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Study of the pathogenic potential of *Dientamoeba fragilis* in experimentally infected mice

Eman K. El-Gayar [a,*], Amira B. Mokhtar [a], Wael A. Hassan [b]

[a] Medical Parasitology Department, Faculty of Medicine, Suez Canal University, Egypt
[b] Pathology Department, Faculty of Medicine, Suez Canal University, Egypt, Ismailia, 41522, Egypt

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ABSTRACT

Dientamoebafragilis (*D. fragilis*) is a protozoan parasite whose pathogenic potential is still disputable. The aim of this study was to illustrate the pathogenicity of *D. fragilis* infection and to determine the infective dose for experimental mice infection. Three groups of mice (8/each) were orally inoculated with *in vitro* cultured *D. fragilis*. The infected groups (G1- G3) received 10^3, 10^5 and 4×10^6 *D. fragilis*/0.5 ml culture, respectively. A control group (G4) only received parasite-free culture. Two weeks post-inoculation all mice were euthanized for histopathological examination. All mice of G3 (100%) and three mice of G2 (37.5%) were infected, and the results were confirmed by PCR and different staining methods. On the other hand, all mice from group G1 showed a completely negative result. Histopathological examination of the colon and caecum of the highly infected group G3 showed active colitis, with infiltration of mixed inflammatory cells such as eosinophils, neutrophils and lymphocytes within the lamina propria of the intestinal wall. The parasite was not invading the colonic mucosa. This study revealed that infection with *D. fragilis* is dose-dependent. Moreover, a dose of 10^5 *D. fragilis*/mouse or higher is necessary to infect mice through the oral route. In addition, this route of infection, although non-invasive, can induce severe inflammatory changes to the colonic and caecal mucosa in experimentally infected mice.

1. Introduction

Dientamoeba fragilis was first described in 1918 as a binucleated, unflagellated protozoan that inhabits the large intestine (Jepps and Dobell, 1918). On electron microscopy, it has been reclassified as an amoeba-flagellate rather than an amoeba (Camp et al., 1974). It has a worldwide distribution in both urban and rural areas with infection rates ranging from 0.5% to 16%, where higher rates were reported in outbreaks and associated to the lack of personal hygiene (Girginkardeşler et al., 2008). In adults, asymptomatic colonization is present in 75-85% of individuals affected by the parasite while in children; disease develops in as many as 90% of those colonized (Crotti et al., 2005). Similar to some other parasites (*e.g.*, *Giardia lamblia*, *Cyclospora cayetanensis*, *Cryptosporidium spp.*), the parasite *D. fragilis* has been showed to cause disease in humans regardless of their immune status. Abdominal pain/discomfort and diarrhea are the symptoms most often described in patients harboring *Dientamoeba*. Other symptoms may include fecal urgency, vomiting, nausea, anorexia, weight loss and fever (Cuffari et al., 1998; Norberg et al., 2003; Stark et al., 2010). The parasite is increasingly recognized as a relatively common cause of human diarrhea and long-term chronic

* Corresponding author at: Ismailia, Suez Canal University, Faculty of Medicine, Medical Parasitology Department, Egypt.
E-mail address: ekamal71@yahoo.com (E.K. El-Gayar).

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infections is commonly observed in infected patients (Stark et al., 2010). *Dientamoeba fragilis* is often associated with other intestinal parasites. A study investigated 1497 confirmed *D. fragilis* cases and found coinfections with *Blastocystis* spp. in 40.3%, with *Endolimax nana* in 24%, and with *Entamoeba coli*, *G. lamblia* and *Entrobius vermicularis* in 6, 5.7 and 5% of the cases, respectively (Ayadi and Bahri, 1999).

D. fragilis cells tend to infect the mucosal crypts of the large intestine from the caecum to the rectum, which is located close to the mucosal epithelium (Levine et al., 1980). In addition, this parasite is not known to be invasive and does not cause cellular damage. It may elicit an eosinophilic inflammatory response in the colonic mucosa. Thus, symptoms are related to the irritation of the superficial colonic mucosal, and it has been reported to be associated with marked peripheral eosinophilia (Gray et al., 2013).

