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I.I. Kyrychenko [1,*], M.S. Biriukov [1], S.I. Pokhil [2], O.M. Tymchenko [2]
[1] Military Medical Clinical Center of the Northern Region of the Ministry of Defense of Ukraine, Kharkiv, Ukraine
[2] State Institution «I. Mechnikov Institute of Microbiology and Immunology of the National Academy of Medical Sciences of Ukraine», Kharkiv, Ukraine
* kiricheigor@gmail.com

CULTIVATION AND CRYOPRESERVATION OF *DIENTAMOEBA FRAGILIS* XENIC CULTURES IN RPMI MEDIUM

Dientamoeba fragilis is one of the most common intestinal protozoan parasites in humans and many animals, which can cause acute and chronic lesions of the gastrointestinal tract. Growing and preservation of D. fragilis cultures in vitro allows studying the features of the life cycle of these parasites, their pathogenic potential, sensitivity to antimicrobial drugs, etc. In this work, the suitability of RPMI nutrient medium for in vitro cultivation and cryopreservation of xenic cultures of D. fragilis was studied for the first time. Based on the results of sowing fresh fecal samples from Ukrainian military personnel (with confirmed monoinvasion of D. fragilis) in RPMI (with 10% heat-inactivated horse serum, without antibiotics), it was established that RPMI is quite suitable for growing both short-term and long-term xenic cultures of D. fragilis. Growth profiles on RPMI (at 37 °C in microaerophilic conditions) of different isolates of D. fragilis are similar. D. fragilis cultures grown on RPMI are highly suitable for studying the morphological structure of these protozoa cells, their division processes, the formation of pseudopodia, pre-cysts and cysts. The most effective cryopreservation of D. fragilis trophozoites is achieved in a composition based on RPMI containing DMSO in a final concentration of 7.0%. The smallest trophozoites (12—15 μm) are the most resistant to freezing and ensure the recovery of growth of D. fragilis cultures after their cryopreservation.

Keywords: *Dientamoeba fragilis,* xenic cultures, cultivation, cryopreservation, RPMI medium.

The name "*Dientamoeba fragilis*" unites a group of intestinal anaerobic unicellular protozoa, ultrastructurally and phylogenetically related to trichomonads and able to parasitize in the large intestine of humans, a number of wild and domestic animals [7, 15, 17, 38]. *D. fragilis* is a cosmopolitan parasite, which is detected in the feces of people all over the world with a frequency of 0.4 to 82.9 % [9, 15, 17, 30]. Invasions of *D. fragilis* have been reported both in clinically healthy individuals and in patients with symptoms disorders of the gastrointestinal tract [3, 6, 9, 15, 20, 30]. The most common manifestations of overt *D. fragilis* infections in humans are abdominal pain and diarrhea, other symptoms include nausea, anorexia, flatulence, malaise, fatigue, weight loss, and unexplained peripheral eosinophilia [3, 15, 17, 33]. Most researchers agree that the «neglected» *D. fragilis* should be considered as the etiologic agent of gastrointestinal disorders, and symptomatic patients should be treated with antimicrobials to reduce their suffering and eradicate the parasites [3, 15, 30, 28, 34]. However, to date, many aspects of the life cycle, morphological structure, physiology, genome, proteome, pathogenic potential and virulence of *D. fragilis* remain unclear [5, 13, 17, 18, 30]. In addition, the methods of laboratory diagnosis and etiotropic therapy of dientamebiasis

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require urgent improvement [15, 21, 30, 34]. *D. fragilis* grown *in vitro* cultures are actively used to solve these scientific problems [3, 8, 13, 16, 18, 19, 22, 26, 27, 31].

The main methodological principles of growing *D. fragilis in vitro* consist of simulating the conditions of the parasite host organism, namely: current *D. fragilis* culture systems are xenic, dixenic, and rarely monoxenic (the parasites are grown together with obligate symbiotic bacteria); the conditions of anaerobiosis necessary for the vital activity of these intestinal protozoa are maintained at all stages of their cultivation (the sowing of the studied material, primary cultures and subcultures of parasites is carried out in a reduced medium, and they are incubated in a microaerophilic or anaerobic atmosphere with an O_2 concentration of 6 % or less); an incubation temperature of 37–42 °C ensures high growth rates of *D. fragilis* trophozoites in cultures (at higher temperatures, parasites reproduce more intensively than at lower temperatures, and at 30 °C and below, their growth does not occur); the nutrient media (NM) used for the cultivation of *D. fragilis* must simultaneously satisfy the physiological (plastic and energy) needs of both the parasites themselves and the symbiont bacteria (this is achieved by introducing serums with growth factors, minerals, amino acids, vitamins, microelements, energy carbohydrates, buffer systems, *etc*. into the composition of the NM) [2, 4, 11, 16, 25, 30]. For long-term maintenance of grown cultures of *D. fragilis*, methods of repeated subcultivation or freezing at temperatures from –20 to –196 °C using cryoprotectants such as glycerol, dimethyl sulfoxide (DMSO), sucrose, glucose are used [4, 24, 29]. The basis of the composition for cryopreservation of *D. fragilis* is usually phosphate buffered saline and liquid NM in which the parasite cultures were grown. It is clear that the difference in the properties of the NMs used requires adaptation of the cryopreservation method: selection of the type and concentration of the cryoprotectant, optimization of the procedures for equilibration, cooling, defrosting, etc.

Over almost 100 years of experience in growing *D. fragilis*, a wide range of cultivation methods and tools has been used, which included NM of Boeck-Drbohlav's, Robinson's, Dobell-Laidlaw's, Loeffler's, Balamuth's, M 199, TYGM-9, as well as media for growing trichomonads (Trichosel Broth and Tritrichomonas fetus medium) and others [2, 3, 8, 11, 13, 16, 18, 22, 30]. For the purpose of growing *D. fragilis* cultures, scientists relatively often prefer the Loeffler's medium and its modifications according to Barratt's or Munasinghe's [4, 11, 16, 18, 19, 25—27, 30, 31]. Due to the lack of industrial production, these types of NM are manufactured in laboratories by the researchers themselves. The manufacturing technology of such NM is complex and labor-intensive, and their composition is multi-component and variable depending on the specific author's modification, which leads to significant differences in the growth qualities of the media used in the cultivation of *D. fragilis* in different laboratories. In addition, the mentioned NM are not universally suitable for the cultivation of other types of intestinal protozoan parasites [2, 11]. The widely available commercial liquid medium RPMI (abbreviation of the full name «Roswell Park Memorial Institute medium 1640») has recently been successfully used for the cultivation of globally widespread pathogens of blastocystosis (*Blastocystis sp*.) and giardiasis (*Giardia spp.*) [14, 39]. This showed the potential for culturing other species of intestinal protozoan parasites, in particular *D. fragilis*, in RPMI medium.

The aim of the research was to study the suitability of RPMI medium for *in vitro* cultivation and cryopreservation of xenic cultures of *D. fragilis*.

MATERIALS AND METHODS

In order to identify intestinal parasites, microscopic studies were conducted on 302 feces samples from military personnel who received inpatient medical care (from February to November 2023) at the Military Medical Clinical Center of the Northern Region of the Ministry of Defense of Ukraine. Each sample was a mixture in a container of three portions of stool collected after a day and fixed with a 10 % formalin solution. Stool samples were enriched by the method of formalin-ethyl acetate sedimentation (with a centrifugation mode of 500 g for 5 min) and examined by microscopy of wet smears stained with D'Antoni's iodine solution (DAIS), and fixed smears permanently stained with Whitley's modified trichrome (mWT) or Heidenhain's iron-hematoxylin (HIH), as well as the modified acid method by Ziehl-Neelsen (mZN) [10, 12].

