed their life cycle and are not in the process of further development. It would be interesting to observe meronts I at the time point of merozoite type I emergence from the parent stage. During this process the expression of receptors might increase as it has been reported for excysting oocysts (Snelling *et al.*, 2007).

**Microvilli Interactions**

*C. parvum* receptors were always found on host cell microvilli, but not on the flat host cell surface itself. This finding supports the hypothesis that *C. parvum* directly interacts with host cell microvilli, possibly utilising microvilli material for its own purpose. Indeed, studies by Bonnin *et al.*, (1999) identified two microvilli-derived components (villin and ezerin) in the protective PV encapsulating invaded parasites. The exclusive location of immunogold labeling in host cell microvilli demonstrates that *C. parvum* “only” interacts with host cell microvilli and not with the flat host cell surface itself.

Fig. 112b shows most clearly that *C. parvum* directly interacts with host cells via *C. parvum* surface receptor-host cell microvilli interactions. Immunogold labeling of *C. parvum* surface receptors, found ubiquitously distributed over the merozoites surface in Fig. 112b, was also found on two elongated host cell microvilli that embraced the merozoite. The parasite had therefore shed its
surface receptors onto these host cell microvilli, possibly with the aim of inducing them to engulf it. On the other hand, the C. parvum surface receptors might be left behind on the host cell microvilli after parasite-host interactions, for example during the course of gliding motility.

Findings of C. parvum surface receptor labeling in C. parvum gliding trails (Fig. 113) demonstrated that gliding invasive stages shed surface receptors onto host cell microvilli. This finding supports previous observations by Tilley and Upton (1994), Cevallos et al., (2000b) and Strong et al., (2000) that identified the shedding of specific C. parvum receptors (CP15 and CP25, GP900 and GP40) during gliding motility of sporozoites. The respective receptors are utilised by sporozoites not only to establish host cell contact (as mentioned earlier), but also to promote gliding motility. Receptors are consequently left behind on their substrate, resulting in trails that were visualised with the help of antibodies. C. parvum receptors expected to be found in gliding trails include the ones mentioned above as well as the CPS500 and Cpa135. Monoclonal antibodies against these and a variety of other known C. parvum receptors, applied in a similar immunogold SEM study, could identify their affiliation with the gliding trails here observed.

**C. parvum stages only Identified via Labeling of Surface Receptor**

The size of the small round structures seen in Figs. 115, 116 and 117 suggests they are microgametocytes. Further, no host cell structures resembling the shape of these described stages were observed, and the small round stages seen in Figs. 115-117 possessed a high number of C. parvum surface receptors. If these small round stages are considered to be microgametocytes,
then the large *C. parvum* stages that they were seen next to, most likely represent microgamonts, their adhesive target. However, TEM of isolated *C. parvum* stages would be needed to identify (macrogamonts) and differentiate stages (microgametocytes from merozoites type II). Unfortunately this was beyond the scope of this thesis, therefore the here presented data requires further testing.

**Negative Controls**

*C. parvum* stages did not show any immunogold labeling in the absence of primary antibodies. As one of the stages examined was a merozoite, which is known to express large numbers of surface receptors, this result confirms that the second antibody, conjugated with the gold atoms, did not bind non-specifically to *C. parvum* stages (Figs. 118 and 119). Thus, results of this study showing immunogold labeling on the surface of parasite stages conclusively identified *C. parvum* surface receptors.

Likewise, the secondary gold-conjugated antibody did not bind non-specifically to host cells (Fig. 121) as no labeling was detected on the host cell membrane at any time, except in close proximity to *Cryptosporidium*. Therefore, any labeling that has been detected in this study was due to specific *C. parvum* receptor-primary antibody, primary antibody-secondary antibody interactions.

Further, no antibody labeling was detected on microvilli in areas of monolayers incubated with both antibodies where no *C. parvum* stages were present (Fig. 120). It was essential to include this negative control to verify the results of this study as *C. parvum* surface receptors had been detected on host cell microvilli associated with *C. parvum* stages. Thus, it can be concluded that the finding of
immunogold labeling on host cell microvilli close to C. parvum stages indicated the presence of C. parvum surface receptors shed by parasite stages.

6.6. Conclusions

Immunogold labeling of surface receptors has been a useful tool to aid in the identification of C. parvum stages with SEM. Without the application of receptor labeling some stages, especially small ones like microgametocytes, are difficult to identify. Labeling of a large variety of C. parvum surface receptors enabled the identification of large molecules on the surface of thin-walled oocysts, the existence of which had only been speculated about before their identification with immunogold labeling. However, immunogold labeling of cell free cultures did not yield the predicted result. Cell free stages that had been observed previously with light microscopy could not be identified with SEM after the application of receptor labeling. The sample preparation procedure required for cell free cultures to be observed with SEM requires centrifugation of already fixed samples, which appears to have a negative impact on the centrifuged C. parvum stages. Further, cell free stages are less stable than stages of cell culture that are encapsulated by the host cell membrane, or in dense host cell contact. Nevertheless, immunogold labeling of cell cultures using polyclonal antibodies was a success. As hypothesised, C. parvum receptors were detected in the host apical membrane surrounding invading and invaded stages. C. parvum receptors were also identified on host cell microvilli, either in close proximity to invaded stages or on microvilli in gliding trails. As no C. parvum receptors were found on other parts of host material, this experiment identified
host cell microvilli to be the primary interaction partner for gliding and invading
*C. parvum* stages.

The usage of polyclonal antibodies to visualise a large array of receptors simultaneously, does not allow identification of specific receptors. Immunogold labeling using monoclonal antibodies against specific receptors (for example the GP40 or Cpa135) would aid to determine the location of specific receptors within *C. parvum* stages, and potentially their involvement in the parasite’s life cycle.