Despite the evidence supporting its pathogenic nature (Stark et al., 2006), it is apparent that a degree of uncertainty still surrounds the pathogenic potential of *D. fragilis* (Ayadi and Bahri, 1999; Banik et al., 2011). To be recognized as a true pathogen Koch's postulate must be fulfilled for *Dientamoeba* as a cause of gastrointestinal illness. Studies conducted to investigate the biology of this parasite are limited by methods of *in vitro* cultivation, with difficulties in establishment of long-term cultures. It is commonly observed that *D. fragilis* grows *in vitro* for only few subcultures before dying out (Clark and Diamond, 2002; Munasinghe et al., 2012). Moreover, the lack of an animal model for dientamoebiasis hinders the ability to demonstrate its pathogenicity (Barratt et al., 2011). However, recently few experimental studies have been carried out (Munasinghe et al., 2013; Eida et al., 2015). This study aimed to investigate the suitability of mice as an animal model for experimental *D. fragilis* infection, and to determine the required infective dose. In addition, we studied the pathological changes caused by *D. fragilis* infection in experimentally-challenged mice.

2. Subjects and methods

2.1. Isolation of D. fragilis

The study was conducted from September 2015 to February 2016, in the Parasitology Department, Faculty of Medicine, Suez Canal University, Ismailia. Fecal specimens were collected from patients seeking medical care for different gastrointestinal complaints such as acute or chronic intermittent diarrhea, or diarrhea alternating with constipation, with or without abdominal pain and attending Outpatient Clinics of Suez Canal University and General Hospitals (Ismailia, Egypt). Fresh stool specimens were immediately examined microscopically by direct smear and Lugol's iodine for detection of *D. fragilis* and to exclude other intestinal parasites followed by formalin ethyl acetate concentration technique (Garcia, 2009). Positive samples for *D. fragilis* were further stained with 10% Giemsa stain (Crotti et al., 2005). Modified acid-fast and trichrome stains were performed to exclude infection of patients with other parasites *e.g. Cryptosporidium*, *Cyclospora*, *Cystoisospora* and *Microsporidium* spp. (Garcia, 2009). Culture for common enteric bacterial pathogens was done to exclude them (LiPuma et al., 2007). Culture on Jones' media without rice starch was done to exclude infection with *Blastocystis* spp. Positive samples for *D. fragilis* were cultivated in the modified Boeck and Drbohlav (MBD) medium supplemented with antibiotics (Sawangjaroen et al., 1993). The culture was incubated at 37°C for 96h. Each day, the sediment of the culture tubes was examined by light microscopy with ×40 objectives for trophozoites. Daily count of the number of *D. fragilis* trophozoites was done using a Neubauer chamber to adjust the inoculation dose to 10^3, 10^5 and 4×10^6 *D. fragilis*/0.5 ml culture medium.

2.2. Experimental animal infection

Throughout the study, thirty-two 5–6weeks old immune-competent albino Balb/c mice, weighted 25–30 g, were purchased from the Veterinary Medicine Animal Lab, Suez Canal University. Mice were housed independently in good ventilated, filter-top cages and provided sterile rodent chow and water *ad libitum*. The cage bedding was changed every day to avoid and reduce the potential for fecal contamination occurring during the experiment. The animals were maintained in animal house at the faculty of Medicine, Suez Canal University at 25°C, and with a relative humidity of 40–60%.The mice were confirmed to be parasite-free by screening for several days by light microscopy, modified acid-fast and trichrome stain fecal smears for protozoa prior to infection with *D. fragilis*. Mice were randomly divided into four groups. The first three groups were inoculated with *D. fragilis* harvested from 4day-old cultures, at different doses: 10^3, 10^5, and 4×10^6 *D. fragilis* trophozoites/0.5 ml culture medium given orally to G1, G2 and G3, respectively. The fourth group G4 (uninfected control) was given parasite-free culture media (0.5 ml/mouse). Oral inoculation was performed *via* 18G ball-tipped feeding needle attached to 1 ml syringe. All mice were monitored daily for weight loss, presence of loose stool or mucous in stool, lethargy and fur loss from day 1 to day 14 post inoculation. All mice were euthanized 2weeks post-inoculation. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed in this study.

2.3. Detection of D. fragilis in feces and intestine

Feces from all mice were examined microscopically by wet mount preparation at the 2nd, 4th and 8th day post-inoculation; also the intestinal content of sacrificed mice was examined. Culture on MBD media was done for negative specimens. In this case, cultures were considered negative if the organism was absent until the 7th day post-challenge.

To confirm the presence of *D. fragilis* in infected mice, genomic DNA was extracted from feces of all mice in the infected groups (G1–G3) using the QiaAMP DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was