The material for the growing of *D. fragilis* cultures was re-selected fresh feces samples from those soldiers in whom monoinvasion of *D. fragilis* was detected according to the results of a preliminary microscopic examination. To establish the ability of *D. fragilis* to undergo primary growth on nutrient media RPMI 1640 (Biosera Inc., France) with added (10 % v/v) heat-inactivated (at 56 °C for 30 min) horse serum (Biosera Inc., France) without antibiotics (hereinafter — RPMI), 100 μl of fecal homogenate in phosphate buffered saline with pH = 7.2 (1:1 v/v) were seeded in 16 × 120 mm tissue culture tubes with a vented screw cap (TPP Techno Plastic Products AG, Switzerland) containing 3.0 ml of the specified medium. The inoculates were cultivated for 5 days at a temperature of 37 °C under microaerophilic conditions in laboratory anaerostat "ANS1" (1-CUBE Ltd., Czech Republic; residual air pressure 50.7 kPa, partial pressure of O_2 about 10.6 kPa). Stabilized (long-term) xenic cultures of *D. fragilis* were obtained from primary parasite cultures after ten successive subcultures (200 μl of inoculum) in a new portion of RPMI. In addition, from suspensions of primary cultures of *D. fragilis*, authentic *Escherichia coli* strains for each isolate of parasites were obtained by generally accepted bacteriological methods. One- or two-day cultures of the latter in meat-peptone broth served as a donor of gram-negative symbiotic bacteria to support the growth of *D. fragilis* in cases where this was required (explained below).

Growth patterns on RPMI were studied on five randomly selected clinical isolates of *D. fragilis* (marked with serial numbers from 1 to 5, respectively) and characterized by the following indicators: parasite cell generation time (Tg) in hours; the maximum concentration of their viable cells (MCVC) in milliliters (ml) of medium; peak day (*i. e.*, time in days) of achieving MCVC in parasite cultures (PTD); the suitability of grown cultures for the study of protozoan cell morphological structure (SSCM); the suitability of the NM for the improvement (simplification) of the growing technique of *D. fragilis* cultures (STGC). Determination of Tg, MCVC and PTD indices was based on the results of counting viable *D. fragilis* cells in microvolumes of their culture suspension. The actual MCVC and PTD values are empirical data, and the Tg values were calculated using the formula:

$$Tg = (T2–T1)/(\log_2(n_2/n_1)),$$

where n_1 — the concentration of cells at the previous stage of parasite cultivation (at time T1), and n_2 — the concentration of cells at the next stage of their cultivation (at time T2).

In our studies, for each culture of *D. fragilis* (T2–T1) = 24 hours. The number of viable *D. fragilis* cells in all test tubes was determined immediately after sowing and daily for five days (24, 48, 72, 96 and 120 hours), which is associated with a clear decrease in the concentration of parasites on the fourth day when they are grown in RPMI medium (own observations). The *D. fragilis* cells were counted in a hemocytometer using the trypan blue dye exclusion test, which was reproduced according to the basic protocol [32] with the difference that the cells were washed from the serum of NM by centrifugation at 500 g for 5 min. The following techniques for counting *D. fragilis* cells and criteria for assessing their viability were used: each counting procedure was performed parallel in two hemocytometers by two different specialists under light microscopy with a total magnification of ×200; cells colored blue were considered non-viable, and unstained (intact) cells — viable; cells with an indeterminate status according to the staining criterion were subjected to microscopy at a magnification of ×400 to establish signs of destruction (destruction of the cell wall and internal structure); cells without signs of destruction were classified as viable (Fig. 1). The SSCM quality index was assessed based on the suitability of *D. fragilis* cultures grown on RPMI medium for visualization under light microscopy (at ×400 magnification) of the morphological structure of protozoan cells at different stages of growth, their division processes, the formation of pseudopodia, pre-cysts, cysts, *etc*. The STGC quality index was assessed based on the results of cultivation experiments (in RPMI) of primary and stabilized cultures of *D. fragilis* in conventional bacteriological test tubes (15.3 × × 120 mm, cotton-gauze plug) under a layer of sterile vaseline oil (PJSC Pharmaceutical Factory "Viola", Ukraine) (5–7 mm high), as well as in "Eppendorf" tubes (F. L. Medical, Italy) with a volume of 1.5 ml (feature of seeding: the total volume of the suspension in the test tube is about 1.6 ml; under the hermetically sealed lid, a layer of air of 3–4 mm; during incubation, the culture

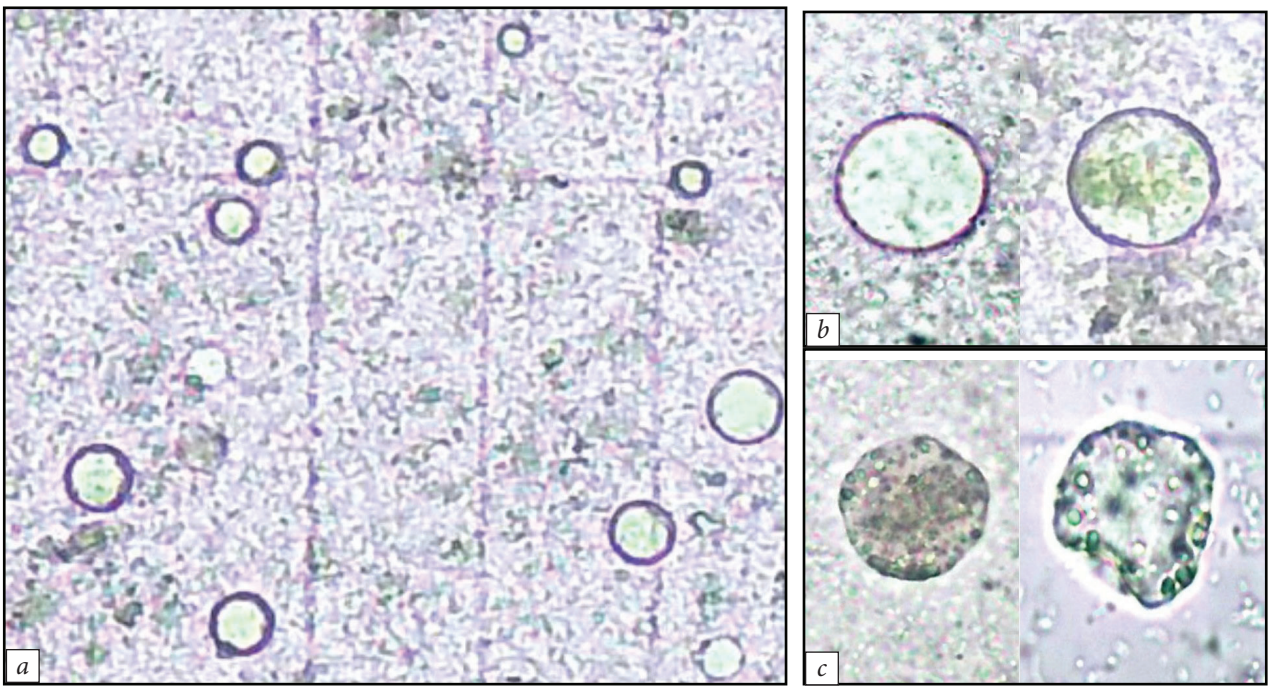

Fig. 1. Counting *D. fragilis* cells in a hemocytometer using the trypan blue dye exclusion test: an example of determining the total concentration of cells in a suspension (*a*); an example of visualization of living cells (*b*); an example of visualization of dead cells (*c*)

tubes were not inverted to avoid saturation of parasite cultures with residual air).

