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Dientamoeba fragilis trophozoites undergoing encystation, apoptosis and necrosis as human parasitic amoeba in clinical stool samples

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Abstract

Dientamoeba fragilis was described as a binucleated trophozoite of amoeba that had no cyst stage and degenerated rapidly once it became outside the human body. Thereafter, it was redescribed as not a true amoeba but an aberrant flagellate that had not flagellated stage and closely related to Histomonas. The current article investigated stool samples utilizing formalin-ethyl acetate concentration method to extract and concentrate the parasites in addition to methylene blue staining to enhance visualisation of the protozoan D. fragilis by light microscopy as well as to confirm the result by electron microscopy. The result found both uninucleated and binucleated trophozoites of D. fragilis capturing fungus or bacterium by pseudopodia together with other bacteria and fungi in cytoplasmic vacuoles of these amoeboid shape trophozoites. Surprisingly precysts and mature cysts of D. fragilis were found also in this article, in addition to many D. fragilis trophozoites undergoing apoptosis and necrosis. In conclusion D. fragilis is an amoeba possessing trophozoite with 1-2 nuclei and it has cyst in its life cycle opposite to previous description. The trophozoite has pseudopodia to capture bacteria and fungi and utilises phagocytosis to ingest food particles in food vacuoles,

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in order to be recognised than the flagellates. Furthermore, the trophozoite undergoes apoptosis and necrosis to explain for first time why it will be found accusingly as non-nucleated trophozoite and why it degenerates rapidly once it will become outside the human body.

Keywords: *Dientamoeba fragilis*, amoeboid trophozoite and cyst, pseudopodia and food vacuoles, apoptosis and necrosis

INTRODUCTION

The parasites include unicellular microorganisms and multicellular organisms that depend expensively upon the hosts for their nutrients. The parasite that lives on the body of its host is called ectoparasite and that lives inside the host is endoparasite. According to anatomy and morphology of the parasites they are differentiated into protozoa, helminths and arthropods (1).

Protozoan is a single eukaryotic cell with relatively complex internal structure and carries out complex metabolic activities (2). The protozoa belong to one of three phyla including Sarcomastigophra, Ciliophora and Apicomplexa. According to their movement they divided into amoebae, flagellates and ciliates in addition to sporozoa that move by pseudopodia, flagella, and cilia or by body flexion, respectively (3).

The amoeba has an active trophozoite and a dormant cyst in its life cycle (4) except Entamoeba gingivalis that has trophozoite only (5). Amoebic trophozoite feeds on bacteria and fungi utilising their pseudopodia and phagocytosis to ingest food in vacuoles or by cup formation (4).

The flagellates take the food through a specialized mouth-like aperture called a cytostome or by absorbing dissolved nutrients through their cell membranes (6). They include different genera such as Chilomastix, Enteromonas, Giardia, Pentatrichomonas, Retortamonas, Trichomonas, and Trypanosoma (7). The life cycles of *Chilomastix mesnli*, *Enteromonas hominis*, *Giardia intestinalis* and *Retortamonas intestinalis* consist of trophozoite and cyst in comparison to *Pentatrichomonas hominis, Trichomonas vaginalis*, *T. tenax* and Trypanosoma species that have only trophozoites and missing cysts (8). Collectively, the amoebae and the flagellates are

considered to be closely related since they belong to the phylum Sarcomastigophra comprising a single large assemblage (9).

