Parasitology International 98 (2024) 102816

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Detection and genotyping of *Dientamoeba fragilis* from symptomatic patients: New insights from Italy into a little-known gastrointestinal protozoan

Isabel Guadano-Procesi [a,1], Federica Berrilli [a,*,1], Margherita Montalbano Di Filippo [b], David Di Cave [a]

[a] Department of Clinical Sciences and Translational Medicine, University of "Tor Vergata", Rome 00133, Italy
[b] Istituto Superiore di Sanità, Rome 00155, Italy

ARTICLE INFO

Keywords:
Gastrointestinal protozoans
Dientamoebiasis
Genotype 1

ABSTRACT

Dientamoeba fragilis (*D. fragilis*) represents a common protozoan in both high and low income countries. Despite this, epidemiological data on dientamoebiasis are still limited, and it is possible that the actual prevalence rates of *D. fragilis* have been underestimated due to the challenges in its detection and identification. In the present study, symptomatic patients from Rome (Central Italy) were surveyed for two years to determine *D. fragilis* percentage of infection and genotypes. Stool samples collection was performed over 864 patients, DNA extracted, and RT-PCR performed by the SeeGene Allplex™ Gastrointestinal Parasite Panel Assays. Seventy-nine resulted positive for *D. fragilis* (9.1%). Co-infections were detected in 22 isolates: 21 displayed *Blastocystis* sp. + *D. fragilis* (27.8%). Based on the sequence of a central fragment of the *SSU rRNA* gene, only genotype 1 was identified. These findings are among the few available data regarding genetic diversity of *D. fragilis* in Italy. Large-scale human and animal research are required to enhance our knowledge of prevalence, host range, genetic variability and zoonotic transmission of this little-known intestinal protozoan.

1. Introduction

Dientamoeba fragilis (*D. fragilis*) is a trichomonad parasitic protozoan inhabitant, in the ameboid form, of the human bowel with a worldwide distribution [1]. Although it was identified at the beginning of the 20th century, a variety of issues related to its life cycle, host range, and pathogenicity remained fairly contentious such as the association between this organism, other pathogens and human illness [2]. Moreover, there has not been provided a comprehensive description on the mechanism of transmission of *D. fragilis* [3]. It has been hypothesized that the pinworm *Enterobius vermicularis* may be a feasible vector of transmission based also on a higher than expected rate of co-infection between *D. fragilis* and *E. vermicularis* [4,5]. The lack of strong evidence to support transmission by helminths, combined with high rates of co-infection with other faecal orally transmitted bacteria and protozoa, would suggest that the most likely mechanism of *D. fragilis* transmission is based on a faecal-oral route [6]. *D. fragilis* possesses a typically binucleate trophozoite stage, usually the only form recovered from the host's faeces. The construction of a rodent model revealed intriguing data on the pathophysiology of dientamoebiasis and the discovery of previously unknown cyst and pre-cyst stage of the life cycle [7,8]. However, in human studies cysts were extremely rare; therefore, they may not be the predominant transmissible stage in humans [9].

Several non-human species among mammals and birds (e.g. pigs, rodents, cats, dogs, budgerigars, cattle) have been shown to play a role as natural host of this protozoa, making them a potential source of infections in humans, but the real epidemiologic significance of these hosts is unknown [10–14].

From a molecular point of view, two genotypes named 1 and 2 have been described based on the variability of different genes (*SSU rRNA*, *actin*, *EF1*). The majority of human and animal isolates belongs to genotype 1, and very few samples have been assigned to the genotype 2 so far [15–17]. However, diversity intra and inter genotypes appears as limited at these loci [9] and further data based on more reliable markers are requested to investigate more deeply the genetic variability of *D. fragilis*.

* Corresponding author.
E-mail address: berrilli@uniroma2.it (F. Berrilli).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.parint.2023.102816
Received 12 July 2023; Received in revised form 2 October 2023; Accepted 10 October 2023
Available online 13 October 2023

Despite all these open issues, *D. fragilis* is emerging as an enteric pathogen, as the incidence of dientamoebiasis has increased in industrialized nations as in areas with substandard sanitation [3]. Patients often appear with gastrointestinal symptoms. The most common clinical manifestations are intermittent diarrhoea, abdominal pain, and fatigue, but also nausea, vomiting, and anorexia often in association with eosinophilia are reported [2,9]. Several studies accounted for the clinical improvement of patients after therapeutic intervention and eradication of the infection thus providing a strong support for the role of *D. fragilis* in gastrointestinal illness [3,18]. However, asymptomatic positivity has been described, and *D. fragilis* has also been considered a commensal of the gut microbiota that coevolved with human hosts [12].

