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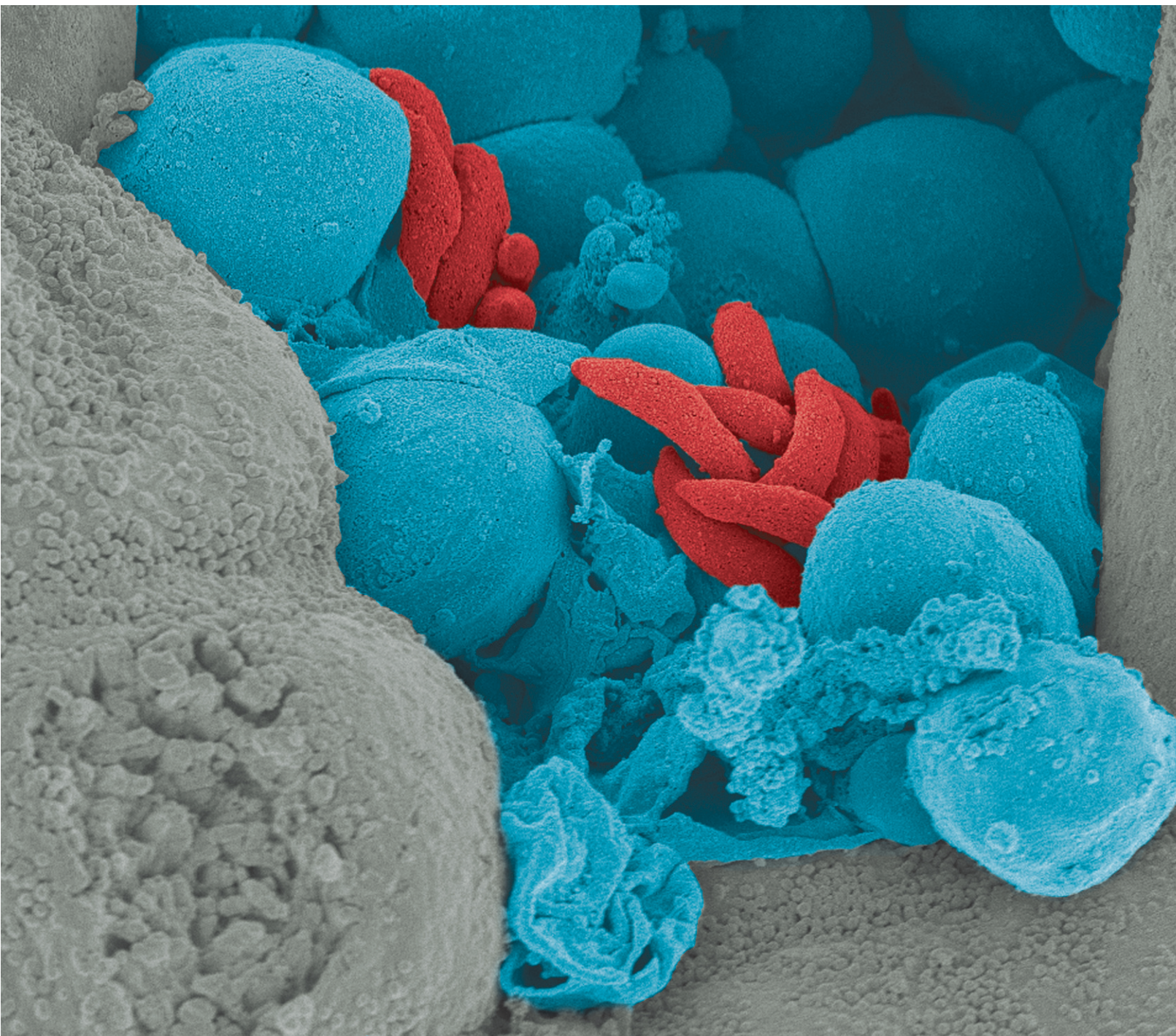

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Cryptosporidia: Epicellular parasites embraced by the host cell membrane

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Abstract

The ultrastructure of two gastric cryptosporidia, *Cryptosporidium muris* from experimentally infected rodents (*Mastomys natalensis*) and *Cryptosporidium* sp. 'toad' from naturally infected toads (*Duttaphrynus melanostictus*), was studied using electron microscopy. Observations presented herein allowed us to map ultrastructural aspects of the cryptosporidian invasion process and the origin of a parasitophorous sac. Invading parasites attach to the host cell, followed by gradual envelopment, with the host's cell membrane folds, eventually forming the parasitophorous sac. Cryptosporidian developmental stages remain epicellular during the entire life cycle. The parasite development is illustrated in detail using high resolution field emission scanning electron microscopy. This provides a new insight into the ultrastructural detail of host–parasite interactions and species-specific differences manifested in frequency of detachment of the parasitophorous sac, radial folds of the parasitophorous sac and stem-formation of the parasitised host cell.

Keywords: *Cryptosporidium*; Host cell invasion; Epicellular; Parasitophorous sac; Ultrastructure

1. Introduction

Apicomplexan organisms of the genus *Cryptosporidium* are typical parasites of the gastrointestinal tract, occasionally affecting other tissues such as the lungs or biliary tract. The disease caused by *Cryptosporidium* spp., cryptosporidiosis, is self-limiting in healthy hosts, nevertheless cryptosporidiosis in immunocompromised hosts is a chronic and debilitating condition (Chen et al., 2002). To date, there is no available treatment for either intestinal or gastric cryptosporidiosis (Thompson et al., 2005).

Numerous *Cryptosporidium* species have been described from diverse hosts including zoonotic species affecting humans (Fayer et al., 1990; Olson et al., 2004; Thompson et al., 2005). *Cryptosporidium parvum* parasitises the intestinal tract, while the larger *Cryptosporidium muris* is described from the stomach of mice. The genus *Cryptosporidium* is characterised by attachment of the developmental stages to the luminal surface of the host cells via a specialised feeder organelle (Tzipori and Griffiths, 1998; Barta and Thompson, 2006). Localisation of cryptosporidian developmental stages in the host epithelium has been the subject of extensive speculation for many years. *Cryptosporidium* spp. are considered to be intracellular but extracytoplasmic, because upon its attachment to an epithelial cell, the motile invasive stage does not enter the host cell cytoplasm (Chen et al., 2002; Thompson et al., 2005; Barta and Thompson, 2006). The invasion process of these parasites and the attachment to the host cell has been studied extensively only in species affecting the intestinal tract;

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in vitro using the cultured cell lines (Lumb et al., 1988; Chen et al., 1998; Huang et al., 2004; O'Hara et al., 2005) and in vivo using experimentally or naturally infected animals (Marcial and Madara, 1986; Yoshikawa and Iseki, 1992; Umemiya et al., 2005). However, the invasion process of the larger gastric *Cryptosporidium* species favourable for ultrastructural analysis has not yet been studied and compared with the intestinal species.