Three *D. fragilis* isolates (numbered 1, 2, and 3) were used in the development of the RPMI cryopreservation method. Each isolate in six tubes of 5 ml RPMI was grown to MCVC. Cultures of the same isolate were transferred to 50 mL conical centrifuge tubes (SPL Life Sciences Co., Ltd., Korea) and mixed (by inverting the tubes) to obtain a homogeneous cell suspension. The *D. fragilis* cells were counted in the pooled suspension as previously described. In suspensions of *D. fragilis* isolates 1, 2, and 3 the concentration of trophozoites was 5.7×10^5 trophozoites / ml, $3.8 \times \times 10^5$ trophozoites / ml and 4.4×10^5 trophozoites / ml, respectively. After repeated mixing, 5 ml of the suspension were transferred to the same 6 test tubes in which parasite isolates were grown. The tubes were centrifuged at 500 g for 5 min. The supernatant was removed, and 5 ml of one of five variants of compositions for cryopreservation (CC), previously prepared on the basis of RPMI, was added to the sediment in five test tubes. All CC included D-glucose (Sigma-Aldrich Co., Germany) in a final concentration of 2.5 % (w / v). Variants CC1-4 differed in the content of DMSO (Labscan Ltd., Ireland) with final concentrations of this ingredient being 1.0, 3.0, 5.0 and 7.0 % (v / v), respectively. The composition of CC5, along with 2.5 % D-glucose and 3.0 % DMSO, included gel sodium alginate (BOC Sciences, USA) with a final concentration of 1.0 % (w / v). Six tubes with cell pellet of each *D. fragilis* isolate, to which 5 ml of RPMI without other additives was added, served as controls. After thorough mixing of the sediment with introduced CC / RPMI (by several single intakes and discharges of the material with a pipette) until the formation of a homogeneous suspension, 500 μl of the latter was poured into cryotubes with a volume of 1.5 ml (Simport Scientific Co., Canada). For cryopreservation of each *D. fragilis* isolate, 55 samples were prepared: 10 — for each variant of CC1–5 and 5 — controls from RPMI.

In the vast majority of experiments, equilibration was carried out at a temperature of 37 °C for 15–20 min, selective studies were performed without an equilibration procedure.

The authors of the study did not have freezing equipment with a controlled cooling rate, so during the studies, six variants of one-, two- and three-stage cooling of samples (cryotubes with *D. fragilis* isolates) were tested. The variants of one-stage cooling of samples included: rapid cooling to –196 °C by immersion in liquid nitrogen in Dewar

flask «X-34 BM», («Kharkiv Transport Equipment Plant Llc», Ukraine), unregulated cooling to –70 °C in a low-temperature chamber «MEDTERM KNT-50-85» («MEDTERM Company Llc», Ukraine) and to –20 °C in a freezer «LIEBHERR CP 4003 210» refrigerator (LIEBHERR Company Ltd, Germany). In two-stage cooling options, samples frozen to –70 °C were immersed in liquid nitrogen after 12 hours, and samples frozen to –20 °C — were transferred to –70 °C. The three-stage cooling of the samples consisted in successively lowering their storage temperature after 12 hours: –20, –70 and –196 °C, respectively. Survival of *D. fragilis* was checked after 10 days for all cryopreservation options, as well as after 1 and 3 months for samples stored at –20 and –70 °C.

Frozen samples were heated in a water bath at 37 °C for 3—5 min. Thawed suspensions of *D. fragilis* were washed once with CC. To do this, 1.0 ml of RPMI was added to the suspensions, mixed (by inverting the closed cryotubes) until they were uniform, then centrifuged at 500 g for 5 min. The supernatant was removed, and the washed sediment was thoroughly diluted with 500 µl RPMI. A drop (50 µL) of the homogenous suspension was used to count viable *D. fragilis* cells in a hemocytometer, and the remainder (about 450 µL) was seeded in 3.0 ml RPMI. Parasite cultures were grown in RPMI after cryopreservation as previously described. The presence of growth of *D. fragilis* isolates in inoculates was assessed after 24 and 48 hours; the growth criteria were an increase in the total number of protozoan cells, visualization of their division stages and motile trophozoites with pseudopodia.

All microscopic studies were performed with light microscope "MIKROmed" Evolution ES-4130 with a set of compensation eyepieces, plan chromatic objects and a universal video camera MDC-500 for image processing on a computer (Mikromed, Trading House Llc., Ukraine).

The obtained data were statistically processed using the licensed software package StatSoft STATISTICA 10.0 (StatSoft Inc., USA), serial number AGFR205F354521FA-5. Calculation and statistical comparison (using the paired test *t*) were subject to absolute (*abs. n.*) and relative values (%) of numerical indices, as well as mean average (χ) with standard deviation (σ) for groups of similar data. The difference between the compared values was considered statistically significant under the condition $p < 0.05$. The descriptive method is used in the analysis of quality indices.

RESULTS OF AND DISCUSSION

According to the results of microscopic studies of 302 stool samples from military personnel of the Northern region of Ukraine, it was established that the prevalence of intestinal parasites among them reaches 10.6 %. All 39 parasites, which were detected in 32 servicemen, are the known unicellular protozoa of the intestinal tract of humans and animals. In total, *D. fragilis* was identified in 14 (4.6 %) soldiers, and in the form of monoinvasion in 8 (2.6 %) individuals. The spectrum of other types of parasites and the frequency of invasions caused by them among Ukrainian servicemen is presented in the previous paper of I.I. Kyrychenko *et al.* [23].

To the authors' knowledge, this is the first study on the suitability of RPMI medium for *in vitro* cultivation and cryopreservation of xenic cultures of *D. fragilis*.

After sowing in RPMI, eight fresh SF from soldiers with monoinvasion of *D. fragilis*, lush growth of primary parasite cultures was observed in 100 % of cases. Primary cultures of *D. fragilis* were not contaminated with other protozoa. Under proper growing conditions, subcultures of *D. fragilis* maintained quite satisfactory permissiveness, which made it possible to easily obtain stabilized xenic cultures for all isolates of *D. fragilis* (Fig. 2).

The growth profiles on RPMI of the studied *D. fragilis* isolates 1–5 were similar, and some differences between parasite isolates at the same growth time concerned only the trophozoites concentration (Fig. 3). Such a difference in concentrations of trophozoites in grown cultures of *D. fragilis* can reach statistical significance, which was established for isolates 1 and 4 ($p < 0.05$).