Interaction between amoebae and helminths was mentioned previously by Theobald Smith in 1895 since he suggested that eggs of the intestinal helminth *Heterakis gallinarum* could transmit the non-encysting *Amoeba meleagridis* the causative agent of blackhead to the turkeys (10). Thereafter, the trophozite *Amoeba meleagridis* was found to have a flagellate character and reclassified to *Histomonas meleagridis* (11). Amazingly, the fatal blackhead in turkeys was produced by feeding embryonated eggs of the helminth *Heterakis papillosa* (12) and *H. meleagridis* transmitted in the eggs *H. gallinarum* (13) as well as *H. meleagridis* was isolated from embryonated eggs of the *H. gallinarum* (14). In the contrary to the previous findings, Tyzzer, EE and Collier, J. (1925) reported about an induced and natural transmission of blackhead in the absence of Heterakis helminths was occurred (15) in agreement with following studies (16-18). Surprisingly, it is worth to mention that some amoebae are found to form transient flagellated stages like what called amoeboflagellates; *Histomonas meleagridis* and *Naegleria fowleri*. Each of *H. meleagridis* and *N. fowleri* has amoeboid cyst and amoeboid trophozoite. These amoeboid trophozoites are also able to transient to flagellate trophozoites (19-21).

Is the protozoan *Dientamoeba fragilis* an amoeba or a flagellate? *D. fragilis* was originally seen in 1909 by Charles Wenyon after the examination of his own stool specimen; however, the organism was not described until 1918 by Margaret Jepps and Clifford Dobell according to the references (22-24). Jepps and Dobell described this protozoan as a binucleated trophozoite of amoeba that had no cyst stage and degenerated rapidly once it became outside the human body. Therefore, they named this amoeba *Dientamoeba fragilis* and placed it in the family Entamoebidae together the known human intestinal genera of amoebae; Entamoeba, Endolimax and Iodamoeba (25, 26). Although Dobell's experiments to induce *D. fragilis* to express a flagellum were not succeeded, he concluded that *D. fragilis* had not flagellate stage and it was not a true amoeba but an aberrant flagellate closely related to *Histomonas*. Thus, Dobell 1940 suggested that the transmission of *D. fragilis* trophozoites from man to man would occur through human parasitic helminths such as *Trichuris trichiura* or *Ascaris lumbricoides* (27) on analogy with that

transmission of *H. meleagridis* trophozoites by the helminth *H. gallinarum* to the turkeys (10).

Many researchers demonstrated that *Dientamoeba*, *Histomonas*, and *Trichomonas* shared many closely related antigens with each other and far fewer with *Entamoeba*, therefore, on the basis of these findings; *D. fragilis* was placed in the order Trichomonadida and the family Monocercomonadidae, subfamily Dientamoebidae (28-33). Furthermore, Munasinghe et. al, 2013 observed cyst stage formation of *D. fragilis* in vitro experiment when *D. fragilis* infected mice and he proved that the isolated cysts could infect rodents experimentally (34).

From practical point view about *D. fragilis* diagnosis in stool samples there were many observations such as:- a) presence of precyst or pseudocyst in a retrospective study of 500 slides from human clinical samples permanently stained smears positive for *D. fragilis* (35) ; b) clear variation in seize of *D. fragilis* cells in same clinical samples together with presence of many non-nucleated cells and difficulties to see nucleus of *D. fragilis* in unstained preparations despite this protozoon found to have one or two nucleus (36); c) presence of smooth and ruffled cells by scanning electron microscope examination of cultured *D. fragilis* together with absence of pseudocysts in these cultures (37). Therefore, this paper was aimed to investigate stool samples utilizing formalin-ethyl acetate concentration method and methylene blue staining to enhance visualisation of the protozoan *D. fragilis* by light microscopy as well as to confirm the result by electron microscopy wishing to clarify and answer the mentioned observations and questions in this article.

MATERIAL AND METHODS OF PARASITE IDENTIFICATION

Two Diarrhoeal stool samples from Karolinska University Hospital, Laboratory of Clinical Microbiology, unit of Parasitology, Huddinge, Stockholm, Sweden, were received to parasitological examination. Sample 16 AE 103302 was from a 45 year male and sample 16 AE 104625 was from a 26 year female.

Sodium acetate, acetic acid and formalin (SAF) fixative is a suitable for preservation and recovery of intestinal parasites by diphasic concentration methods and permanent staining (38). Formalin-ethyl acetate centrifugation method that strains, extracts

and preserves the parasites is utilised to examine the prepared samples microscopically for demonstrating trophozoites and cysts of protozoa in addition to ova and larvae of helminths (39).