In this study, we have used field emission scanning electron microscopy (FE SEM) to study the ultrastructural features of sporozoite and merozoite attachment to the gastric host cell. The use of FE SEM provided new insight into the host–parasite interaction and dramatically exceeded the resolution capacity of the traditional SEM studies (i.e. Vítovec and Koudela, 1988; Fayer et al., 1990; Umemiya et al., 2005). Using the detailed ultrastructural information from two *Cryptosporidium* species affecting host gastric cells, we describe morphological features of the host–parasite interaction and identify *Cryptosporidium* spp. conserved features as well as species-specific differences associated with cryptosporidiosis.

2. Materials and methods

The *C. muris* Tyzzer, 1907 isolate was originally obtained from naturally infected captive mountain goats (*Oreamnos americanus*) and is now passaged in multimammate rats (*Mastomys natalensis*). Developmental stages of *C. muris* were studied in experimentally infected multimammate rats (*M. natalensis*) as described elsewhere (Valigurová et al., 2007). *Cryptosporidium* sp. in the stomach of a naturally infected black-spined toad (*Duttaphrynus melanostictus*) from the Malay Peninsula is derived from the original material (unpublished data). While the description of this species is yet to be published, we will refer to this isolate of *Cryptosporidium* sp. from *D. melanostictus* as *Cryptosporidium* sp. 'toad'.

For histological sections, the samples were processed following standard procedure and embedded in Histoplast II; 6 μm thick sections were stained with H&E and examined using an Olympus AX 70 microscope. For transmission electron microscopy, the tissue samples were fixed overnight at 4 °C in freshly prepared 3% glutaraldehyde in 0.2 M phosphate buffer, washed for 1 h in phosphate buffer (pH 7.0), postfixed in 1% osmium tetroxide (OsO_4) in the same buffer for 3 h and finally dehydrated in an alcohol series and embedded in Epon (Polybet 812). Sections were cut with glass knives and stained with uranyl acetate and lead citrate. Observations were made using a JEOL 1010 TEM. For scanning electron microscopy, the specimens were fixed overnight at 4 °C in 3% glutaraldehyde in cacodylate buffer, washed 3×15 min in the buffer, postfixed in 2% OsO_4 in cacodylate buffer for 2 h at room temperature, and finally washed 3×15 min in the same buffer. After dehydration in a graded acetone series, specimens were critical point-dried using CO_2, coated with gold and examined using a JEOL JSM-7401F – FE SEM. JEOL JSM-7401F is a semi-in-lens cold cathode FE SEM capable of high resolution of up to 1.0 nm.

Throughout this paper, we use the term parasitophorous sac to describe the peculiar localisation of cryptosporidian developmental stages. Parasitophorous sac is the preferable term introduced by Paperna and Vilenkin (1996) for the host-derived structure enveloping cryptosporidian developmental stages.

3. Results

Both studied cryptosporidian species are restricted to the surface of the epithelium of the glandular part of the gastric mucosa. The most conspicuous difference between the two species is that *C. muris* primarily parasitises epithelial cells within crypts of the gastric glands (Fig. 1A and B), whereas *Cryptosporidium* sp. 'toad' is evenly distributed throughout both the luminal surface of the gastric epithelium and crypts of the gastric glands (Fig. 1C and D).

3.1. Host cell envelopment of C. muris by circular membrane folds

In the early phase of *C. muris* invasion, zoites are attached to the apical surface of gastric cells, among the microvilli. Contact between the apical part of the invading zoite and the surface of the host cell induces modulation of the host cell membrane. The membrane loses its microvillous nature and forms a circular membrane fold, tightly encircling the apical end of the zoite (Fig. 2A). This membrane fold gradually rises up along the zoite. The advancement of the rim of membrane fold is often uneven and therefore eventual envelopment of the zoite, i.e. formation of parasitophorous sac, is completed in various parts of the zoite, not necessarily on its distal end (Figs. 2A–H and 3A and B). Occasionally, seemingly more than one rim of rising membrane folds was observed (Fig. 2D and F). The base of the parasitophorous sac enveloping developmental stages is usually somewhat raised, if compared with the surrounding host epithelium (Figs. 2G, H and 3A–E). The surface of the parasitophorous sac is usually smooth, with irregular radial folds being occasionally observed around its base (Fig. 3D). Pore-like structures are occasionally present in the surface of an apparently fully developed parasitophorous sac (Fig. 3B). The outer membrane of the parasitophorous sac is continuous with the plasma membrane of the host cell and there is usually an indication of a dense band area on its base (Fig. 3E). Meronts are often enveloped by a ruptured parasitophorous sac, or they are completely naked showing the merozoites budding from a large residual body (Fig. 3F). We refer to these changes as artefacts of sample processing; nevertheless they allowed us to observe merozoite morphology. Merozoites are short and plump when budded, appearing longer and slender in advanced meronts. Mature oocysts released from parasitophorous sacs are ellipsoidal in shape, possessing a typical semicircular longitudinal suture in the oocyst wall