The regularity of changes in Tg index at growth stages of *D. fragilis* isolates 1–5 in RPMI were the same and did not depend on the initial concentration of trophozoites in the inoculums used. Under these circumstances, the balanced value of Tg was 11.5 ± 1.2, 8.1 ± 0.6, 9.3 ± 0.7 and 21.1 ± 2.9 hours on the first, second, third and fourth days of cultivation of parasite isolates, respectively. The MCVC value in stabilized *D. fragilis* cultures fluctuated within the range from 5.7×10^5 trophozoites / ml (isolate 1) to 2.1×10^5 trophozoites / ml

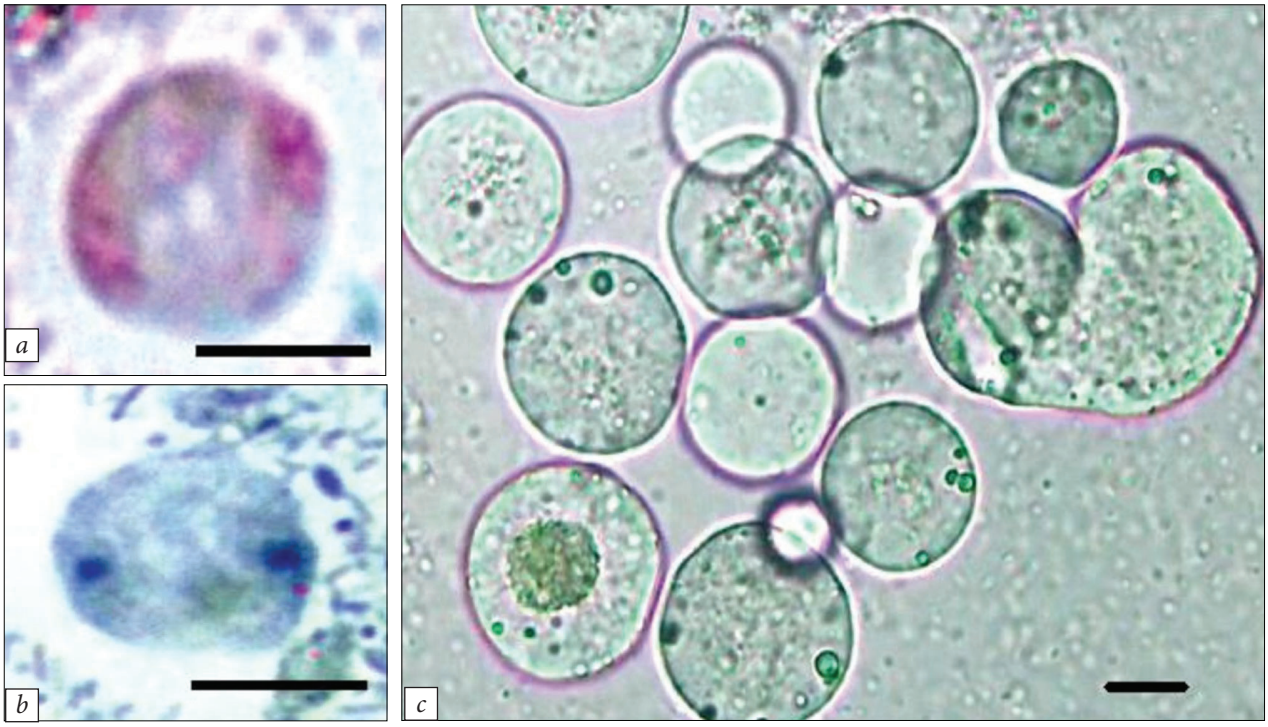

Fig. 2. Microphotographs of D. fragilis trophozoites: *a* and *b* — in fecal smears stained with mWT and HIH; in a crushed drop of a stabilized culture grown on RPMI (*c*); reference mark 10 μm

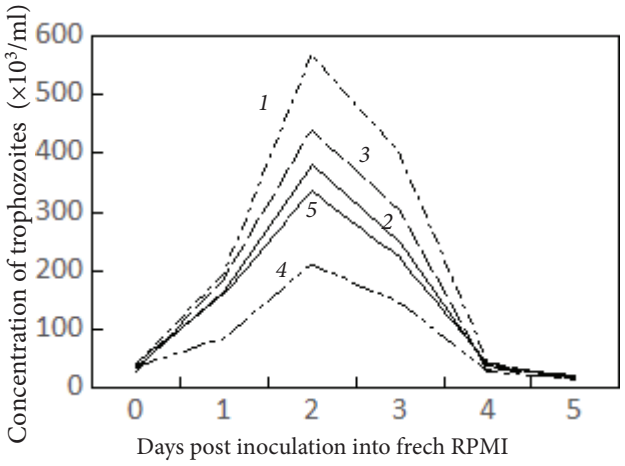

Fig. 3. Growth profiles of five D. fragilis isolates on RPMI: 1 — isolate 1, 2 — isolate 2, 3 — isolate 3, 4 — isolate 4, 5 — isolate 5

(isolate 4), and the balanced value of this index (calculated for all studied isolates) reached (3.9 ± ± 1.8) × 10^5 trophozoites / ml. The actual value of PTD (with reached of MCVC) for all *D. fragilis* isolates is the second day of their cultivation. Thus, taking into account the data of growth profiles and Tg, MCVC, PTD indices, it can be stated that when growing *D. fragilis* cultures on RPMI, the first day is the phase of adaptation and the beginning of parasite growth, the second — the phase of their exponential reproduction, the third is the phase of stationary growth and the beginning of trophozoites death, and the fourth — the phase of their exponential dying off.

The growth patterns of *D. fragilis* cultures in RPMI that we established are similar to the growth dynamics of these protozoa on modified NM of Boeck-Drbohlav's, Loeffler's, Robinson's, and TYGM-9 [4, 25]. However, in our opinion, the use of RPMI for culturing *D. fragilis* has a number of advantages over the above mentioned NM. First of all, the production of RPMI for such a purpose is technically simple and fast, and the growth properties of the medium are more valid, given the commercial production of the required components. In addition, in RPMI achieves lush growth of *D. fragilis* trophozoites without rice starch, which is usually added to the media (2—25 μg for one parasite culture) as an energy carbohydrate [4, 11, 22, 26, 29]. Trophozoites with ingested starch grains serve as an additional identification feature of *D. fragilis*, but at the same time starch grains cause turbidity of the medium and shield elements of the internal structure of the parasite cells. In contrast, RPMI's transparency ensures its high SSCM rating. In cultures of *D. fragilis* grown on RPMI, the size (12—65 μm),