Due to its fragile nature, the recovery of *D. fragilis* significantly depends on quick suitable fixation after defecation, and the low prevalence values reported in prior research may be also the consequence of inadequate sample preparation and inspection methodology [3]. In the last years, with the use of improved diagnostic methods for the detection and identification of intestinal pathogens by more sensitive molecular tools, the power of *D. fragilis* diagnostic has been significantly implemented [18,19].

As regards Italy, in the last decades few epidemiological studies on the prevalence of *D. fragilis* were conducted [10,18,20–23] and only one investigation also aimed to genotype the isolates [10].

In order to provide additional data from Italy on this poorly investigated protozoan, a two years round study was conducted by faecal sampling from a cohort of symptomatic patients with gastrointestinal symptoms in order to: (i) investigate the presence of *D. fragilis* by means of a multiplex real-time RT-PCR assay; ii) assess the percentage of infection; (iii) characterize the parasite at the *SSU rRNA* gene. The comparison of the isolates with those previously published from human and non-human hosts was also accomplished.

2. Materials and methods

The study was conducted in the Unit of Parasitology of the Azienda Ospedaliera Universitaria Policlinico Tor Vergata (PTV) of Rome, Central Italy, which handles about 48,000 accesses annually, and acting as First Aid for a population of 800,000.

From February 2021 to December 2022 faecal samples from 864 symptomatic patients, both hospitalised and outpatients, with suspected gastrointestinal illnesses as reported in the medical order were collected. The main symptoms, when indicated from the medical order, were abdominal pain, diarrhoea, and nausea.

Stool samples were analyzed within twenty-four hours after their collection. DNA was extracted from each of the samples using the Starlet extraction automate (SeeGene) and then processed following the SeeGene Allplex™ Gastrointestinal Parasite Panel Assays for the diagnosis of gastrointestinal parasitic pathogens as described by the manufacturer instructions. The panel contains a multiplex real-time PCR that amplifies genes for small subunit ribosomal RNA. This panel allows for the detection of the following parasites: *Blastocystis* sp. (labelled as *Blastocystis hominis* (BH), *Cryptosporidium* spp. (CR), *Cyclospora cayetanensis* (CC), *Dientamoeba fragilis* (DF), *Entamoeba histolytica* (EH), *Giardia duodenalis* (labelled as *G. lamblia*) (GL) (See Fig. S1).

The following information were recorded for each patient: i) positivity or negativity for *D. fragilis*; ii) co-infections with other gastrointestinal protozoans; iii) sex; iv) age. Diagnostic laboratory results were combined and anonymized before analysis, as patient data before being processed. All procedures performed in this study were in accordance with the 1964 Helsinki Declaration and its later amendments.

Given the higher RT-PCR sensitivity, only isolates tested as positive for *D. fragilis* and showing cycle threshold (Ct) values lower than 30 were selected and processed by end-point PCR for sequencing and genotyping. Molecular characterization was performed amplifying a fragment of 850 bp of the *SSU rRNA* gene using the primer DF400 and DF1250 as described [24]. This gene target was selected also considering that most of the *D. fragilis* sequences deposited in GenBank were obtained by *SSU rRNA* amplification, thus allowing to compare our results with those available in literature. Amplicons were purified through the mi-PCR Purification Kit (Metabion International AG) and then sent an independent laboratory for sequencing (Bio-Fab Research, Rome, Italy).

In order to locate any potential double peaks, the resulted chromatograms were manually analyzed using FinchTV 1.4 software (Geospiza, Inc., Seattle, WA, USA). For the purpose of comparison, consensus sequences were matched with those previously published on GenBank database. This resulted in a set of sequences that went under detailed screening; some of them were excluded as not adequately overlapped with ours due to the use of different primers. The final dataset included 17 sequences (plus our isolates) labelled with the following accession numbers (Acc. No.): MN560149–50, ON242172, MN914083, AB692771–72, FJ649228, OP375682–83, OP375691, OP375693, OM250406, JQ677147–49, and AY730405, for genotype 1; U37461 for genotype 2. All out of one (Acc. No. ON242172, isolated from cattle in Turkey) referred to humans, and only one was from Italy (Acc. No. JQ677149). Details on the final dataset are reported in Table 1.