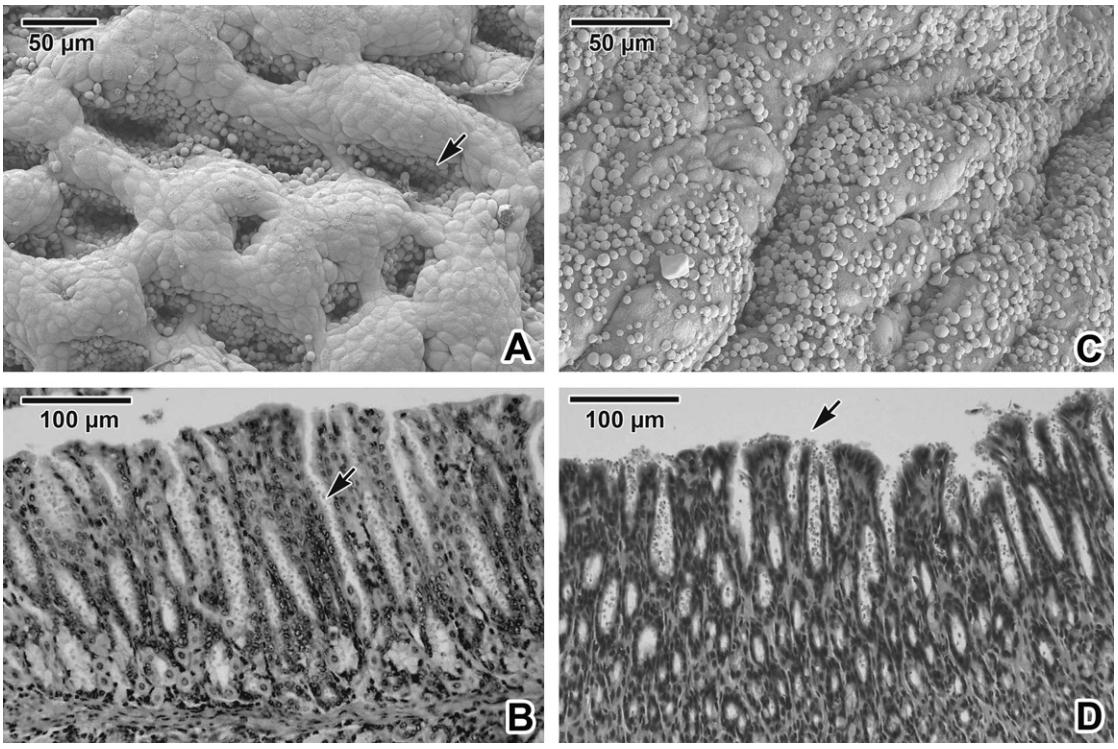

Fig. 1. General aspects of gastric cryptosporidian infections. (A) Rodent gastric epithelium with developmental stages of *Cryptosporidium muris* within crypts of gastric glands (arrow); field emission scanning microscopy (FE SEM). (B) Paraffin section of rodent gastric epithelium, showing crypts of gastric glands infected with *C. muris* (arrow); H&E. (C) Anuran gastric epithelium evenly infected with developmental stages of *Cryptosporidium* sp. 'toad'; FE SEM. (D) Paraffin section of anuran gastric epithelium infected with *Cryptosporidium* sp. 'toad' (arrow); H&E.

(Fig. 3G). Numerous developmental stages, still completely enveloped by an intact parasitophorous sac, are seen detached from the epithelium, and their attachment site can be observed from below (Fig. 4A). In addition, empty parasitophorous sacs are occasionally seen detached from the host epithelium and revealing their dense band areas (Fig. 4B and C). Invariably, the area just below the dense band is part of the host–parasite system, where the detachment takes place (Fig. 4A–C). A filamentous projection, morphologically typical of *C. muris*, forms a bulky protuberance encircling the dense band area (Fig. 4A–D). A distinct dense line limits the feeder organelle and separates it from the filamentous projection (Fig. 4C, D, and F). Ruptured, empty parasitophorous sacs show residua of the feeder organelles, which are encircled by distinct Y-shaped membrane junctions, i.e. annular rings (Fig. 4E).

3.2. Detachment of *Cryptosporidium* sp. 'toad' from the epithelial surface

During the invasion process of *Cryptosporidium* sp. 'toad', a tight-fitting membrane fold gradually rises up along the zoite (Fig. 5A), finally enveloping the zoite with the parasitophorous sac. Occasionally, young bell-shaped trophozoites, surrounded by elongated microvilli, are present on affected epithelium (Fig. 5B). The resulting undifferentiated young trophozoites are round and often surrounded by slightly elongated microvilli (Fig. 5C). From the outset of trophozoite formation, structurally uniform radial folds are formed, evenly distributed around the base of each parasitophorous sac. The radial folds become more distinct as the contained developmental stage increases in size (Figs. 5E and F and 6A, E, and F). Mature trophozoites and successive stages are completely enveloped by the parasitophorous sacs with an apparently smooth outer membrane (Fig. 5D–H). The base of most stages is raised, if compared with the surrounding host epithelium (Figs. 5H and 6A). The elevated base of the parasitophorous sac is generally devoid of microvilli. Some stages are located on a long stem of host cell origin, the surface of which is either smooth or possesses microvilli (Fig. 5E–G). Parasitophorous sacs enveloping the meronts are often ruptured and reveal merozoites (Fig. 5D). The oocysts are generally spherical to subspherical and possess a typical semicircular longitudinal suture in their wall (Fig. 6A and B). Ruptured and empty parasitophorous sacs, very common in *Cryptosporidium* sp. 'toad', possess a frilled base with the feeder organelle encircled by the Y-shaped membrane junction retained after disintegration or oocyst liberation (Fig. 6B–D). Numerous developmental stages are found detached from the epithelial surface, showing the frilled base of the parasitophorous sac (radial folds) inserted under the button-like area of the dense band (Fig. 6A, E, and F). No circular protuberance of the filamentous projection is visible. As shown in the transmission electron micrographs, the detached developmental stages