Immunogold labeling using monoclonal antibodies could further aid to locate specific receptors on parasite and host surfaces, and enable the identification of receptors, the existence of which has been speculated upon. *C. parvum* receptors that still remain to be identified include: *C. parvum* surface receptors essential to mediate microgametocyte adherence to macrogamonts and oocyst surface receptors, which facilitate re-attachment of thin-walled, auto-infective oocysts. Using monoclonal instead of polyclonal antibodies in a similar study would answer the question whether already known receptors are mediating these functions or if yet undescribed receptors are involved.

The disruption of zoite-ligand interactions offers a potential drug target. Visualising specific *C. parvum* receptors in cell culture with SEM could give a novel insight into *C. parvum*-host cell interactions and the disruption of these.
7. General Discussion and Conclusions

An extensive study which monitors and morphologically describes the complete life cycle of *C. parvum* with electron microscopy had not been performed previously. The complete life cycle of *C. parvum* had only ever been observed with light microscopy (for example Hijjawi *et al.*, 2001). However, the present study demonstrated that light microscopy is not sufficient to visualise and morphologically characterise life cycle stages. Only certain aspects of *Cryptosporidium*’s life cycle have been examined before with electron microscopy (Huang *et al.*, 2004; Valigurova *et al.*, 2008); currently, the life cycle of *C. parvum* is incompletely understood. The present study is the first to morphologically examine and describe all known life cycle stages of *C. parvum*, and their relationships with host cells using electron microscopy. In addition, novel life-cycle stages and host-parasite interactions were observed and their place in *C. parvum*’s life cycle postulated.

7.1. Comparisons of Results obtained with Different Methods

7.1.1. Light Microscopy

Due to its limitations in resolution, light microscopy was not sufficient on its own to present a reliable picture of *C. parvum* life cycle stages in *in vitro* culture. Light microscopy did not allow a magnification higher than 1000x, and this only possible under oil immersion. Viewing samples under oil immersion required sample centrifugation and therefore impacted on the results. The depth of focus through a parasite stage also had an impact on the result. As images obtained with light microscopy presented two dimensional cross-sections through
parasite stages, parasite stages may have been misinterpreted. Dependent on
the plane of focus a cross-section through a parasite stage revealed different
images. However, some features of *C. parvum* in *in vitro* culture could only be
observed with light microscopy but not with TEM or SEM, for example
extracellular stages which formed in the supernatant above cells. Therefore,
light microscopy was essential to this study. It also enabled a simple and robust
method to establish the most suitable culture conditions for further *in vitro*
studies on *C. parvum*. With light microscopy live parasite stages can be
monitored in the same culture over subsequent days. In this study, light
microscopy appeared to be the most suitable microscopic method to examine
cell free samples. Attempts to examine cell-free culture with SEM (Chapter
6.4.2) did not yield the large variety of life cycle stages that were expected,
through observations made with light microscopy (Chapter 3, Figs. 8 and 9).
The sample preparation required to examine cell-free culture with SEM probably
damages the more fragile parasite stages, which are not surrounded by outer
protective membranes. Thus, light microscopy might be the only suitable
microscopic method to examine cell-free samples of *C. parvum*.

However, light microscopy is not sufficient to examine and describe *C. parvum*
life cycle stages in cell culture. Intracellular *C. parvum* stages in cell culture are
difficult to distinguish with light microscopy, their nature difficult to identify and
their surface morphology nearly impossible to determine (see Hijjawi *et al.*, 2001). In this thesis, light microscopy on cell culture was therefore only used to
provide a sequential overall assessment of infectivity, monitor the development
of monolayers and to establish optimal culture conditions (Chapter 3). To
identify all *C. parvum* life cycle stages that occurred in culture, and visualise their morphology, high resolution electron microscopy was essential.

### 7.1.2. TEM

TEM is the most suitable microscopic method to perform detailed morphological examinations on interior structures of parasite stages and gain information on the host-parasite interaction zone.

In contrast to SEM and light microscopy, TEM is very time consuming, and more importantly does not guarantee that all possible life cycle stages are found. Even in densely infected monolayers, there is a higher chance to miss than to locate a *C. parvum* stage when preparing TEM sections. Many life cycle stages might therefore be missed if TEM was the only method to examine a parasite culture. Also, TEM showed a limitation similar to light microscopy. Dependent on the plane of the cross-section (similar to the depth of focus in light microscopy) the size, shape and presence of intracellular organelles and structures of the same parasite stage appeared differently. Thus, parasite stages examined with TEM could be misinterpreted. The most relevant results of this study were achieved with SEM.

### 7.1.3. SEM

High resolution SEM allows for all parasite stages that are present in a monolayer to be identified, within one study. At low resolution an overview of the entire monolayer can be achieved, which is important for the location of parasite stages as infections are unevenly distributed in culture. SEM enables full resolution of single parasite stages measuring less than 1 μm, and reveals a
clear image of the entire parasite, as well as distinct morphological features. One limitation of SEM is that it does not enable the visualisation of internal organelles (unless a parasite is at the stage of endogenous stage release), which can make definitive identification difficult, especially when considering new stages. The application of a high electron acceleration voltage permits the visualisation of some interior structures, however, of thin-walled parasite stages only. Therefore, TEM images were matched with SEM images whenever possible. Due to the time limitations of TEM, not all parasite stages imaged with SEM could be matched with TEM images.

Unfortunately all SEM and TEM work had to be performed on conventionally fixed material. Reason for this was the unwillingness of the CMCA to allow viable infectious parasite samples to be brought into common access preparatory laboratories to perform high pressure freezing techniques for TEM, and the unavailability of CryoSEM to study frozen material in Western Australia. Conventional fixation techniques including critical point drying cause changes and shrinkage in cellular structures, which must be considered in all observations.

7.1.4. Immunogold Labeling

Immunogold labeling of *C. parvum* receptors had been performed previously (for example Huang *et al.*, 2004; Matsubayashi *et al.*, 2008). However, these studies were based on the labeling of specific receptors only for examination with TEM. For the present study a protocol was established to visualise a wide range of *C. parvum* receptors in an entire *C. parvum* culture for examination
with SEM. This method visualised the distribution of *C. parvum* receptors expressed on the surface of free parasite stages, and on the surface of the apical membrane engulfing intracellular parasite stages. This method also enabled visualisation of parasite receptors left behind on host cell material. The disadvantages and advantages of polyclonal versus monoclonal antibodies have been detailed in Chapter 6.