Throughout the paper, the sequence Acc. No. AY730405 small subunit ribosomal RNA gene complete sequence was used as reference sequence for numbering the nucleotide positions.

SSU rRNA identities at the genotype level were verified using the Basic Local Alignment Search Tool (BLAST). Then, phylogenetic analysis of the dataset was performed in MEGA X using the Maximum likelihood (ML) method based on Tamura 3-parameter model (as selected by ModelTest, AIC criterion) [25]. Bootstrapping with 1000 replicates was used to determine support for the generated clades.

In the phylogenetic analysis, sequences from various trichomonads, including *Histomonas meleagridis* (Acc. No. EU647884–86), *Trichomonas vaginalis* (Acc. No. U17510, KM603347), *Tritrichomonas nonconforma* (Acc. No. AY055803), and *Tritrichomonas foetus* (Acc. No. M81842, U17509) were included to infer the evolutionary relationship between *D. fragilis* isolates and related species.

Table 1
Dataset used for the construction of the phylogenetic tree. Information on genotype, host, country of origin, isolate, sequence length and Genbank Acc. No. are presented.

Genotype	Host	Country	Isolate	Sequence length (bp)	GenBank Acc. No.
genotype 1	*H. sapiens*	Italy	DFHS1It (present study)	748	OQ345680
“	“	Iran	Tehran 9	842	AB692771
“	“	“	Tehran 10	856	AB692772
“	“	Australia	–	1661	AY730405
“	“	“	Sydney 2	792	FJ649228
“	“	United Kingdom	Df379	1501	JQ677147
“	“	“	Df1085	1501	JQ677148
“	“	Italy	DfH1	1085	JQ677149
“	“	Turkey	AduDf14	806	MN560149
“	“	“	AduDf138	806	MN560150
“	“	Germany	ISS-8_L2921	1621	MN914083
“	“	Turkey	DF10	788	OM250406
“	“	Czech Republic	B82	859	OP375682
“	“	“	B113	872	OP375683
“	“	“	B338	888	OP375691
“	“	“	B341	864	OP375693
“	Cattle	Turkey	Dfc09	810	ON242172
genotype 2	–	–	strain Bi/PA (ATCC 30948)	1676	U37461

3. Results

A total of 864 patients has been evaluated and tested for suspected gastrointestinal symptoms. Seventy-nine individuals were positive for *D. fragilis*, showing cycle threshold (Ct) values ranging from 19.4 to 41.3, and resulting in a percentage of infection of 9.1%. The age of the positive patients ranged from 1 to 75 years old, the median age was 21 years, with the average age coming in at 28.8 years. Forty-six patients were females, and 27 patients were males; for 6 people the gender was not available. Patients' population data are summed up in Table 2.

In 56 out of 79 cases (70.9%), *D. fragilis* was the only parasite detected. Co-infections with other parasites were detected in 22 isolates: 21 displayed *Blastocystis* sp. + *D. fragilis* (27.8%), and in one case (1.3%) *D. fragilis* was found in combination with *Cryptosporidium* spp.

3.1. Molecular identification

Among the positives, 33 isolates were selected having Ct values <30. Fourteen (named DFHSnIt) provided good quality sequences resulting in a 748 bp fragment spanned the position 450–1198 of the reference sequence Acc. No. AY730405. Being all sequences from the present study identical, only one representative isolate was selected (DFHS1It) and compared with other *D. fragilis* sequences from different hosts obtained from GenBank. We deposited in GenBank the representative sequence (DFHS1It) under the accession number OQ345680.

The analysis of the entire dataset, counting a total of 18 sequences, revealed strong conservation within the *SSU rRNA* fragment. BLAST analyses of DFHS1It sequence indicated 100% of identity (748/748 bp; 100% query coverage) with *D. fragilis* Acc. No. JQ677149 *Homo sapiens* isolate from Italy, and with isolates described from other countries (Genbank Acc. No. MN560149–50 from Turkey; JQ677147–48 from UK; AB692771–72 from Iran; FJ649228 from Australia; OP375693 from Czech Republic), all from humans and described as genotype 1. The same 100% identity was also encountered with isolate DF10 from Turkey (OM250406), with a 96% of query coverage and, noteworthy, with a *D. fragilis* isolate from cattle (Acc. No. ON242172) from Turkey with a query coverage of 99%.