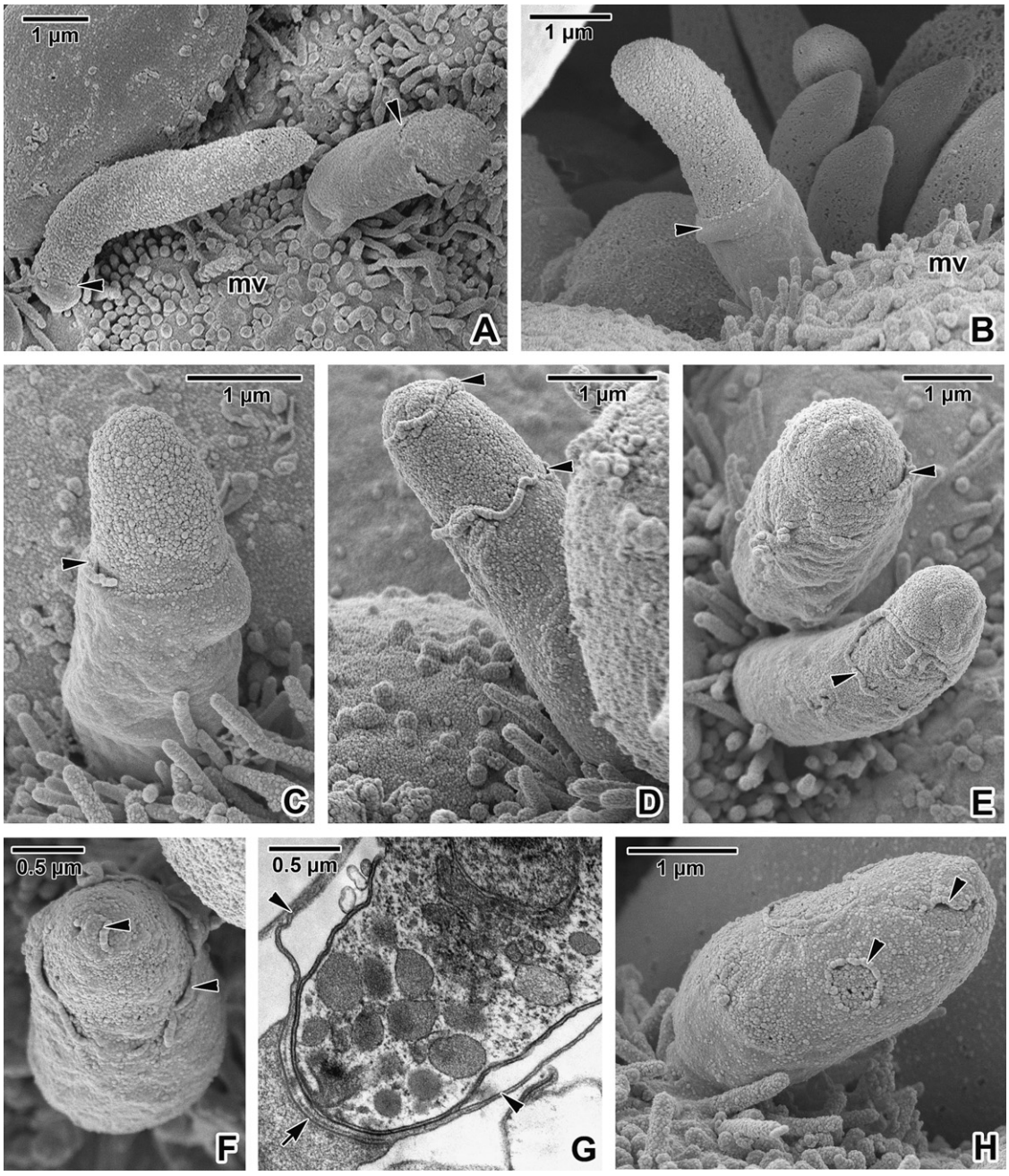

Fig. 2. Early developmental stages of *Cryptosporidium muris*. (A–D) Zoites being gradually enveloped by tight-fitting membrane fold(s); fold rims (arrowheads), microvillous surface (mv). (E–F) Progress in the envelopment process and formation of parasitophorous sac; membrane fold rims (arrowheads). (G) Attachment site of early trophozoite; dense band (arrow), parasitophorous sac (arrowheads). (H) Zoite almost completely enveloped by parasitophorous sac; multiple fusion areas (arrowheads).

are liberated by disruption of the host cell in the zone just below the dense band (Fig. 6E–G).

4. Discussion

This study compares ultrastructural features during the development of two evolutionarily distinct gastric cryptosporidia. We describe the parasite invasion process, formation of the parasitophorous sac and the attachment strategy, using scanning and transmission electron microscopy. Two gastric species, *C. muris* and *Cryptosporidium* sp. 'toad', from a mammal and an amphibian, respectively, allowed us to generalise our observations for gastric *Cryptosporidium* spp. and compare those with published information on intestinal species.

Both studied cryptosporidia exhibited a comparable strategy of host cell invasion, in which contact between the invading zoite and the host cell induced recruitment of the microvillar membrane. The recruited part of the epithelial cell membrane loses its microvillous appearance and