In contrast to molecular methods which have been used to examine host-parasite interactions (Chen *et al.*, 2000; Zeng *et al.*, 2006), immunogold labeling of *C. parvum* receptors for SEM achieves results which are visual and thus reliably interpretable.

### 7.1.5. Cell-Free Culture

According to the results obtained with light microscopy (Chapter 3, Figs. 8 and 9), *C. parvum* life cycle stages of cell-free culture exhibited a different morphology than stages in cell culture. Whether these reflect extracellular stages that occur *in vivo* remains to be determined and further work is required to determine the value of cell-free culture as a suitable model for the life-cycle *in vivo*. Nevertheless, it is possible that in cell-free culture the same life cycle stages occur as in cell culture, or even *in vivo*, but their appearance could be different in the absence of host cell interaction and associated structural changes; for example, apical membrane formation. Studies by Hijjawi *et al.*, (2004) had already concluded that *C. parvum* is capable of completing its life cycle in cell-free culture. Recent studies by Kartashev *et al.*, (2009) and Zhang *et al.*, (2009) support Hijjawi’s observation that *Cryptosporidium* is able to propagate in cell-free medium, for a limited duration. This ability would certainly
be advantageous for the parasite. If thick-walled oocysts are shed into a moist reservoir that is rich in nutrients, *C. parvum* could propagate to achieve higher numbers for infection of the next host. Replication of *C. parvum* in purified water has indeed been observed by Annika Boxell (personal communication). Thus, cell-free culture may be of value in studying *C. parvum* development in drinking water, and would be of interest to the water industry to better understand waterborne outbreaks of cryptosporidiosis.

Some stages seen in cell free culture were also found in cell cultures, for example, extracellular trophozoite-like stages were seen in the supernatant above cells as well as in cell-free culture (Fig. 8b). However, on the basis of light microscopy these stages could not be conclusively identified. Merozoites type II may have been mistaken for trophozoite-like stages given the limited resolution of light microscopy. It is also difficult to identify stages of cell-free culture as they can not be distinguished on the basis of their relationship with host cells.

However macrogamont-like stages were found in cell-free culture (Fig. 9c) which resembled extracellular macrogamont-like stages seen in cell culture with SEM (Chapter 5, Figs. 71a and 71b). Likewise, large gamonts seen in cell-free culture (Figs. 8l and 9d) were also detected in the supernatant of cell culture with SEM (Chapter 5, Figs. 71c, 72b and 73a-c). Macrogamonts and large gamonts described in this thesis appeared similar in their morphology (Fig. 9, Figs. 71-73). It is possible that they possess two morphological forms, yet fulfil the same function in *C. parvum*’s life cycle. The presence of these gamont-like
stages were reported in studies of Hijjawi et al., (2002), which isolated gamont-like stages of *C. andersoni* from the intestine of mice.

Another stage identified with light microscopy, in both cell-free culture and the supernatant above cells, was the so called extracellular meront (Figs. 4f, 5i-k, 8m), as described previously by Hijjawi et al., (2004). Although extracellular meronts appeared as accumulations of small stages in the present study, they were identified as one parasite stage because they possessed a cell wall surrounding the entire stage, which was clearly visible under the light microscope. Unfortunately, with EM no such extracellular meronts were detected as the sample preparation procedure for SEM and TEM requires the removal of the supernatant. Examinations of supernatants with SEM were attempted but did not reveal any identifiable *C. parvum* stages.

### 7.1.6. Cell Culture

Cell culture was found to be the only suitable *in vitro* model for *in vivo* infections. In contrast to cell-free culture, cell culture could be observed with light microscopy, SEM and TEM. Detailed discussions are found in Chapters 4 to 6. Images of *C. parvum* infections in cell culture obtained in this study, complemented images of an *in vivo* infection of a toad with *C. muris* (Valigurova et al., 2008). Comparison of the results obtained in this study to the results by Valigurova not only verifies the findings of this thesis, but also demonstrates the morphological similarities of life cycle stages between *Cryptosporidium* species.
7.1.7. Oocyst Stock

Thin-walled oocysts were present in the inoculant and detected 6hrs post inoculation because the whole mouse gut was harvested for the isolation of oocysts. Faeces are thought to only contain thick-walled oocysts (O'Donoghue, 1995). Thin-walled and thick-walled oocysts seem not to be produced at the same time point in vivo. The oocyst stock used for the inoculation of experiments in Chapter 5 contained thick-walled and thin-walled oocysts (Figs. 21 and 82). However, the oocyst stock used for experiments in Chapter 6 appeared to only contain thin-walled oocysts (Fig. 91). The experiments in the two chapters were performed with oocysts stocks harvested from different mice, during different studies. As thick-walled oocysts have to form a thick oocyst wall, in contrast to thin-walled ones, they can be expected to take longer for their development. Thus, at the time point of oocyst harvest for the experiments in Chapter 6, numerous thin-walled oocysts were present in the intestinal lumen, whereas thick-walled oocysts had not yet matured and resolved host cell contact.

Immunogold labeling of C. parvum surface receptors applied in this study has shown that thin-walled oocysts do express receptors to adhere to tissue. Surface receptors that mediate adhesion to target tissue, but hinder adherence to other tissues (Kuznar and Elimelech, 2006), are a necessity for thick-walled oocysts that are ingested by a potential host. Thin-walled oocysts, however, never leave their host and excyst again in the intestine of the same host, to keep the infection going. That they express surface receptors suggests that they reattach to host tissue, so that they persist within their original host. As
receptors are first expressed during maturation of internal sporozoites (Fig. 91), the oocysts have time to travel in the lumen of the intestine and infect the host elsewhere, thus spreading the infection.