Identification of parasites was based on microscopic morphology of the found parasites compared with those in standard texts, literature and micrographs according to Centers of Disease Control and Prevention standard methods for diagnosis of intestinal parasites (40).

Further examinations were made for *D. fragilis* trophozoites positive samples. After the centrifugation, the samples were stained by methylene blue for light microscopy and photography in addition to ultrathin sections for electron microscopy and ultramicrography for morphological identification and visualisation of *D. fragilis*.

Methylene staining

Methylene blue in a 1% aqueous solution (Science Company) has many uses as a biological stain that stains nuclei more strongly than cell cytoplasm. This stain was used for diagnosis of *D. fragilis* by light microscopy and microphotography in this study.

Electron microscopy

D. fragilis trophozoites positive pellet samples obtained from concentration method were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3), with 0.1 M sucrose and 3 mM CaCl2, for 30 min at room temperature. Samples were then washed in sodium cacodylate buffer and post-fixed in 2% osmium tetroxide in the same buffer for 1 h. The samples centrifuged and the pellets were dehydrated and embedded in Epoxy resin (LX-112). The embedded samples were cut into ultrathin sections, placed on grids and stained with uranyl acetate and lead citrate. Sections were examined under a transmission electron microscope (TEM).

RESULTS

Stool samples were prepared by the formalin-ethyl acetate concentration method and the pellets were examined by light- and electron microscope.

Light Microscopy

The result found either amoeboid or round cells that could not be identified without staining, but methylene blue staining uncovered trophozoites of *D. fragilis* that were eukaryotic cells having amoeboid shapes measuring from 8 to 13 µm in diameter that might contain 1 or 2 nuclei, pseudopodia and vacuoles as shown in the following figures. A trophozoite of *D. fragilis* measured 11 x 14 µm was found to contain 2 nuclei and have an amoeboid shape with pseudopodia (Fig. 1, left panel). Another trophozoite of *D. fragilis* measured 10 x 20 µm was found to contain one nucleus and have an elongated amoeboid shape with two vacuoles. Surprisingly, two precysts of *D. fragilis* with 7 µm and 8 µm in diameters were found also in this sample (Fig. 1, right panel).

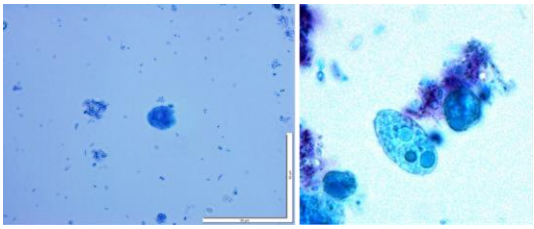

Figure 1: Left and Right panels: *D. fragilis* trophozoites and precyst.
Left panel: Binucleated *D. fragilis* trophozoite 13 x 15 µm has an amoeboid shape and pseudopodia. Magnification 1000X.
Right panel: Uninucleated trophozoite 10 x20 µm with 2 vacuoles, precyst with 7 µm diameter and other precyst with 8 µm diameter. Magnification 3000X.

Moreover, other precyst measured 7 µm in diameter was found in (Fig. 2 left panel). Finely, a mature 7 µm diameter cyst was found together with a 3 µm diameter precyst in upper corner and a *Blastocystis hominis* cell measuring 4x5 µm lower as shown in (Fig. 2 right panel).

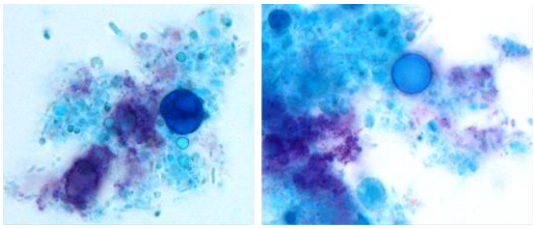

Figure 2: left and right panels: Precyst and mature cyst of *D. fragilis*.
Left panel: precyst with 7. µm in diameter. Magnification 3000x.
Right panel: Mature cyst with diameter of 7 µm, 3 µm precyst in upper corner and 5 µm Blastocystis hominis cell in lower corner. Magnification 3000X.