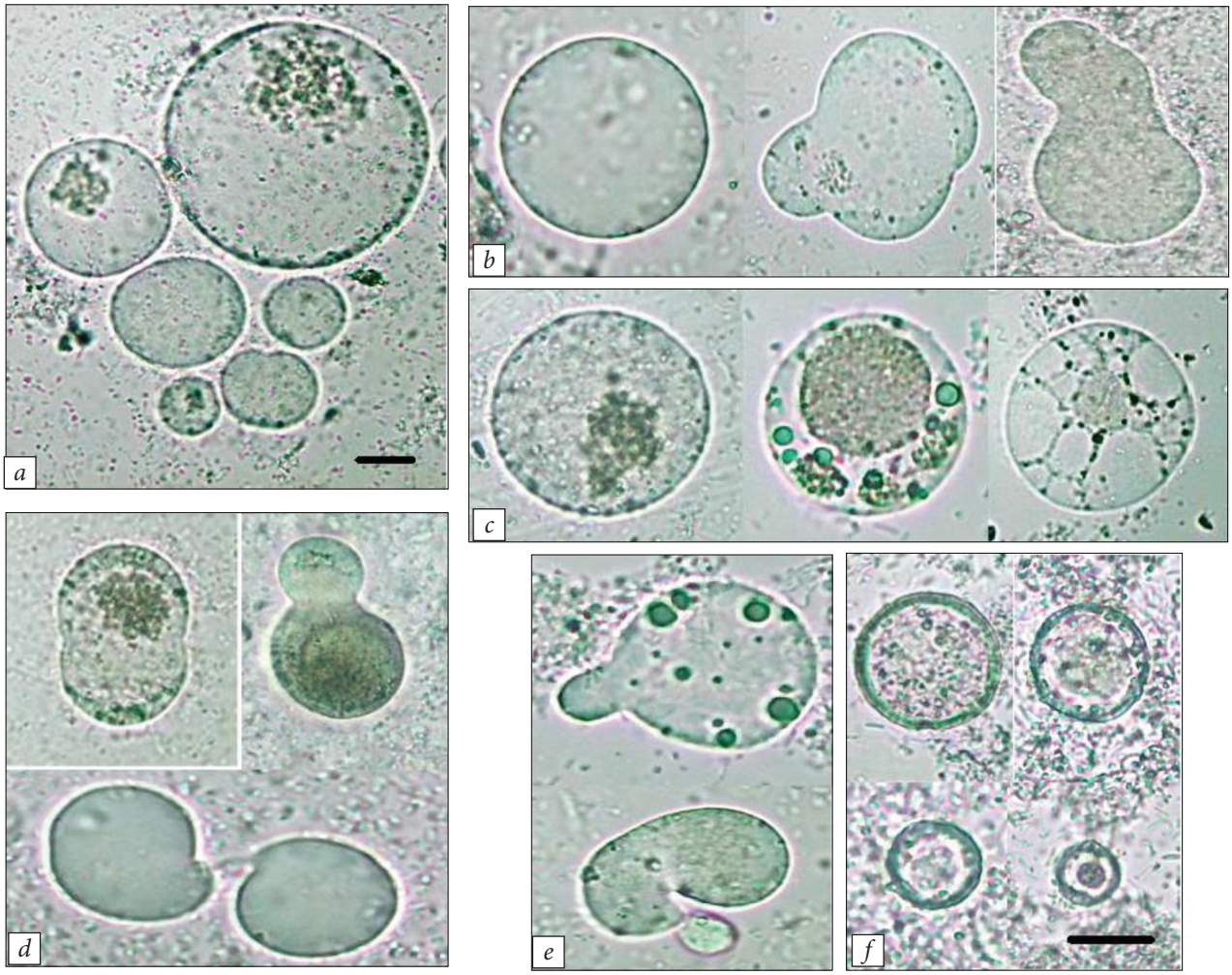

Fig. 4. Microphotographs of *D. fragilis* cells in a crushed drop of cultures grown on RPMI, with examples: size of trophozoites (*a*); forms of trophozoites (*b*); structures of trophozoites (*c*); stages of trophozoite division (*d*); formation of pseudopodia by trophozoites (*e*); stages of cyst formation (*f*); reference mark 10 μm

shape (spherical, amoeboid, irregular) and structure of trophozoites (cell envelope, peripheral and central cytoplasm, internal granules, vacuoles, filaments, *etc.*), as well as the stages of their division, the occurrence of pseudopodia, the formation of pre-cysts and cysts in aging cultures of parasites (Fig. 4) are clearly visualized.

We assessed the quality index of STGC with two rating values: moderate and high suitability of RPMI for growing *D. fragilis* cultures in bacteriological test tubes under a layer of vaseline oil (PJSC Pharmaceutical Factory "Viola", Ukraine) and in "Eppendorf" test tubes (F. L. Medical, Italy) with a small volume of residual air, respectively.

All primary cultures of *D. fragilis* were abundantly grown in test tubes under a layer of oil. However, subcultures of trophozoites died after 3 (isolate 4) or 4 passages (isolates 1, 2, 3 and 5). That is, the way of growing *D. fragilis* in bacteriological test tubes under a layer of vaseline oil is quite suitable for obtaining short-term cultures of parasites. It was possible to support the growth of long-term subcultures of trophozoites under oil by enriching the inoculums with authentic strains of *E. coli*. For this purpose, 100 μl of one- or two-day-old broth cultures of bacteria were added to 3 ml of RPMI at each successive passage of trophozoites, starting from the third to fourth for specific isolates of *D. fragilis*. These results of our studies confirm the conclusion of the authors [16] about the decisive role of gram-negative enterobacteria as obligate symbionts that ensure the growth of *D. fragilis* cultures *in vitro*.

The way of growing parasites in "Eppendorf" tubes (F. L. Medical, Italy) turned out to be more effective, less costly and technically simple. This

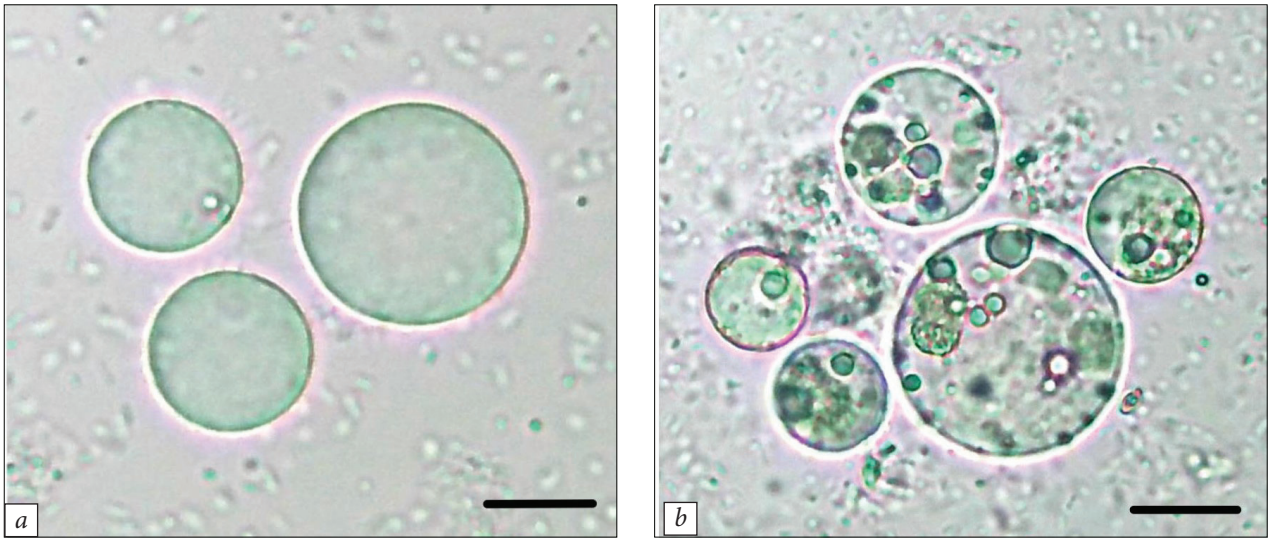

Fig. 5. Microphotographs of *D. fragilis* after the equilibration procedure: in the samples with C1-C4 (*a*); in the sample with CC5 (*b*); reference mark 10 μm

way was used to obtain both short-term and long-term trophozoites cultures of all *D. fragilis* isolates without the need for additional introduction of symbiotic bacteria. The economic advantage of this way is due to the relatively low cost of "Eppendorf" tubes (F. L. Medical, Italy) and a decrease (almost 2 times) in the volume of RPMI used. A simple technique for reseeding *D. fragilis* subcultures involved removing (on the second day of cultivation) the suspension from the test tube to the sediment, which contains the vast majority of trophozoites and symbiotic microflora, and adding the required volume of fresh RPMI (releasing the medium into the test tube ensures the desired mixing). The practical availability of the way of growing *D. fragilis* in "Eppendorf test tubes" (F. L. Medical, Italy) substantiates the prospect of its use for laboratory diagnosis of dientamebiasis, studying the sensitivity of pathogen isolates to antiparasitic agents and evaluating the effectiveness of parasite eradication after etiotropic therapy.

The part of the work on developing the method for *D. fragilis* cryopreservation in RPMI was the most labor-intensive with a large amount of obtained experimental data.