7.2. Summary of Results

The introduction of this thesis reviewed the sequence of molecular mechanisms and pathways that *C. parvum* employs to invade host cells (see Publications: *Trends in Parasitology*, 2009). Understanding the host-parasite interactions is crucial in order to develop a successful treatment against cryptosporidiosis. Even though cure rates of more than 90% have been reported, clinical trials utilising anti-cryptosporidial agents have not yet achieved complete eradication of the pathogen (Allam and Shehab, 2002). However, the life cycle, biology and mechanisms of *C. parvum*-host cell interaction remain incompletely understood. It is essential to understand all of these factors to be able to develop an effective treatment. To aid this purpose, the present study was performed.

7.2.1. Infected Host Cell Layers

As an important prerequisite for this study, morphological features of the host cell line were examined with SEM, so that they could be distinguished from parasite stages in culture (Chapter 4).

In the course of subsequent examination of infected cell cultures interesting observations were made:

SEM images of infected monolayers revealed that infections with *C. parvum* caused host cell necrosis, rather than apoptosis. Previous research had mainly focused on the modulation of host cell apoptosis by *C. parvum* (Chen *et al.*,
1999, Ojcius et al., 1999; O’Hara et al., 2007). Only one study identified *C. parvum* induced host cell necrosis (Elliott and Clark, 2003), which supports the findings of the present study. The observations of the present study are further supported by previous studies by Widmer et al., (2000) that found host hell apoptosis impairs *C. parvum* development. It is likely that the host employs apoptosis as a mechanism to eradicate infections. Therefore, it appears advantageous for the parasite to inhibit apoptosis rather than to induce it. Indeed, for the first time studies by Mc Cole et al., (2000) suggested that *C. parvum* might inhibit apoptosis. Further studies by Chen et al., (2001) and Liu et al., (2008) proved that *C. parvum* does inhibit host cell apoptosis, for its own advantage. Today it is believed that *C. parvum* modulates host cell apoptosis by inhibition of apoptosis when host cell death would hinder its development, and induction of apoptosis once the parasite’s function is fulfilled (Mele et al., 2004; Hashim et al., 2006, Liu et al., 2009). The results of the present thesis, however, only support the first of these two points. Host cell apoptosis in infected areas was decreased in the present study, supporting previous research that *C. parvum* inhibits apoptosis, to prevent its eradication from the host. However, cells infected with *C. parvum* were always observed to die of necrosis and not of apoptosis, as explained in Chapter 4. *Cryptosporidium* infection of monolayers occurred in concentrated areas rather than ubiquitously throughout the culture. *C. parvum* stages in one area were observed to represent identical life cycle stage (for example several meronts type I), or at least be at the same stage of development within the parasite’s life cycle (for example microgamonts and macrogamonts). How *Cryptosporidium* gains advantage through this behaviour can only be speculated. Perhaps the
presence of similar life cycle stages in close proximity enhances the pairing of stages during syzygy, such as gamonts, as described for gregarines by Lacombe et al., (2002) and for C. andersoni by Hijjawi et al., (2002). Stages in one area of infection being at the same stage of development especially becomes relevant once the sexual life cycle is active, for fertilisation and possibly the exchange of genetic material via syzygy to occur.

7.2.2. Oocyst Excystation and Host Cell Infection

Excystation of inoculated oocysts was observed to occur during a 48 hr period following the trigger for excystation and inoculation into host cell culture. It appears that C. parvum gains advantage by not excysting all oocysts simultaneously. If oocysts excyst during a 48 hr period, a higher variety of life cycle stages is present at any one time point. Also, newly formed thick-walled oocysts would be passed in the faeces throughout a 48 hr period, rather than at one single occasion, which would distribute infective oocysts throughout a much larger environmental habitat and reach a larger number of potential hosts. Future studies, characterising the underlying regulatory mechanisms within the C. parvum genome via genetic analysis, could support these findings.

The size and morphology of both thick-walled and thin-walled oocysts was determined in this study and compared to the literature (see Chapters 5 and 6). This study identified that the presence of surface receptors on thin-walled oocysts depends on the presence of sporozoites (either developing or fully developed) and does not depend on the presence of host cells. Receptor expression was detected in the absence of host cells (Chapter 6). Similarly, the size and morphology of excysted sporozoites was determined and compared to
the literature (see Chapter 5). The results from the present study suggest that two morphologically different types of sporozoites exist, dependent on their origin; either thin- or thick walled oocyst. Sporozoites seen in this study hatching from thick-walled oocysts measured 0.8 x 5 μm, whereas studies by Reduker et al., (1985), which also used SEM, determined the size of sporozoites from thin-walled oocysts to be 0.6 x 3.8 μm. Thus, it was hypothesised that two different types of sporozoites might exist, and why the apical ends of sporozoites appear to be elongated when in proximity to host cells was discussed.

7.2.3. Host Cell Invasion

Novel information on the sporozoite host cell invasion process was presented. Elongated host cell microvilli were seen to embrace invading sporozoites (Fig. 23). A thin membrane engulfing the invading sporozoites was seen to develop from these microvilli. Previous studies by Bonnin et al., (1999) had already identified microvilli components in the parasitophorous membrane. Thus, it is possible that the protective inner membranes surrounding C. parvum stages are formed from microvilli material, and not as a protrusion of the host cell apical membrane. Microvilli which were found incorporated into the apical membranes surrounding many trophozoite stages can therefore be concluded to have been incorporated during this process (see for example Fig. 27b). The outermost membrane, however, is most likely to form as a protrusion of the host cell apical membrane as Figs. 24 and 29 suggest. These findings concur with the results of Valigurova et al., (2007 and 2008), which identified 2-3 separate membranes surrounding invaded C. muris stages.
Co-invasion of two or more invasive parasite stages into one host cell was monitored and found to be a result of *C. parvum* invasive stages adhering to each other. All free *C. parvum* stages seen in this study were able to randomly adhere to each another, presumably via *C. parvum-C. parvum* surface receptor interactions.