Utilising Methylene stain in this study, the light microscopy figures (1-2) detected 2 (28%) trophozoites compared to 5 cysts (72%) of *D. fragilis* to uncover a relatively high percent of cysts was observed by the light microscopy.

Electron Microscopy

Electron microscope analysis showed uninucleated trophozoite of *D. fragilis* characterised by amoeboid shape with 9 µm diameter. This eukaryotic cell has bacterium and fungus in cytoplasmic vacuoles and pseudopodia capturing fungus (Fig. 3 left panel). Binucleated *D. fragilis* trophozoite with a diameter of 7.5 µm having bacteria and fungi in cytoplasmic vacuoles is found (Fig. 3 right panel).

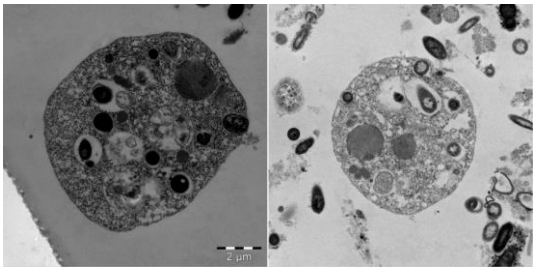

Figure 3: left and right panels: Uninucleated and binucleated *D. fragilis* trophozoites.

Left panel: *D. fragilis* uninucleated trophozoite with 9 µm diameter capturing fungus cell by pseudopodia. There were bacteria and fungi in cytoplasmic vacuoles of this amoeboid shape trophozite. Right panel: Binucleated *D. fragilis* trophozoite with 7 µm having bacteria and fungi in cytoplasmic vacuoles.

Moreover, there are a mature cyst with 3 µm in diameter in figure 4 left panel and a precyst (immature) measured 6 µm in diameter in the figure 4 right panel.

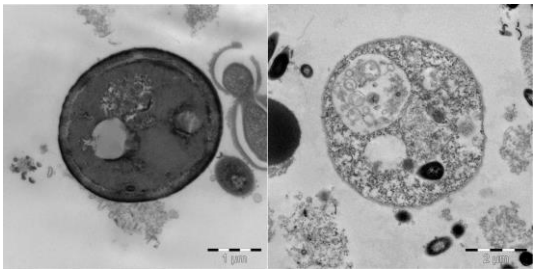

Figure 4: *D. fragilis* cysts: Left panel mature cyst 3µm diameter and Right panel early precyst with a 6 µm in diameter.

D. fragilis exhibits apoptosis and necrosis

Distinguishing features of apoptosis are chromatin condensation, reduced size of the nucleus (shrinkage) and DNA fragmentation (41, 42). Features of necrosis are plasma membrane disruption and nuclear disintegration (41, 43).

In this study, a binucleated 4 µm diameter trophozoite of *D. fragilis* was found undergoing apoptosis and exhibiting exocytosis vesicles (Fig. 5 left panel). Moreover, in figure 5 right panel, there were four trophozoites. The largest measured 6 µm in diameter, undergoing apoptosis characterized shrinkage and exhibited exocytosis vesicles. However, to the right of the large trophozoite there was another trophozoite that undergoing both late necrosis and apoptosis since it lost its plasma membrane and just a little part of the cytoplasm around the apoptotic nucleus was left. Also there were two small trophozoites to the left of the large trophozoite, the upper trophozoite underwent early necrotic apoptosis that characterized by plasma membrane disruption and nuclear disintegration (karyolysis) compared to the lower that underwent apoptosis as all these events shown in Fig. 5, right panel.