The main results of studies characterizing the effectiveness of cryopreservation of *D. fragilis* trophozoites in RPMI are as follows.

The used equilibration procedure (15–20 min at 37 °C) is necessary and sufficient in duration. Its implementation (compared to the data from studies without it) increased by 4.5 ± 1.3 times the survival of trophozoites of *D. fragilis* in suspensions of isolates 1, 2 and 3, cryopreserved with CC2–4 under conditions of one-stage freezing to –20 and to –70 °C ($p < 0.05$). Upon completion of equilibration, no signs of destruction of trophozoites were detected in all suspensions treated with CC1–4 (Fig. 5A). That is, the short-term effect of DMSO concentrations from 1.0 to 7.0 % at 37 °C does not cause a significant decrease in the viability of *D. fragilis* in RPMI. On the contrary, in all samples treated with CC5, excessive granulation and vacuolization of trophozoites were observed (Fig. 5B).

Regardless of the composition of CC used, all samples that were rapidly cooled to –196 °C in one-stage did not resume growth in RPMI after thawing, washing and seeding (Table 1). Microscopically, such samples visualized the total destruction of trophozoites, which was probably related to the well-known fragility of their outer shell and excessively intense formation of ice crystals in parasite cells. After any other variant of freezing in all samples containing CC1 and CC5, it was also not possible to restore the growth of *D. fragilis* cultures. It is obvious that 1.0 % DMSO is generally an insufficient concentration of this cryoprotectant both in CC1 and in compositions with another base used for *D. fragilis* cryopreservation [4]. If, when examining thawed samples from CC1 using microscopy, we found only destroyed trophozoites, then in samples from CC5 the latter retained the integrity of the outer membrane, which prevented TB from entering cells

when performing tests with this dye. Such an unexpected dissonant result of cryopreservation of parasite cultures in CC5 against the background of certain success in restoring their growth in some samples from CC2 (the composition of which also contained 3.0 % DMSO, and differed from the previous one only in the absence of 1.0 % sodium alginate) prompted us to perform additional studies to investigate the effect of sodium alginate on the growth of *D. fragilis*.

It should be noted that in recent years, Ukrainian and foreign scientists have shown the usefulness of introducing sodium alginate into the composition of CC for increasing the efficiency of cryopreservation of various types of eukaryotic cells (*Saccharomyces spp*., endotheliocytes) [35, 37]. We have found that the addition of alginate (to a final concentration of 0.2 to 2.0 %) to RPMI negatively affects the growth of *D. fragilis*. During the first day of cultivation in the presence of sodium alginate, the following were microscopically detected: excessive granulation and vacuolization of trophozoites (Fig. 5B), cessation of their proliferation and the formation of pseudopodia,

Table 1. **Growth of *D. fragilis* cultures after their cryopreservation with different cooling modes and CC1–5 variants**

Term of evaluation of the result after cryopreservation	Cooling mode	Variants of CC				
		CC1	CC2	CC3	CC4	CC5
10 days		–	+	+	+	–
1 month	One-stage to –20 °C	–	–	×	+	–
3 months		–	–	–	×	–
10 days		–		+	+	–
1 month	One-stage to –70 °C	–		×	+	–
3 months		–		×	+	–
10 days	One-stage to –196 °C	–			–	–
10 days		–		+	+	–
1 month	Two-stage to –20 °C/–70 °C	–		+	+	–
3 months		–		×	+	–
10 days	Two-stage to –70 / –196 °C	–		×	+	–
10 days	Three-stage to –20 /–70 /–196 °C	–		+	+	–

"+" — growth was obtained in all samples with different isolates of *D. fragilis*; "–" — growth was not obtained in all samples with different isolates of *D. fragilis*; "×" — growth was obtained only in some samples with the same type of CC variant without taking into account the differences in the growth of specific *D. fragilis* isolates

a marked decrease in the mobility of external and ingested symbiotic bacteria, an increase in the thickness of the outer shell of *D. fragilis* cells (Fig. 4E). In the next two days, the formation of parasite pre-cysts and cysts occurs (earlier and more intensively than in the control) (see Fig. 4F). To date, there is insufficient data to adequately explain the mechanism of action of sodium alginate on growth inhibition of *D. fragilis*. We found no publications on the direct antiprotozoal effect of sodium alginate on *D. fragilis* or other intestinal protozoa. In addition, the antibacterial activity of alginate hydrogels in combination with various enhancers [36], as well as the direct bactericidal (moderate) effect of sodium alginate on *E. coli* [1], is already widely known. Moreover, increasing the viscosity of RPMI with 1.0 % alginate gel (to about 0.02 Pa·s) reduces the motility of *D. fragilis* trophozoites and their uptake activity of symbiotic bacteria necessary for survival. Whatever the mechanism of sodium alginate inhibition of *D. fragilis* growth, its consequences were irreversible. Although after exposure to alginate, the parasite cells retained their integrity throughout the entire observation period (5 days), their growth was not restored in fresh RPMI medium either under conditions of single or double washing of alginate residues (carefully carried out at different times), or under conditions of enrichment of the medium with glucose (2.5 and 5.0 %) and / or broth cultures of symbiotic *E. coli* (100 µl and 200 µl per inoculation).

The results of growth of *D. fragilis* cultures after different cryopreservation options with the used CC1–5 are presented in Table 1, the data of which outline the general trend of an increase in the frequency of obtaining growth with an increase in the concentration of DMSO in the CC variant.

According to the total performance of growth recovery in RPMI of all three *D. fragilis* isolates after their cryopreservation with different cooling modes, CC4 was recognized as relatively more effective ($p < 0.05$). That is, among the studied concentrations of DMSO in CC2–4, the optimal one is 7.0 %. The use of CC4 with this concentration of DMSO allows maintaining the growth capacity of *D. fragilis* cultures for at least 1 month in samples frozen to –20, –70 and –196 °C (see Table 1). These findings are close to the data of Sawangjaroen et al. [29], who reported successful cryopreservation of *D. fragilis* trophozoites at a final DMSO concentration of 7.5 %. In contrast, our results are not consistent with the conclu-

Table 2. **Viability parameters of *D. fragilis* trophozoites in CC1–5 after freezing under different cooling modes**

Cooling mode	Survival rate of *D. fragilis* trophozoites in % of control (size of cells in µm)*				
	CC1	CC2	CC3	CC4	CC5
One-stage to –20 °C	0	16.0 ± 8.5 (12–28)	23.5 ± 11.0 (12–35)	54.0 ± 12.5 (12–45)	0
One-stage to –196 °C	0	0	0	0	0
One-stage to –70° C	0	54.0 ± 12.5 (12–45)	11.8 ± 6.0 (12–18)	18.9 ± 7.5 (12–18)	0
Two-stage to –20 / –70 °C	0	2.5 ± 1.0 (12–18)	17.5 ± 7.5 (12 –22)	27.5 ± 11.0 (12–26)	0
Two-stage to –70 / –196 °C	0	0	8.4 ± 5.5 (12–15)	15.8 ± 7.2 (12–15)	0
Three-stage to –20 /–70 / –196 °C	0	0	10.9 ± 6.5 (12–15)	20.1 ± 7.5 (12–15)	0

* — survival data of *D. fragilis* trophozoites 10 days after cryopreservation are given

sion of J.L. Barratt *et al.* [4], where 1.375 % DMSO was found to be its optimal concentration for cryopreservation of most *D. fragilis* isolates.