This study extensively investigated *C. parvum*’s interactions with, and impact on, host cell microvilli. Gliding trails of elongated microvilli were frequently observed on the host cell monolayer, ranging from an excysted parent stage to newly formed daughter stages. Such gliding trails were caused by a sporozoites or merozoites gliding through the apical brush border before host cell invasion. Immunogold labeling for SEM confirmed that invasive zoites interacted with host cell microvilli (for example in gliding trails). Immunogold labeling identified that *C. parvum* receptors were left behind on host cell microvilli by gliding parasites, consistent with the shedding of material reported by Tilley *et al.*, (1994), Arrowood *et al.*, (1999) and Wetzel *et al.*, (2005).

Similar to gliding trails, meshworks of microvilli were commonly observed around trophozoites. Microvilli within meshworks were found to be elongated and their expression greatly increased, in contrast to non-infected areas. This finding supports the hypothesis that *C. parvum* utilises microvilli material for its own purpose, as suggested by studies of Bonnin *et al.*, (1999). *C. parvum* may induce the host cell to produce even more microvilli material as increased numbers of microvilli around trophozoites, in comparison to non-infected areas, were seen to occur (see Fig. 35).
A large variety of *C. parvum* receptors had been identified to play a role in the host cell attachment and invasion process (Tilley and Upton, 1994; Langer and Riggs, 1999; O’Connor *et al.* , 2007a). The function of these receptors has already been reviewed and discussed in Chapters 1, 5 and 6.

**7.2.4. Trophozoite Receptor Expression**

Trophozoites were found to express receptors within their apical membranes (Fig. 108), which otherwise consist of host cell microvilli material (Huang *et al.*, 2004; Valigurova *et al.*, 2008; Fig. 23). The expression of surface receptors increased with maturity of the trophozoite. Receptor expression into the outer membrane probably occurs to enable contact with other *C. parvum* stages, such as the adherence of microgametocytes onto macrogamonts. Thus, the expression is the highest in mature stages.

Recent studies by Jakobi and Petry (2006) observed that the expression of certain *C. parvum* receptor genes (CP15, CP17, P23 and GP900) follows a unique time schedule during intracellular development. This suggests that the functions of these receptors are not restricted to invasive life cycle stages. It would be interesting to use monoclonal antibodies directed against respective *C. parvum* antigens to test whether some of these receptors are expressed on the surface of mature life cycle stages, oocysts and other cell free stages. As the present study also detected receptor expression in the membranes surrounding mature *C. parvum* stages, it can be concluded that *C. parvum* receptors play an important role throughout the entire life cycle of the parasite.
In some developing trophozoites the formation of a thick parasite membrane (PM) was observed with TEM. It was proposed that the PM may form the outer membrane of developing oocysts.

### 7.2.5. Feeder Organelle

The nature of the so called feeder organelle has been critically examined. This study demonstrated that the actual feeder organelle must be carefully differentiated from the rudimentary body, a parasite structure (identified in this study) of which merozoites and/or gametocytes are formed, and released by budding. The feeder organelle, however, is located underneath this rudimentary body and was seen to form a deep connection into the host cell. Tunnel-like feeding connections into the host cell, already described previously in studies by Huang et al., (2004), were also observed in this study (Fig. 77c). *C. parvum* is known to rely on the host cell in a feeding-dependent fashion as it lacks several de novo synthesis pathways (Barta and Thompson, 2006). TEM cross-sections suggested that the parasite may utilise host cell protein and fatty acid production and assembling machinery (consisting of nucleus, endoplasmic reticulum and golgi) to satisfy its feeding dependency. The hypothesis was made that *C. parvum* might thus establish a connection to the host cell nucleus to obtain nutrient supply from the host cell, using the host cell’s own transport mechanism.

### 7.2.6. Binary Fission and Syzygy

Results of this study suggest that *C. parvum* does not only replicate via the two pathways known to date, but that it also applies binary fission (Fig. 36) as an
additional means of asexual replication, and syzygy (Fig. 37), to aid the exchange of genetic material. Syzygy of two gamonts is a well documented occurrence in gregarines (Lacombe et al., 2002). Thus, it can be concluded that C. parvum probably employs syzygy for a similar purpose, potentially the exchange of genetic material between gamonts of the same sex or to aid fertilisation of macrogamont/microgamont-pairs.

The attachment of two stages to each other via discs was monitored for microgamonts and discussed in Chapter 5. TEM micrographs revealed that some adjacent parasites in syzygy attached on to each other via the feeder organelle structure, instead of to a host cell. In a host cell environment, C. parvum does not depend on feeding off each other. Thus, the most likely explanation for this observation is that mature C. parvum stages apply syzygy to exchange genetic material as observed and described for gregarines by Lacombe et al., (2002) and Toso and Omoto (2007).

7.2.7. Meronts I

Surprisingly, the formation of meronts I was observed after only 24 hrs of culture. This stands in contrast to previous research (Hijjawi et al., 2001). In addition, the size and morphology of meronts I was determined more accurately in this study than it was possible to do previously with the light microscope (Hijjawi et al., 2001). Merozoites type I were observed to be formed by budding of a rudimentary body located at the base of the meront. For the first time, the size and morphology has been presented in one study (see Chapter 5). Merozoites type I possessed a lateral protrusion which was identified via immunogold labeling to be rich in receptors. This lateral protrusion was
suggested to mediate host cell attachment instead of the apical tip. Reorientation of apical organelles to initiate the invasion process was thought to occur following initial contact, as the invasion machinery known to mediate invasion process is located at the apical end (Huang et al., 2004).

7.2.8. Meronts II
Two different types of meronts II were observed in this study. The first type resembled the meront II accepted in the life cycle today (as described previously by Hijjawi et al., 2001): possessing a thicker cell wall than a meront I, being slightly larger in size, releasing round merozoites, but otherwise appearing very similar. The second type however, had a different morphology. Without detaching from the parent stage, each merozoite I formed a daughter stage (Fig. 59). The finding of the second type of meront II is new to this study. Since different types of merozoites were also observed in this study, different types of meronts II may exist. Consequently, C. parvum might exhibit a much larger variety of life cycle stages than previously suggested (Hijjawi et al., 2001). A complex life cycle facilitates a parasite’s survival in a host by maximising the chances of escaping the host’s immune responses.