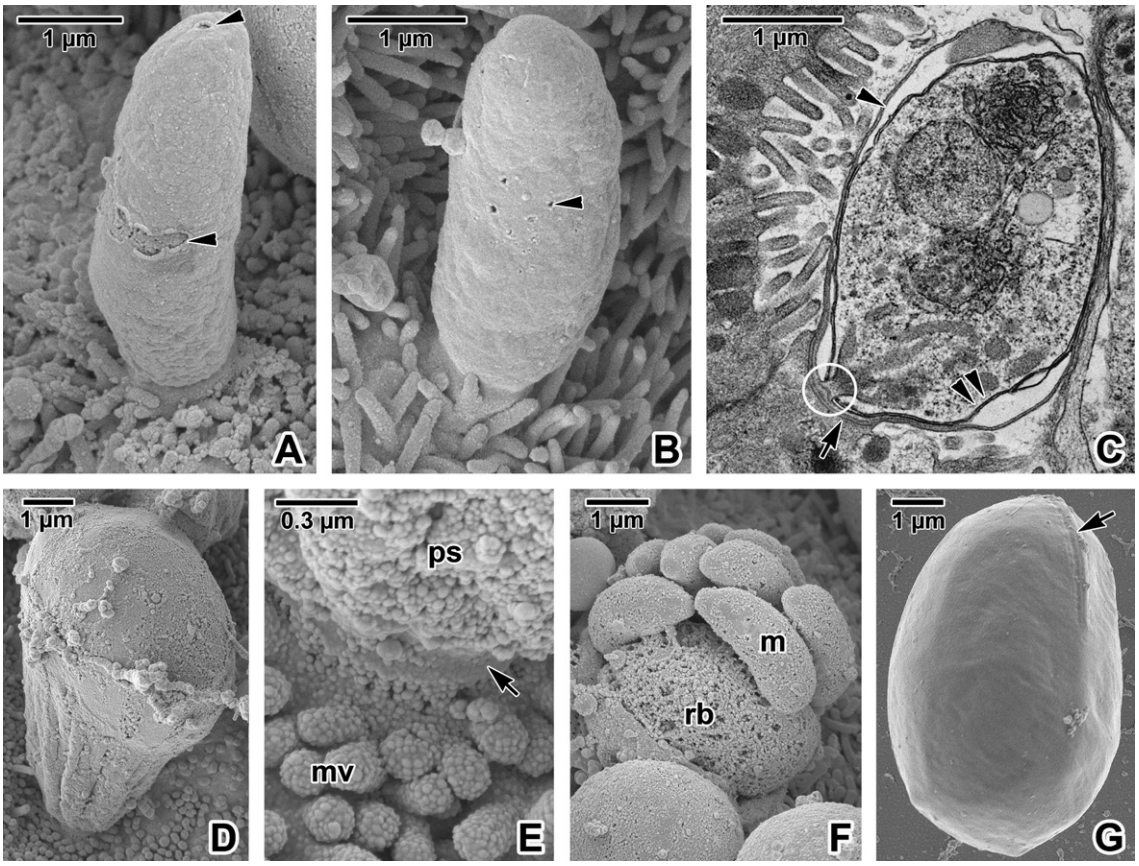

Fig. 3. Maturing trophozoites and successive stages of *Cryptosporidium muris*. (A) Zoite almost completely enveloped by parasitophorous sac; fusion areas (arrowheads). (B) Maturing trophozoite completely enveloped by parasitophorous sac; pore-like structures (arrowhead). (C) Maturing trophozoite; parasitophorous sac (arrowhead), parasite pellicle (double arrowhead), tunnel connection (in circle), dense band (arrow). (D) Undetermined developmental stage with fully developed parasitophorous sac. (E) The base (arrow) of parasitophorous sac (ps) with surrounding microvilli (mv). (F) Meront; merozoites (m) budding from residual body (rb). (G) Mature oocyst from faeces; longitudinal suture (arrow).

forms a tight-fitting elastic membrane fold, encircling the contact zone between zoite and host epithelial cell, which gradually rises along the zoite (Figs. 2 and 5). Our observations are in general agreement with the previous studies on intestinal cryptosporidia either in enterocytes (Umemiya et al., 2005) or in cholangiocytes (Chen et al., 1998; Chen and LaRusso, 2000). Although these studies show details using scanning electron microscopy, we further investigated the ultrastructural detail of the parasite invasion to elucidate the fate of the membrane ruffling and the parasite localisation.

In both affected hosts, we clearly illustrate the advancement of the membrane fold rim. In contrast to the model of zoite envelopment by Umemiya et al. (2005), the extensive foreskin-like membrane ruffling is uneven, and the zoite envelopment is consequently completed in various parts of the invading zoite. Importantly, the inner and outer membrane of the parasitophorous sac and intervening cytoplasm are derived from the host cell.

Our FE SEM observation, together with detailed comparative transmission electron microscopy of *Gregarina steini* and *C. muris* revealing evolutionary homology of these two parasites (Valigurová et al., 2007), shows that cryptosporidia do not penetrate under the host cell membrane, nor do they come into close contact with the host cell cytoplasm. Therefore, the cryptosporidian developmental stages are not intracellular. The only exception is a region of early tunnel connection between the parasite and the host cell; nevertheless, even this transient connection disappears when the feeder organelle forms and becomes limited by a dense line separating it from the filamentous projection, i.e. from the parasitophorous sac of host cell origin. The feeder organelle is known to be essential in salvaging nutrients (Perkins et al., 1999; Striepen et al., 2004) and directly separates the parasite cytoplasm from the affected host cell (Tzipori and Griffiths, 1998). The parasite remains attached to the host cell surface, only enveloped by the host cell membrane folds. Doflein (1929) coined the term epicellular as being attached to the limiting cell membrane. Our observations support the term epicellular to reflect the cryptosporidian localisation, rather than the term intracellular-extracytoplasmic, used throughout the literature for cryptosporidian localisation. In addition, the cryptosporidian attachment strategy is very similar to that of the eugregarines, which are also epicellularly located on the microvillous surface of the host epithelial cells although not enveloped by the host cell folds (Barta and Thompson, 2006; Valigurová et al., 2007).