Usually, the assessment of the effectiveness of the proposed method of cryopreservation of *D. fragilis* (as well as many other species of protozoa) is limited to the criterion of restoration of growth of their cultures after defrosting [4, 24, 29]. However, for further improvement of cryopreservation procedures for *D. fragilis*, in addition to selecting the most effective CC (in our case, CC4), it is important to take into account the degree (%) of survival of parasite cells under different sample cooling conditions and the resistance of different-sized trophozoites populations to freezing (Table 2).

Based on the results of microscopy of thawed samples (cryopreserved with CC2-4), we found that regardless of the *D. fragilis* isolate and the initial concentration of trophozoites in its suspension, the most significant losses of viability of the latter occur at the stages of cooling to –20 and –70 °C ($p < 0.05$). Previous freezing of samples to –20 °C slightly increases (by 1.3—1.5 times) the % survival rate of trophozoites at subsequent stages of cooling to –70 and –196 °C, but without achieving statistical significance ($p > 0.05$). The least resistant to freezing is the population of large-sized trophozoites (with a diameter of >45 μm), which dies (destroys) already at the stage of cooling to -20 °C (Table 2). Among trophozoites of medium size (15—45 μm), which constitute the vast majority in two-day cultures of *D. fragilis* grown on RPMI, only cells with a relatively smaller diameter (≤ 26 μm) are able to survive freezing to –70 °C. The highest resistance to freezing (at all cooling modes) is characteristic of the trophozoites population of the smallest size (12—15 μm), which probably ensures the recovery of growth of *D. fragilis* cultures after their cryopreservation. Thus, a promising direction for improving the method of cryopreservation of *D. fragilis* in RPMI is to increase the survival of trophozoites at the critical stage of freezing by controlled regulation of the cooling rate and the use of young parasite cultures with a larger population of small-sized cells.

CONCLUSIONS

1. RPMI medium (with 10 % heat-inactivated horse serum, without antibiotics) is quite suitable for *in vitro* growing of both primary (short-term) and stabilized (long-term) xenic cultures of *D. fragilis*. Growth profiles on RPMI (at 37 °C in microaerophilic conditions) of different isolates of *D. fragilis* are similar. In the phase of exponential reproduction of parasites (the second day of cultivation), the generation time of trophozoites is 8.1 ± 0.6 hours, and the equilibrium index of their maximum concentration reaches (3.9 ± 1.8) × × 10^5 cells / ml.

2. *D. fragilis* cultures grown on RPMI are highly suitable for studying the morphology of these protozoa cells, their division processes, the formation of pseudopodia, pre-cysts and cysts. Cultures of *D. fragilis* can be grown on RPMI in a cost-effective and technically simple way in 1.5 ml "Eppendorf" tubes (F. L. Medical, Italy).

3. The most effective cryopreservation of *D. fragilis* trophozoites in RPMI is achieved when using a composition that, along with 2.5 % D-glucose, contains DMSO in a final concentration of 7.0 %. This composition allows maintaining the growth capacity of *D. fragilis* cultures for at least 1 month in samples frozen to –20, –70 and –196 °C.

4. During cryopreservation, a significant decrease in viable cells of *D. fragilis* occurs at the stages of their cooling to –20 and –70 °C due to the destruction of large and medium-sized trophozoites. The smallest trophozoites (12–15 μm) are the most resistant to freezing and ensure the recovery of growth of *D. fragilis* cultures after their cryopreservation.

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REFERENCES

1. Abdellatif Soliman SM, Sanad MF, Shalan AE. Synthesis, characterization and antimicrobial activity applications of grafted copolymer alginate-g-poly(N-vinyl imidazole). RSC Adv. 2021; 11(19): 11541-8.
2. Ahmed NH. Cultivation of parasites. Trop Parasitol. 2014; 4(2): 80-9.

3. Aykur M, Calıskan Kurt C, Dirim Erdogan D, *et al.* Investigation of *Dientamoeba fragilis* prevalence and evaluation of sociodemographic and clinical features in patients with gastrointestinal symptoms. Acta Parasitol. 2019; 64(1): 162-70.
4. Barratt JL, Banik GR, Harkness J, *et al.* Newly defined conditions for the *in vitro* cultivation and cryopreservation of Dientamoeba fragilis: new techniques set to fast track molecular studies on this organism. Parasitol. 2010; 137(13): 1867-78.
5. Barratt JL, Cao M, Stark DJ, Ellis JT. The transcriptome sequence of Dientamoeba fragilis offers new biological insights on its metabolism, kinome, degradome and potential mechanisms of pathogenicity. Protist. 2015; 166(4): 389-408.
6. Bruijnesteijn van Coppenraet LE, Dullaert-de Boer M, *et al.* Case-control comparison of bacterial and protozoan microorganisms associated with gastroenteritis: application of molecular detection. Clin Microbiol Infect. 2015; 21(6): 592.e9-19.
7. Cacciò SM. Molecular epidemiology of Dientamoeba fragilis. Acta Trop. 2018; 184: 73-7.
8. Calderaro A, Buttrini M, Montecchini S, *et al.* MALDI-TOF MS as a new tool for the identification of *Dientamoeba fragilis*. Parasit Vectors [Internet]. 2018 Jan 04 [cited 2024 Aug 10]; 11: 11. Available from: https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-017-2597-3
9. Calderaro A, Buttrini M, Montecchini S, *et al.* Prevalence of intestinal parasitoses in a non-endemic setting during a 10-year period (2011-2020): a focus on *Dientamoeba fragilis*. Microorganisms [Internet]. 2022 Feb 12 [cited 2024 Aug 10]; 10 (2), 426. Available from: https://doi.org/10.3390/microorganisms10020426
10. CDC: DPDx – laboratory identification of parasites of public health concern. Centers for disease control and prevention. Sensors [Internet]. 2022 Dec 14 [cited 2023 Jun 30]. Available from: https://www.cdc.gov/dpdx/diagnosticprocedures/index.html
11. Clark CG, Diamond LS. Methods for cultivation of luminal parasitic protists of clinical importance. Clin Microbiol Rev. 2002; 15(3): 329-41.
12. Clinical and Laboratory Standards Institute (CLSI). Procedures for the recovery and identification of parasites from the intestinal tract; Approved guideline – Second Edition. CLSI document M28-A2 [Internet]. 2005 Jun 30 [cited 2022 Dec 11]; 25(16). Available from: https://clsi.org/ media/1460/m28a2_sample.pdf
13. El-Gayar EK, Mokhtar AB, Hassan WA. Study of the pathogenic potential of *Dientamoeba fragilis* in experimentally infected mice. Parasite Epidemiol Control. 2016; 1(2): 136-43.
14. Elnazeer A.M., Elmalik K.H., Elshikh A.A. Isolation, excystation and in vitro culture of Giardia-spp from fecal samples of suspected patients in RPMI media Europ Academic Res. 2016; 3(10): 10670-89.
15. Garcia LS. *Dientamoeba fragilis*, one of the neglected intestinal protozoa. J Clin Microbiol. 2016; 54(9): 2243-50.
16. Gough R, Barratt J, Stark D, Ellis J. Diversity profiling of xenic cultures of *Dientamoeba fragilis* following systematic antibiotic treatment and prospects for genome sequencing. Parasitol. 2020; 147(1): 29-38.
17. Guadano-Procesi I, Berrilli F, Montalbano Di Filippo M, Di Cave D. Detection and genotyping of *Dientamoeba fragilis* from symptomatic patients: new insights from Italy into a little-known gastrointestinal protozoan. Parasitol Int. [Internet]. 2024 Feb [cited 14 May 2024]; 98: 102816. Available from: https://www.sciencedirect.com/science/article/pii/S1383576923000946
18. Hall LM, Munasinghe VS, Vella NGF, *et al.* Observations on the transmission of *Dientamoeba fragilis* and the cyst life cycle stage. Parasitol. 2024; 151(3): 337-45.
19. Hamidi N, Meamar AR, Akhlaghi L, *et al. Dientamoeba fragilis* diagnosis by fecal screening: relative effectiveness of traditional techniques and molecular methods. J Infect Dev Ctries. 2018; 12(1): 52-9.
20. Hawash YA, Ismail KA, Saber T, *et al. Dientamoeba fragilis* infection in patients with digestive and non-digestive symptoms: a case-control study. Korean J Parasitol. 2020; 58(2): 129-34.
21. Intra J, Sarto C, Besana S, Tiberti N, Brambilla P. The importance of considering the neglected intestinal protozoan parasite *Dientamoeba fragilis*. J Med Microbiol. 2019; 68(6): 890-2.
22. Kurt Ö, Girginkardeşler N, Özbilgin A, Ok ÜZ. Choosing culture for *Dientamoeba fragilis* detection in stool when PCR is not available: assessment of its efficacy and comparison of three culture media. Infect Dis Clin Microbiol. 2019; 1(1): 34-41.
23. Kyrychenko II, Biriukov MS, Pokhil SI, Tymchenko OM. Protozoan intestinal invasions in military personnel of the northern region of Ukraine. Modern Gastroenterol. 2024; 137(3): 39-45.
24. Miyake Y, Karanis P, Uga S. Cryopreservation of protozoan parasites. Cryobiol. 2004; 48(1): 1-7.
25. Munasinghe VS, Stark D, Ellis JT. New advances in the *in vitro* culture of *Dientamoeba fragilis*. Parasitol. 2012; 139(7): 864-9.
26. Munasinghe VS, Vella NG, Ellis JT, *et al.* Cyst formation and faecal-oral transmission of *Dientamoeba fragilis* - the missing link in the life cycle of an emerging pathogen. Int J Parasitol. 2013; 43(11): 879-83.