Large merozoites type II showed more surface receptors than small merozoites type II, as revealed with immunogold labeling. To better understand this observation further experiments, including intensive observations of infected cultures (or preferably isolated in vivo stages), are needed. Further characterisation of C. parvum meronts and merozoites would also help explain observations made in this study.
7.2.9. Gamonts

The further a *C. parvum* stage progressed in the life cycle, the less host cell contact it maintained. Gamonts showed different types of host cell attachment. Tunnel like-feeding connections as described by Huang *et al.,* (2004) were also observed in this study (Fig. 84c). Most micro- and macrogamonts however attached to host cells via a stalk-like structure, as previously described by Valigurova *et al.,* (2008), which enabled them to easily break host cell contact. Stalks were seen to be attached to thick-walled oocysts after breaking of host cell contact, but never thin-walled oocysts. Thin walled oocysts seemed not to produce stalk-like structures (Fig. 81). As thin-walled oocysts are produced to re-infect the same host, it can be concluded that host cell detachment is not as important for thin-walled oocysts as for thick-walled oocysts which have to be passed in faeces. Many micro- and macrogamonts possessed stalks. However, some macrogamonts and large gamonts severed host cell contact without the aid of a stalk-like organelle (see Fig. 71a). This finding supports the hypothesis that different subtypes of gamonts do exist.

7.2.9.1. Microgamonts

For the first time, the morphology of microgamonts and microgametocytes has been comprehensively visualised. After their release, microgametocytes were observed to develop a ring-like structure which was thought to play a role in the attachment to macrogamonts. Some microgamonts were seen to break host cell contact before the time point of gametocytes release to travel free in culture media. For this purpose, microgamonts with a stalk-like structure broke contact between the stalk and the host cell, thereby retaining the stalk. Other
microgamonts severed membranes adjacent to ring-like structures to break contact with their attachment zones. Immunogold labeling, to identify microgamonts and gametocytes as stages of *C. parvum*, was essential as the morphology of microgamonts has never been described before and, for example, meronts have been mistaken for microgamonts (see Fayer *et al.*, 1997).

### 7.2.9.2. Macrogamonts and Large Gamonts

Five different subtypes of macrogamont-like stages were identified in the life cycle of *C. parvum*. Two subtypes develop into either thin-walled- or thick-walled oocysts. It would be advisable to describe these subtypes as zygotes instead of macrogamonts, because three other subtypes exist, which develop internal zoites without developing into an oocyst. One of these three macrogamont subtypes was found to stay attached to its host cell and release infective zoites into culture (Fig. 70). Another subtype was found to break host cell contact (Fig. 71a) to travel in the intestinal lumen and release internal zoites (Fig. 78c and d). The last subtype resembled the large gamont-like stage described by Hijjawi *et al.*, (2002 and 2004) (Fig. 71c). Large gamonts appeared to be a stage similar to a macrogamont, resolving host cell contact and travelling through the lumen of the intestine. Large-gamonts, similar to the ones described by Hijjawi *et al.*, (2002), were detected with light microscopy and SEM on separate samples. Comparison to similar *C. parvum* stages aided their identification. Their existence, proposed for *C. parvum* by Hijjawi *et al.*, (2004), was confirmed in the present study and their morphology more closely examined.
As noted earlier, this complexity of the life-cycle is likely to aid the parasite’s survival and reproductive success.

7.2.10. Completion of the Life Cycle

The life cycle of *C. parvum* was found to be completed in culture, with the formation of oocysts after only 5 days. This observation stands in contrast to studies by Hijjawi *et al.*, (2001), which described the completion of *C. parvum*’s life cycle after 7 days. Some *Cryptosporidium* species are known to complete their life cycle after as little as 2 days (O’Donoghue, 1995). Thus, it was considered possible that *C. parvum* might complete its life cycle earlier than the 7 days proposed by Hijjawi.

The early occurrence of microgamonts after only 48 hrs in culture can be explained if the inoculant consisted of other stages than just the expected thin-walled and thick-walled oocysts. However, if *C. parvum* completes its life cycle after fewer days, this would explain the early occurrence of microgamonts as well as the occurrence of oocysts after 5 days. The present study found that oocyst excystation occurs within 48 hrs following inoculation. Oocysts excysting after 48 hrs may account for the majority of microgamonts being detected, as expected, after 5 days (Fayer *et al.*, 1997; Hijjawi *et al.*, 2001). A small amount of oocyst excystation immediately after inoculation may have resulted in the early microgamonts seen after 2 days.

On the other hand, the occurrence of two different types of sporozoites was hypothesised earlier, dependent on their origin from either thick-walled or thin-walled oocysts. Host cell invasion of sporozoites resulting in meronts I, which after one day release merozoites type I to invade cells and form gamonts, would
explain the occurrence of microgamonts after 2 days. Thin-walled, as well as thick-walled oocysts were found to have been inoculated in the experiments detailed in Chapter 5. It would be plausible that sporozoites excysting from thin-walled oocysts initiate the sexual life-cycle earlier than sporozoites from thick-walled oocysts. Thin-walled oocysts, in contrast to thick-walled oocysts, re-infect a host which is already infected. Therefore it would be an advantage to progress to the sexual life cycle as soon as possible to produce more thick-walled oocysts for the release in the faeces. Thick-walled oocysts in contrast, when ingested, first need to establish an infection in a host. Therefore, sporozoites excysting from thick walled oocysts might first produce meronts I, which release merozoites type, to propagate in the asexual life cycle and increase the infection within the first host. The finding of different morphological forms of merozoites (type I) further supports this hypothesis.

7.2.11. Cell-Free Development

Studies by Boxell (personal communication), suggested the formation of trophozoites directly within oocysts. Similar observations were made in this study. Macrogamonts that broke host cell contact and travelled free in the media were seen to form and release round trophozoite-like stages without prior host cell contact (Fig. 78c and d).