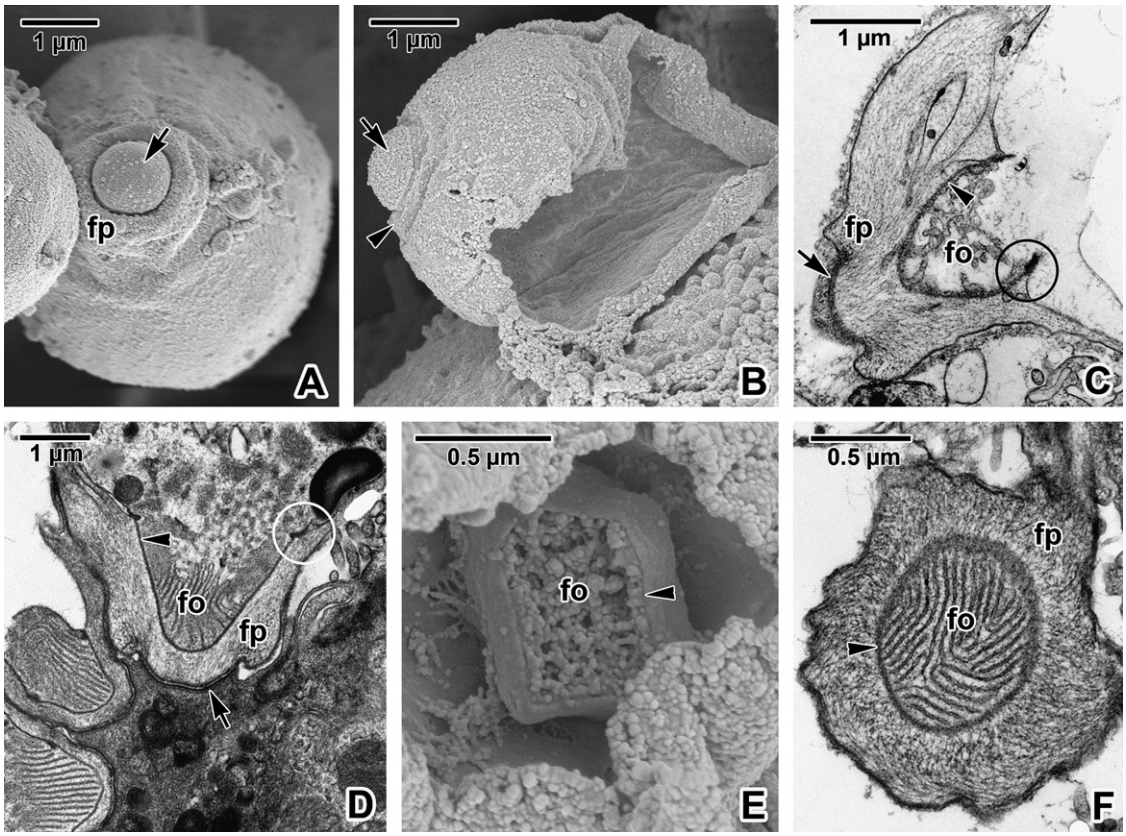

Fig. 4. Attachment site of *Cryptosporidium muris*. (A) Developmental stage showing base of parasitophorous sac; dense band area (arrow), filamentous projection (fp). (B) Detached parasitophorous sac; dense band area (arrow) encircled by filamentous projection (arrowhead). (C) Detached parasitophorous sac; feeder organelle (fo), Y-shaped membrane junction (in circle), filamentous projection (fp), dense line (arrowhead), dense band (arrow). (D) Feeder organelles (fo) in longitudinal section; filamentous projection (fp) limited by dense line (arrowhead), Y-shaped membrane junction (in circle), dense band (arrow). (E) Interior of parasitophorous sac; feeder organelle (fo), Y-shaped membrane junction area viewed from above (arrowhead). (F) Cross section of the feeder organelle (fo) encircled by filamentous projection (fp); dense line (arrowhead). Image D taken from Fig. 44 in Valigurova, A., Hofmannova, L., Koudela, B., Vavra, J., 2007. An ultrastructural comparison of the attachment sites between Gregarina steini and Cryptosporidium moris. J. Eukaryot. Microbiol. 54, 495-510 with permission from The International Society of Protistologists and Blackwell Publishing.

Detailed observation of the developmental stages of *C. muris* and *Cryptosporidium* sp. 'toad' revealed the existence of pore-like structures in the surface of the parasitophorous sac. We have also seen these structures in older developmental stages, thus they are not a priori considered initial non-fused areas before the rims of membrane folds completely coalesce. These incompletely fused areas revealed by FE SEM are tentatively thought to be the same areas reported by Umemiya et al. (2005). These authors have suggested the existence of incomplete fusion of the folds surrounding the developmental stages using transmission electron microscopy and some ruthenium red-positive parts located between the lining of the membrane fold suggesting the presence of connection between the interior and exterior of the parasitophorous sac (Figs. 16 and 17 in Umemiya et al., 2005). Our observation using FE SEM directly supports these findings and it provides further evidence for the similarity of cryptosporidian attachment to eugregarines where membrane folds are absent (Valigurová et al., 2007).

Epicellular localisation is not a primitive ancestral characteristic, but rather an advanced feature independently occurring in apicomplexan parasites (Davies and Ball, 1993). Morphological adaptations are key to the understanding of the evolutionary modification leading to *Cryptosporidium* spp. (Leander and Keeling, 2003; Barta and Thompson, 2006).

It is now well established that the origin of the parasitophorous sac is the host cell. Using electron microscopy, Vetterling et al. (1971) were the first to suggest that the parasitophorous sac derives from the microvillar outer membrane of the host cell. Further support came from the freeze fracture technique and cell cultured cryptosporidian infection detailing the envelopment of zoites by redundant folds of apical membrane (Marcial and Madara, 1986; Lumb et al., 1988; Yoshikawa and Iseki, 1992).

Our observations and other available information support the hypothesis that the parasitophorous sac is derived from folding of the host cell membrane. It has been experimentally documented that the intestinal cryptosporidia modify a glucose and water influx involved in the formation of the hostcell membrane protrusion and subsequent parasite engulfment (Chen et al., 2005). Our ultrastructural observation extends this host cell membrane protrusion