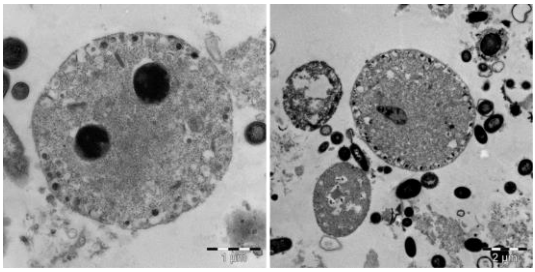

Figure 5: *D. fragilis trophozoites* undergoing apoptosis and necrosis.
In left panel *D. fragilis* binucleated trophozoite underwent apoptosis and exhibited exocytosis vesicles. In right panel *D. fragilis* trophozoites underwent apoptosis, early necrotic apoptosis (upper left) and late necrotic apoptosis (upper right).

Generally, in all electron microcopy figures (3-5) it was found 2 (25%) binucleated trophozoites, 2 (25%) uninucleated trophozoites, 2 (25%) non-nucleated (apoptotic and necrotic) trophozoites in addition to 2 (25%) cysts of *D. fragilis*. Relatively high percentage (75%) of trophozoites was observed by TEM compared to 29% found by LM. In the other side, relatively high percentage (71%) of cysts was observed by LM compared to 25% by TEM table 1.

Table1: Numbers and percentages of *D. fragilis* trophozoites verses cysts found by Light (LM) - and transmission electron microscope (TEM).

D. fragilis	**Microscopy**		**Total**
	TEM	**LM**	
Trophozoite	**6**	**2**	**8**
Cyst	**2**	**5**	**7**
Total	**8**	**7**	**15**
Trophozoite %	**75**	**29**	
Cyst %	**25**	**71**	
Total	**100**	**100**	

DISCUSSION

Previously, *D. fragilis* was described as protozoa with a binucleated trophozoite of amoeba that had no cyst stage and degenerated rapidly once it became outside the human body (25, 26). Confusedly, this description was modified to *D. fragilis* had not flagellate stage and it was not a true amoeba but an aberrant flagellate closely related to *Histomonas* (27).

In contrast, the result of current article has found uninucleated *D. fragilis* trophozoites capturing fungus cell by pseudopodia together with other bacteria and fungi in cytoplasmic vacuoles of this amoeboid shape trophozite (Fig. 3 left panel)), in addition to a binucleated *D. fragilis* having bacteria and fungi in cytoplasmic vacuoles of trophozoites also (Fig. 3 right panel).This information has clarified clearly that *D. fragilis* is an amoeba that feeds on bacteria and fungi by phagocytosis (4) and it differs completely from flagellates that feed by aid of cytostome or absorbing (6).

The result of this current article also has found that *D. fragilis* trophozoite is not binucleated only but has 1-2 nuclei (Figs.1, 3, 5 left panel) likes the amphizoic amoeba *Acanthamoeba castellanii* as shown in (Fig. 6), but it differs from the flagellate *Giardia intestinalis* trophozoite that has 4 nuclei and from the ciliate *Balantidium coli* trophozoite that has 2 nuclei. Therefore it is better to describe *D. fragilis* is an amoeba and its trophozoite has 1-2 nuclei instead of binucleated amoeba as described before by (25), furthermore, non-nucleated (apoptotic and necrotic) trophozoites could be found also as in the figure 5 right panel.

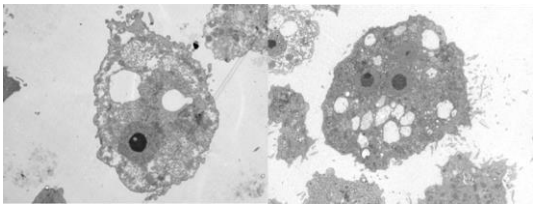

Figure 6: *Acanthamoeba castellanii* trophozoites: uninucleated trophozoite (left panel) and binucleated trophozoite (right panel).
Magnification 2250x TEM from the author

Surprisingly, from morphological point of view, the result has found that *D. fragilis* is a real amoeba that has trophozoite with pseudopodia, precyst (Figs. 1 right panel, 2 both panels, 3 right panel) and cyst (Figs. 2 right panel, 3 left panel) in its lifecycle as other amoebae specially *A. castellanii* (44, 45) and differs from these flagellates; *Chilomastix mesnli*, *Enteromonas hominis* and *Giardia intestinalis* that have trophozoites with flagellae and cysts compared to *Pentatrichomonas hominis, Trichomonas vaginalis*, *T. tenax* and Trypanosoma species that have only trophozoites with flagellae and missing cysts (8).