27. Nagata N, Marriott D, Harkness J, Ellis JT, Stark D. *In vitro* susceptibility testing of *Dientamoeba fragilis*. Antimicrob Agents Chemother. 2012; 56(1): 487-94.
28. Pietilä JP, Häkkinen TA, Pakarinen L, *et al.* Treatment of *Dientamoeba fragilis*: a retrospective finnish analysis of faecal clearance and clinical cure comparing four antiprotozoal drugs. New Microbes New Infect [Internet]. 2023 Sep 23 [cited 2024 12 Jun]; 54: 101179. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC10542007/
29. Sawangjaroen N, Luke R, Prociv P. Diagnosis by faecal culture of *Dientamoeba fragilis* infections in australian patients with diarrhoea. Trans R Soc Trop Med Hyg. 1993; 87(2): 163-5.
30. Stark D, Barratt J, Chan D, Ellis JT. *Dientamoeba fragilis*, the neglected trichomonad of the human bowel. Clin Microbiol Rev. 2016; 29(3): 553-80.
31. Stark D, Barratt JL, Roberts T, Marriott D, *et al.* Activity of benzimidazoles against *Dientamoeba fragilis* (Trichomonadida, Monocercomonadidae) *in vitro* and correlation of beta-tubulin sequences as an indicator of resistance. Parasite [Internet]. 2014 Aug 25 [cited 2024 Aug 12]; 21: 41. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC4141546/
32. Strober W. Trypan blue exclusion test of cell viability. Curr Protoc Immunol. 2015; 111: A3.B.1–A3.B.3.
33. van Gestel RS, Kusters JG, Monkelbaan JF. A clinical guideline on *Dientamoeba fragilis* infections. Parasitol. 2019; 146(9): 1131-39.
34. van Kalleveen MW, van Gool T, Klarenbeek N, *et al. Dientamoeba fragilis* in children: a systematic review on diagnostic considerations and efficacy of treatment. Expert Rev Gastroent Hepatol. 2020; 14(4): 231-42.
35. Vysekantsev IP, Martsenyuk VP, Buriak IA, Gurina TM. Freezing regimens and gel carrier composition influence safety of *Saccharomyces boulardii* immobilized yeast cells. Probl Cryobio Cryomed. 2021; 31(4): 343-52.
36. Wathoni N, Suhandi C, Ghassani Purnama MF, *et al.* Alginate and chitosan-based hydrogel enhance antibacterial agent activity on topical application. Infect Drug Resist. 2024; 17: 791-805.
37. Xiang X, Liu Z, Zhao G. Sodium alginate as a novel cryoprotective agent for cryopreservation of endothelial cells in a closed polytetrafluoroethylene loop. Biopreserv Biobank. 2020; 18(4): 321-8.
38. Yetismis G, Yildirim A, Pekmezci D *et al.* First report and genotyping of *Dientamoeba fragilis* in pet budgerigars (*Melopsittacus undulatus*), with zoonotic importance. Zoonoses Public Health. 2022; 69(5): 572-8.
39. Zhang X., Qiao J., Wu X., Da R. *et al. In vitro* culture of *Blastocystis hominis* in three liquid media and its usefulness in the diagnosis of blastocystosis. Int J Infect Dis. 2012; 16(1): e23-e28.

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І.І. Кириченко [1], М.С. Бірюков [1], С.І. Похил [2], О.М. Тимченко [2]*
[1] Військово-медичний клінічний центр Північного регіону Міністерства оборони України, м. Харків, Україна
[2] Державна установа «Інститут мікробіології та імунології ім. І.І. Мечникова Національної академії медичних наук України», м. Харків, Україна
* kiricheigor@gmail.com

ВИРОЩУВАННЯ ТА КРІОКОНСЕРВУВАННЯ КСЕНІЧНИХ КУЛЬТУР *DIENTAMOEBA FRAGILIS* У СЕРЕДОВИЩІ RPMI

Dientamoeba fragilis — один з найпоширеніших кишкових найпростіших паразитів людини і багатьох тварин, здатний викликати гострі та хронічні ураження шлунково-кишкового тракту. Вирощування та збереження культур *D. fragilis in vitro* дозволяє вивчити особливості життєвого циклу цих паразитів, їх патогенний потенціал, чутливість до антимікробних препаратів тощо. У роботі вперше вивчено придатність живильного середовища RPMI для вирощування *in vitro* та кріоконсервування ксенічних культур *D. fragilis*. За результатами посіву в RPMI (з 10% термоінактивованої сироватки коня, без антибіотиків) свіжих зразків фекалій від українських військовослужбовців (з підтвердженою моноінвазією *D. fragilis*) встановлено, що RPMI є цілком придатним для вирощування як короткострокових, так довгострокових ксенічних культур *D. fragilis*. Профілі росту на RPMI (при 37 оС в мікроаерофільних умовах) різних ізолятів *D. fragilis* є подібними. Культури *D. fragilis*, вирощені на RPMI, є високопридатними для вивчення морфологічної структури клітин цих найпростіших, процесів їх поділу, утворення псевдоподій, формування пре-цист і цист. Найефективніше кріоконсервування трофозоїтів *D. fragilis* досягається в композиції на основі RPMI, що містить ДМСО в кінцевій концентрації 7,0 %. Трофозоїти найменшого розміру (12—15 мкм) є найбільш стійкими до замороження і забезпечують відновлення росту культур *D. fragilis* після їх кріоконсервування.

***Ключові слова*:** *Dientamoeba fragilis*, ксенічні культури, вирощування, кріоконсервування, середовище RPMI.