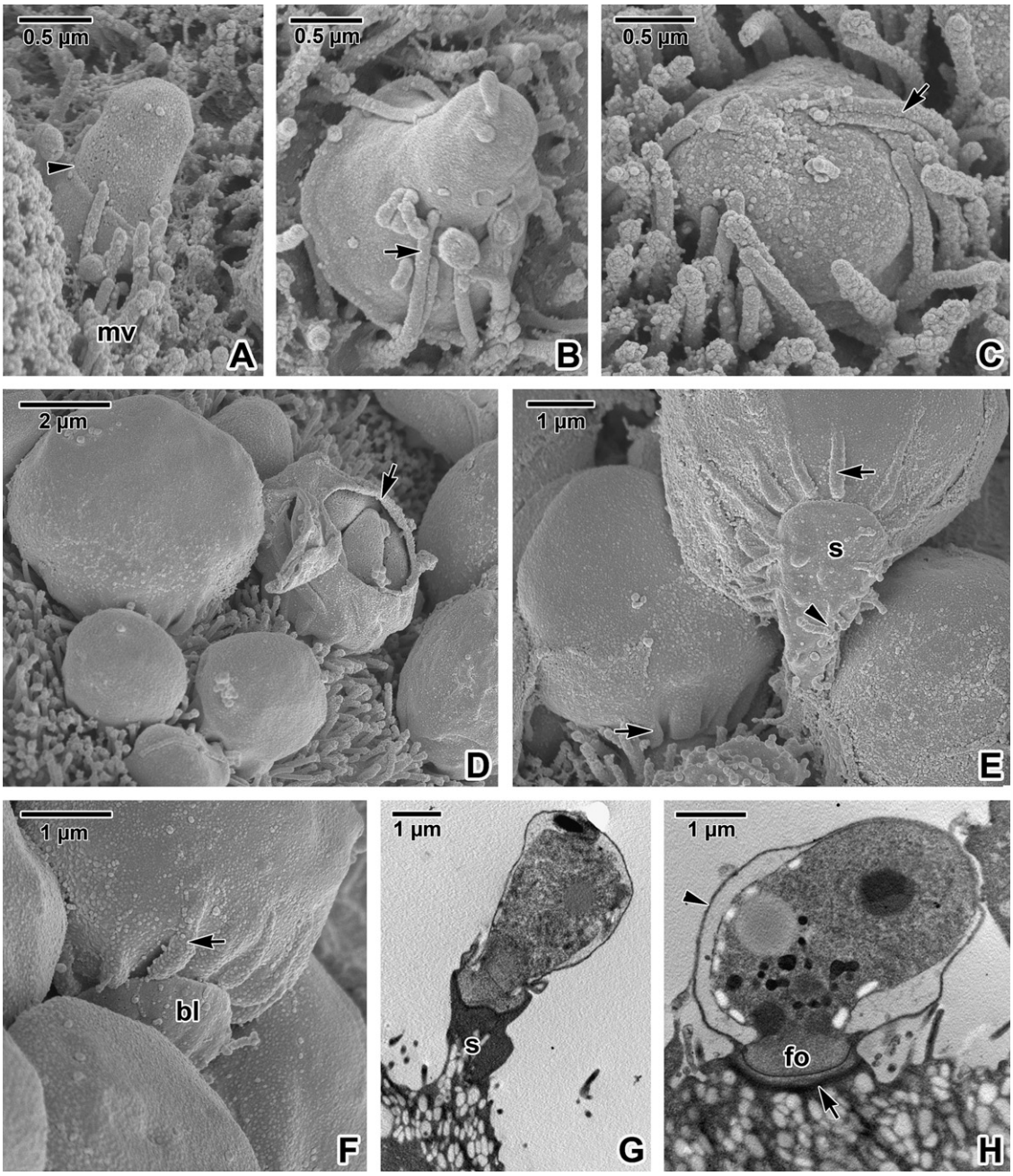

Fig. 5. Developmental stages of *Cryptosporidium* sp. 'toad'. (A) Zoite gradually enveloped by tight-fitting membrane fold; fold rim (arrowhead), microvillus surface (mv). (B) Bell-shaped trophozoite surrounded by elongated microvilli (arrow). (C) Trophozoite surrounded by slightly elongated microvilli (arrow). (D) Developmental stages and meront; ruptured parasitophorous sac with merozoites (arrow). (E) Developmental stages without stem (left) and with stem (s) bearing microvilli (arrowhead); radial folds of parasitophorous sacs (arrows). (F) Stem with frilled base of parasitophorous sac; radial folds (arrow), button-like area of dense band (bl). (G) Microgamont; stem (s). (H) Macrogamont; parasitophorous sac (arrowhead), feeder organelle (fo), dense band (arrow).

formation to the entire genus including gastric *Cryptosporidium* spp. However, the nature of the involvement of the microvilli adjacent to the zoite in the formation of the parasitophorous sac remains elusive. A recent transmission electron microscopic study has provided convincing evidence for the elongation of microvilli surrounding the contact zone between the parasites and the host cell membrane regardless of their involvement in the formation of the parasitophorous sac both in murine intestinal cells and in the cholangiocyte cell culture (Chen et al., 1998; Umemiya et al., 2005). Only a few elongated microvilli surrounded *Cryptosporidium* sp. 'toad' and we have not observed the densely packed or elongated microvilli previously reported during intestinal cryptosporidiosis. In our preparations of the gastric cryptosporidia, microvilli did not play any role in the process of parasitophorous sac for-