7.2.12. Unclassified Stages

Last but not least this study identified two atypical stages with immunogold as being *C. parvum*, for which the place and role in the life cycle of *C. parvum*, as well as their nature, remains unclear and requires further investigation (Figs.
84e, 85 and 86). These observations emphasise that the life cycle of *C. parvum* has not yet been completely described, with some stages yet to be discovered. Microscopic examinations either *in vitro* or preferably *in vivo* would be the best methods to serve this purpose.

### 7.2.13. Phylogenetic Affinity

Until recently, *Cryptosporidium* species have been falsely classified as belonging to the phylum of coccidia (see Butaeva *et al.*, 2006). However the insensitivity to anti-coccidial drugs cast doubt on this affinity (Thompson *et al.*, 2005). Consequently, Barta and Thompson (2006) compiled evidence from biological and molecular studies (Carreno *et al.*, 1999), which demonstrated that *Cryptosporidium* species are closer related to the apicomplexan gregarines than coccidia. Thus, it comes as no surprise that the morphological studies based on TEM by Butaeva *et al.*, (2006) identified strong similarities in host cell attachment and feeding mechanisms between gregarines and *Cryptosporidium*, even though *Cryptosporidium* was thought of as being closely related to coccidia.

Results from the present study show strong similarities between *C. parvum* and gregarines, and distinct differences to coccidia. The present study identified that, similar to gregarines, *C. parvum* resides extracytosolic, protected by the host cell membrane, yet connected to the host cell interior via a feeding connection similar to the transmembrane feeding connection of gregarines. Coccidia, however, reside intracytosolic, similar to plasmodium (see Barta and Thompson, 2006). The findings of the present study also concur with findings of
Valigurova et al., (2007), which compared the attachment site of *Gregarina steini* and *C. muris*, based on TEM.

In summary, the present study confirms *Cryptosporidium's* affinity with gregarines.

### 7.3. Suggestions for Future Research

More intensive microscopic studies would be needed to discover remaining stages in the life cycle of *C. parvum*. It is possible that not all parasite stages found *in vivo* are also present *in vitro*, as *C. parvum* might not be required to exhaust all of its options in an *in vitro* model in the absence of, for example, the host's immune defence. Therefore, *in vivo* studies examining life cycle stages in biopsy specimens of, for example infected cows, would be most valuable. As the present study shows, comparison of results between scientists, and the application of *C. parvum* specific labeling to culture, aids the identification and verification of atypical *C. parvum* stages.

For future microscopic examinations of *C. parvum*, a combination of light microscopy and EM would be advisable. TEM is time consuming, but the best choice to gain an insight into the parasite's internal structures and obtain information about the host-parasite relationship. SEM enables the morphological examination of the entire range of life cycle stages and should enable the discovery of any missing life cycle stages of *C. parvum*. SEM also yields valuable information on the host-parasite relationship, allowing insight into the parasites interior in the case of stages at the time-point of endogenous zoite release or, if high electron acceleration voltages are applied, to thin-walled stages. However, stages that are free in the supernatant and/or lumen of the
intestine may not be visualised. In this study several attempts were made to concentrate such stages onto coverslips for viewing by SEM (Fig. 21 of a sporozoite isolated from the supernatant above cells). However, the necessary sample preparation destroyed nearly all of these, more fragile stages. Thus it seems important to involve light microscopy in future microscopic investigations. This study has demonstrated that immunogold labeling of C. parvum surface receptors can be used to identify cell-free, as well as invaded C. parvum stages, with SEM. Thus, the immunogold protocol established in this study provides a way to identify C. parvum with SEM directly, without having to apply other techniques such as isolation of single stages and/or PCR (Hijjawi et al., 2002).

In future, the protocol established in this study can be applied to label specific receptors with immunogold particles for SEM. This will enable the study of specific host-parasite interactions in an entire sample, examining all life cycle stages within one experiment. Until today, immunogold labeling of C. parvum receptors for TEM only allowed for the study of single parasites (Matsubayashi et al., 2008). The immunogold labeling for SEM protocol established in the present study, however, enables the examination of a whole group of parasites in context, including host cell surroundings. The protocol developed in this study offer economical, flexible and less cost intensive method to identify C. parvum stages (as compared to for example Hijjawi et al., 2002) and a time saving method to examine parasite receptors of interest (as compared to Matsubayashi et al., 2008).

To obtain more reliable results, future studies should try to minimize artefacts due to sample preparation procedures. Standard fixation techniques including critical point drying, which were unavoidable in this study as better methods
were not available, cause shrinkage and damage to cellular structures. The utilisation of CryoSEM and high pressure freezing techniques for TEM would result in less sample damage and therefore more reliable results.

The discovery of new life cycle stages and morphological characterisation of already accepted life cycle stages opens new windows into *Cryptosporidium* research. Researchers working in the field of Parasitology may search for the newly described life stages in other species of *Cryptosporidium*. Other researchers might test whether the newly identified extracellular stages and features (macrogamonts, large gamonts, free microgamonts, adhesive disc on microgametocytes and the lateral protrusion seen on merozoites type I) represent susceptible drug targets. As extracellular stages are not protected by the host cell apical membrane they are potentially more susceptible to drug therapy. The finding of this study that oocyst are not the only stages developing from macrogamonts which break host cell contact and/or produce infective zoites might therefore provide new avenues for *Cryptosporidium* research, drug targeting and disease prevention.