However, from molecular biology point of view, findings of Cox FEG 1998 declared that *D. fragilis* was closely related to three species of Trichomonas affecting humans (*T. vaginalis, T. tenax, T. hominis*) and on the basis of rRNA evidence quite distinct from the other flagellates genera *Giardia, Enteromonas, Chilomastix* and *Retortomonas* (46).

In this context, recently, despite the protozoa have different four groups according to their movements, a polymerase chain reaction (PCR) and sequence analysis utilising protozoa-specific forward primer (P-SSU-342f) and Eukarya-specific reverse primer (Medlin B) targeting 18S rRNA gene succeeded to identify 66 species of pathogenic protozoa in water samples. Surprisingly, the identified protozoa species represented amoebae, apicomplexa and ciliates in addition to flagellates (47). This surprise agrees with the fact assuming that amoebae and flagellates are considered to be closely related since they belong to the phylum Sarcomastigophra comprising a single large assemblage (9). Unfortunately on the other side, the sequenced PCR positive samples were 57 samples but only 38 samples passed sequencing and 19 samples failed to pass the sequencing (47) uncovering that every diagnostic method has its limitation.

In the current study, encystation of *D. fragilis* trophozoites in clinical stool samples was uncovered by light- and electron microscopy. Surprisingly, both mature cysts and precysts of *D. fragilis* were described in more details in comparison with other studies that observed cyst stage formation of *D. fragilis* in vitro experiment when *D. fragilis* infected mice (34) and presence of precyst or pseudocyst of *D. fragilis* in a retrospective study of 500 slides from human clinical samples was performed on all permanently stained smears positive for *D. fragilis* (35).

In the current study, more trophozoites and fewer cysts was identified by TEM compared to LM according to table 1. Such information might indicate that sensitivity of light microscopy was negatively affected by the previous misinformation about *D. fragilis* had not cyst (25), taken together with apoptosis and necrosis that change morphology of this parasite by destruction of cell nucleus and plasma membrane as found in the current article.

The current article found that *D. fragilis* cyst is small in size that can not be seen easily by low and high power lens therefore *D. fragilis* was described carelessly that had not cyst stage in 1918 (25). Anyway, the cyst can be seen easily by the oil emersion lens after staining with methylene blue stain as shown by this article.

Moreover, it is worth to mention that the result of current article suggests firstly and strongly a rule of *D. fragilis* cyst as a mod for faecal-oral transmission of this amoebic protozoan between hosts as other intestinal parasites to document the biphasic life cycle of *D. fragilis* in clinical samples.

Newly, for the first time and for my knowledge, the transmission electron microscopy in this current article showed that many *D. fragilis* cells underwent cell death that was described as apoptosis and necrosis, in agreement with what occurred for unicellular eukaryotes under certain conditions (48) such as Trypanosoma species, *Acanthamoeba polyphaga* (49) and *A. castellanii* (50).

Astonishly, for the first time, this undergoing of apoptosis and necrosis has explained why *D. fragilis* degenerates rapidly outside the human body (Fig 5) and together with the encystation (Figs 2 and 4) also uncovered the reason of the presence of non-nucleated cells of *D. fragilis*.

Moreover, the encystation of *D. fragilis* trophozoites has explained the presence of smooth and ruffled cells by scanning electron microscopy of cultured *D. fragilis* according to (37). Since the ruffled cells are trophozoites possessing pseudopodia and the smooth cells are cysts having no pseudopodia as described in the current article and clarified by scanning electron microscopy (SEM) for the amoeba *A. castellanii* (Fig.7).