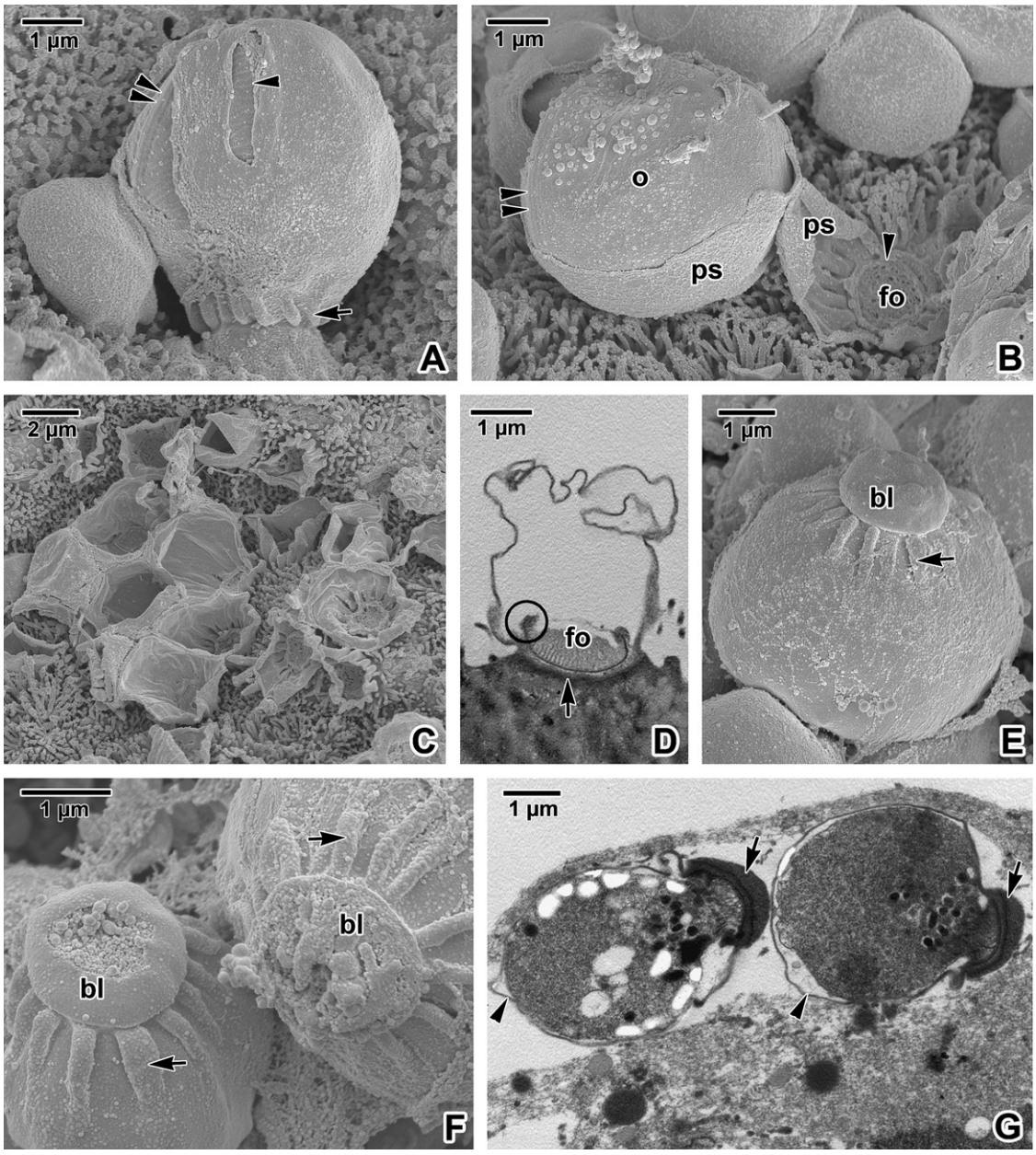

Fig. 6. Attachment site of *Cryptosporidium* sp. 'toad'. (A) Mature oocyst still enveloped by ruptured parasitophorous sac (arrowhead); partly visible longitudinal suture (double arrowhead), radial folds (arrow). (B) Released oocyst (o) and ruptured parasitophorous sac (ps); partly visible longitudinal suture (double arrowhead), feeder organelle (fo), Y-shaped membrane junction area (arrowhead). (C) Ruptured, empty parasitophorous sacs showing their bases. (D) Empty parasitophorous sac; feeder organelle (fo), residuum of the Y-shaped membrane junction (in circle), dense band (arrow). (E and F) Detached developmental stages showing the frilled bases of parasitophorous sacs; button-like dense band areas (bl), radial folds (arrows). (G) Detached developmental stages; parasitophorous sacs (arrowheads), zones of detachment from unmodified part of host cell, i.e. zones below dense band (arrows).

mation. Whether the massive recruitment of microvilli is dependent on the parasite localisation or is specific for intestinal species remains to be further elucidated.

The term parasitophorous vacuole for location of *Cryptosporidium* spp. is misleading, because parasitophorous vacuole refers solely to the vacuolar space bordered by a membrane (Scholtyseck, 1979). Instead the parasitophorous sac (Fig. 7) is composed of (i) a host–parasite space, (ii) a host cell membrane fold continuous with the microvillar membrane of the gastric cell (membrane of the parasitophorous sac), (iii) a thin layer of host cell cytoplasm enclosed by this membrane fold and (iv) a dense band dividing the unmodified and modified part of the host cell, i.e. parasitophorous sac including the filamentous projection (Valigurová et al., 2007 and this manuscript). The parasitophorous sac is unique in its origin, development and structure, distinguishing the genus *Cryptosporidium* from all other apicomplexans.

Ultimately, the parasitophorous sac ruptures and releases either merozoites or oocysts. Observed oocysts

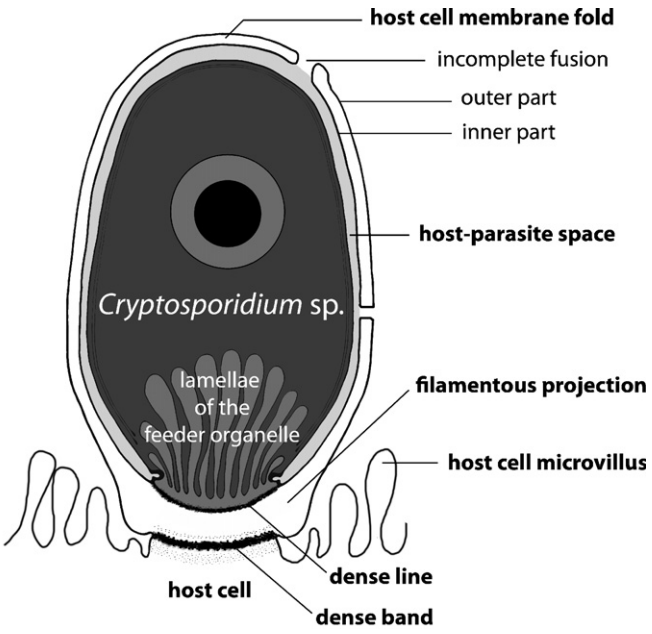

Fig. 7. Schematic representation of the *Cryptosporidium* spp. parasitophorous sac. The dense band labelled in the figure separates the host cell into two domains; (i) the modified part of the host cell, i.e. parasitophorous sac, above the dense band and (ii) the unmodified part of host cell below it.

possessed a typical environmentally resistant oocyst wall (Figs. 3G and 6A,B). The oocyst wall is characterised by a unique single suture at one pole spanning one-third of the circumference of the oocyst. During excystation the suture dissolves and forms a slit-like opening used by the sporozoites to exit the oocyst (Reduker et al., 1985).

Both studied species parasitise gastric mucosa, yet there are obvious species-specific differences between *C. muris* and *Cryptosporidium* sp. 'toad'. A prominent distinguishing trait of *Cryptosporidium* sp. 'toad' is the regular presence of prominent radial folds at the base of the parasitophorous sac. Furthermore, unlike in *C. muris*, early and maturing trophozoites of *Cryptosporidium* sp. 'toad' were often surrounded by multiple individually elongated microvilli. Nonetheless, with the evolutionary difference between the two parasitised hosts and host specificity of each of the two *Cryptosporidium* spp. it remains unknown whether these features reflect only the nature of the host tissue or if there is a parasite component involved in the morphological appearance.

The stem-formation observed in tissues infected with *Cryptosporidium* sp. 'toad' is likely a result of spatial requirements and growth of neighbouring individuals, eventually resulting in the detachment of the developmental stages. It is noteworthy that a similar event was described in *Cryptosporidium wrairi* parasitising a guinea pig (Vetterling et al., 1971). The detachment revealed the fragile part of the host–parasite interface below the dense band composed of a plaque of host filamentous actin (Lumb et al., 1988; Elliott et al., 2001; Chen et al., 2005), which is thought to anchor the parasite and prevent its occasional detachment (Beyer et al., 2002). In contrast to the voluminous filamentous projection of *C. muris*, which forms a circular protuberance surrounding the dense band area, the filamentous projection in *Cryptosporidium* sp. 'toad' is inconspicuous and hidden behind the dense band. Such morphological difference suggests species-specific adaptations at this critical area of host–parasite interface. The ultrastructural details of the dense band area segregating the modified and unmodified parts of the host cell are the foundation of a better understanding of parasite host specificity at the molecular level and the development of strategies eliminating the attachment of the parasite to the host cell (Perkins et al., 1999; Chen et al., 2005).

In conclusion, using *Cryptosporidium* spp. affecting gastric cells in vivo, we have mapped ultrastructural features of the parasite attachment to the host cell. In our micrographs, particularly using high resolution FE SEM, we have shown the epicellular localisation of the parasite enveloped by a parasitophorous sac originating from host cell membrane folds. Ultrastructural observation extends our understanding of host epithelium folding and zoite envelopment to the entire genus of *Cryptosporidium* regardless of the parasitised host cell. These findings will permit elucidation of the molecular mechanisms behind these conserved morphological features as potential targets for cryptosporidian therapy.

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