Stages developing from macrogamonts have been observed to travel free in culture media and form internal trophozoites without host cell contact. Such stages might form in environments other than within hosts. Therefore, findings of this thesis might be useful to researchers working in the field of water reservoir quality, for the prevention of disease outbreaks. The best way of treating a disease is preventing its outbreak in the first place.
This study has identified and morphologically characterised structures in merozoites and microgametocytes which mediate the invader’s attachment to host cells, versus macrogamonts. The characterisation of these specific organelles on merozoites rich in *C. parvum* receptors may offer a suitable drug target to eradicate a *C. parvum* infection in its very early days before the sexual life cycle has started at day 2-4. As *Cryptosporidium* depends on merozoite invasion to maintain an infection for longer than a day, it would be possible to target extracellular invasive stages (sporozoites from thick-walled oocysts, merozoites type I and II and sporozoites from thin-walled oocysts) only in therapeutic attempts. Many receptors have been found on the surface of invasive stages and were given a role in the host cell attachment and invasion process (for example gp900 - Barnes *et al.*, 1998; CSL – Langer and Rigs, 1999; gp40/15 - Cevallos *et al.*, 2000b; Cpa135 - Tossini *et al.*, 2003; p30 - Bhat *et al.*, 2007;). Disruption of a combination of receptors may be necessary to prevent infection. Future SEM studies utilising the immunogold protocol established in this study will assist in identifying the combination of receptors active in the invasion process of a distinct parasite stage. The possibility to disrupt zoite ligand – host cell receptor interactions (Langer *et al.*, 2001), to prevent extracellular invasive stages of *Cryptosporidium* from host cell invasion, offers a new direction in anti-cryptosporidial drug development.

For infections in a later stage however, stages of the sexual life cycle may need to be targeted as well. Here, the attachment organelle found on microgametocytes may represent a potential drug target. Immunogold SEM of selected receptors could elucidate which receptors are involved in microgametocyte adherence to macrogamonts.
Surface macromolecules on oocysts are known to mediate selective attachment to target tissue (Kuznar and Elimelech, 2006). A receptor, the Cp12, has been identified in studies by Yao et al., (2007) to mediate oocyst adherence. It is possible that more, yet undescribed, receptors are involved in oocyst adherence to target tissue. Applying specific immunogold labeling against Cp12 to oocyst culture, compared to another culture labeled with non-specific C. parvum labeling, would answer this question. If more receptors were visible with SEM in the case of non-specific immunogold labeling, than specific Cp12 labeling, other receptors would be involved in oocyst adherence. It might be possible to prevent a C. parvum infection through early intervention by disrupting oocyst adherence to target tissue by blockade of receptor-ligand interactions, if an infection is thought to have occurred, for example during a laboratory accident.

To rationally design a drug against cryptosporidiosis it is essential to understand the parasite's biology, especially its interactions with host cells. The present findings on oocyst, merozoite and microgametocyte morphology and host cell interactions provide a prerequisite for developing such therapeutic interventions.

Although cryptosporidiosis in humans is an uncommon disease in the Western world, infection causes losses to the meat and dairy industries. It would thus be of economic benefit to focus research on drug development and treatment on the species C. parvum which affects both humans and livestock, rather than C. hominis which only affects humans. Intensive in vivo studies employing light microscopy, SEM and TEM will be essential to discover the complete life cycle of Cryptosporidium, as it occurs in clinical infections. It would be of special
interest to follow up on the development of extracellular stages, as they represent an easier drug target than intracellular stages. An alternative to preventing infection of host cells, by disrupting zoite-ligand interactions, would be the elimination of extracellular stages. Antibiotics such as azithromycin, paromomycin and nitazoxanide have been proven to strongly reduce Cryptosporidium infections (Giacometti et al., 1999). Further research into the efficacy of different antibiotics, and combinations appears promising. In bacteria, antibiotics are known to suppress protein synthesis. Even though their mode of action in Cryptosporidium remains unknown, it is possible that they fulfil a similar function. Unlike bacteria, extracellular merozoite-like stages do not produce a cell wall. However, they are the most susceptible as they are not protected by the apical host cell membrane. It is possible, therefore that antibiotics inhibit the synthesis of surface proteins in C. parvum extracellular stages which are essential for the completion of the life cycle. Thus, antibiotics might either destroy extracellular stages of C. parvum or inhibit their re-infection of host cells via disrupting the expression of C. parvum surface receptors essential for host cell adherence.

The development of a vaccine sounds a promising option at first glance. However, incomplete immunity may still lead to losses. It would be desirable to have a treatment on hand in cases of an outbreak in either humans or cattle. To treat cryptosporidiosis, anti-cryptosporidial agents will either have to pass through the apical membrane engulfing the parasite, or reach the parasite through the feeder-organelle via host cell transport mechanisms to target intracellular stages. Results of this study suggested that C. parvum may
reprogramme the host cells machinery, utilising the host cells transport mechanisms to obtain essential fatty and amino acids, which *C. parvum* is unable to synthesis itself. Further research into the parasite’s feeding dependency, including TEM, and molecular studies to identify *C. parvum* genes involved in fatty acid and amino acid utilization, could identify whether the parasite’s feeding pathways offer potential routes for drug delivery.

However, as *Cryptosporidium* depends on merozoite invasion to maintain an infection for longer than a day, and depends on sporozoite invasion to initially establish an infection, it would be possible to target extracellular invasive stages only via the disruption of zoite-ligand interactions, as described above and proposed by Langer *et. al.*, (2002), potentially via antibiotic treatments (Giacometti *et al.*, 1999).

### 7.4. Concluding Remarks

With the *in vitro* culture used in this study all life cycle stages accepted in the life cycle of *C. parvum* today were observed after 5 days. Thus, *C. parvum* culture in HCT-8 cells is a useful model to represent *in vivo* infections. Life cycle stages of *C. parvum* in culture appeared similar to *C. muris* life cycle stages *in vivo*, as described by Valigurova *et al.*, (2008), verifying the HCT-8 cell culture as a suitable model for a clinical infection. However, it remains a model, because intestinal components like mucus layers, digestive fluids, enzymes and the host’s immune system, which all play a role in the establishment and eradication of a *C. parvum* infection in the host, are not present.
The present study is the first to morphologically describe all known, as well as de-novo life cycle stages of *C. parvum* and the relationships of most life cycle stages with host cells. Hence, this study provides a basis for future research on the parasite *Cryptosporidium*.

It is easier to fight your enemy when you know all of his 100 faces.
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