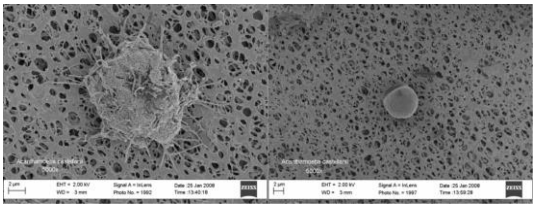

Figure 7: *Acanthamoeba castellanii* trophozoite and cyst: the trophozoite is ruffled cell because its pseudopodia (left panel) and the cyst is smooth cell having no pseudopodia (right panel)
Magnification 5000x SEM

Many biological findings such as blood leucocytes, erythrocytes and fungi that will be found in the stool clinical sample can not be detect by molecular biology and immunological methods. But microscopy of general stool examination reports all finding seen in the specimen like parasite, bacteria and fungi, in addition to leucocytes and erythrocytes and their useful meaning in diagnosis. Importantly, the microscopy reports all parasites found in the sample and gives more information about each parasite if it is a helminth or protozoan, in addition to the presence of larva, ovum, trophozoite or cyst. Moreover, adult helminth such as *Entrobius vermicularis* or *Ascaris lumbricoides* and mature segments of Tinea species are found by macroscopic examination (necked eye of the microscopist).

In the other side, the microscopy in the hematology especially blood film gives valuable quantitative and qualitative information about blood cells and their disorder in addition to presence of blood parasites such as Babesia species and plasmodium species together with their stages and percentage of infected erythrocytes (parasitaemia). How such all the important information can be gained by any automated instrument rather than microscopy?

In routine detection of parasites, microscopy is the standard method having high specificity and sensitivity especially detection of blood parasites (malaria), urinary tract parasites and intestinal

parasites but the microscopy has less limitation. In comparison molecular methods identify just one parasite because limits of the PCR primer and limits of sequencing (47). Furthermore, the available commercial panels PCR assay; RIDA®GENE Parasitic Stool Panel I) cannot detect more than four microorganisms of the diarrheal protozoa (51) and Novodiag® Stool Parasites; qPCR and microarray technologies that detect 26 parasites out 300 (52). Lastly, the direct smear of the microscopy was shown to have a sensitivity of 61% and Kato Katz (60%) compared to formal ether concentration (92%) for the detection of a variety of parasites including protozoa and helminths (53).

In this article, microscopy has shown that *D. fragilis* trophozoite possesses pseudopodia, utilises phagocytosis and undergoes encystation as a second phase of amoebic lifecycle. Thus, the microscopy uncovers for the first time that trophozoite of the protozoan *D. fragilis* is an amoebic trophozoite that has a cyst stage found in clinical samples. These important findings enhance that microscopy is a powerful method in diagnostic parasitology together with molecular biology and immunology methods that complete the microscopy in some cases as differentiation between *E. histolytica* and *E. dispar* as well between Cryptosporidium species by PCR or indirect detection by serology.

CONCLUSIONS

Dientamoeba fragilis is an amoeba possessing trophozoite with 1-2 nuclei and not only binucleated. The trophozoite has pseudopodia to capture bacteria and fungi and utilises phagocytosis to ingest food particles in vacuoles that will differentiate *D. fragilis* away from the flagellates.

D. fragilis is an amoebic protozoon that has a biphasic life cycle since the ruffled trophozoite encysts to build the smooth cyst in clinical samples opposite to Jepps and Dobell description.

The encystation of *D. fragilis* found in this article strongly explains for first time why it will be found both non-nucleated cells and smooth cells (cysts) in same samples.

In this article, *D. fragilis* trophozoite found to undergo apoptosis and necrosis as a real eukaryotic cell such as macrophages and amoebae.

Present of the apoptotic and necrotic cells of *D. fragilis* strongly explain for first time why it degenerates rapidly once it will become outside the human body and why it will be found as non-nucleated trophozoites (necrotic and apoptotic cells).

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