Two nucleotide variations (an insertion of a single G at position 629 and a substitution C > T at position 696) differentiated our isolates from the reference sequence Acc. No. AY730405 derived from a symptomatic Australian patient (99.73% of similarity). A 99.87% of similarity, given by a gap in position 1049, was observed with the sequence Acc. No. MN914083, isolated from human faeces, Germany. Differences were also detected comparing our sequence with several isolates from humans from Czech Republic (Acc. No. OP375682, OP375683, OP375691), showing values of similarity from 99.73% to 99.87%.

Finally, along the amplified fragment, BLAST analyses of our new sequence indicated 95.29% of identity (100% query coverage) with the *D. fragilis* strain Bi/PA (ATCC 30948) Acc. No. U37461, identified as genotype 2. This was given by several nucleotide substitutions and two insertions (one of them involving 15 nucleotides) that allow for an easy differentiation between genotype 1 and genotype 2.

3.2. Phylogenetic analysis

Phylogenetic analysis was performed to generate a maximum likelihood tree using partial-length *SSU rRNA* gene sequences: one generated from this study and representative for 14 identical isolates, 17 references sequences retrieved from Genbank and 8 sequences from various trichomonads, included as close taxa and used as out-groups (Fig. 1). Since the 17 nucleotide sequences used in this study differ in length, the dataset was trimmed at positions 475 to 1198 of the reference sequence AY730405 to maximize the overlap between our and *D. fragilis* sequences retrieved from GenBank. This produced valid alignments of 723 positions for all the sequences in the final dataset. Our isolate(s) were unambiguously assigned to genotype 1, being included within a clade together with other genotype 1 isolates from humans and animals from different countries. This clade is clearly separated as a distinct branch, supported by a bootstrap proportion of 96 from the isolate Acc. No. U37461, the only currently available in the GenBank identified as genotype 2.

The related species included in the analysis (*H. meleagridis*, *T. vaginalis*, *T. nonconforma*, *T. foetus*) were identified as separated branches.

In Table 3 data regarding *D. fragilis* occurrence and genotype in this and in previous relevant studies (2010−2023) from different areas of Italy are shown.

Table 2
Gender, percentage of infection and age of the patients positive for *D. fragilis*.

Gender	N positive	% positive	Age (mean)	Age (median)
M	27	34.2	26.1	18
F	46	58.2	30.3	22
NA	6	7.6	–	–
tot.	79	–	28.8	21

4. Discussion

According to a recent classification scheme, the genus *Dientamoeba* Jepps & Dobell, 1918 falls within the Supergroups Excavata, in the clade of the Metamonada, phylum Parabasalia [26]. Several parabasalids are important parasites of both animals and humans, e.g. *T. vaginalis*, *T. foetus* and *H. meleagridis* [27]. Within the genus *Dientamoeba* the only species *fragilis* is recognized so far.

There is now strong evidence that *D. fragilis* represents likely one of the most prevalent protozoan parasites infecting humans [2,18]. Despite this, it is often described as a "neglected parasite", given that several aspects of its biology, such as an in-depth picture of the transmission pathways, remain controversial [9].

Overall, epidemiological data on dientamoebiasis are still scarce, and an underestimation of the real prevalence rates of *D. fragilis* is feasible due to the difficulties in its detection and identification. The trophozoite forms degenerate quickly, and the morphological characteristics are rapidly lost if the peculiar techniques used for its identification, e.g. a prompt fixation followed by permanent staining of the specimen, are not performed [2]. However, the sensitivity of microscopy remains low. In recent years, laboratory diagnosis of *D. fragilis* has been gradually replaced by more sensitive and specific tests, such as those based on conventional and real-time polymerase chain reaction (RT-PCR), although these tools are not employed routinely by most of the diagnostic centres [9]. It makes difficult to compare prevalence data available in literature, being them produced through different diagnostic approaches, and it also does not allow a proper understanding of dientamoebiasis epidemiology.

The implementation of the conventional methods with other diagnostic techniques could significantly improves the rates of detection. Syndromic molecular testing provides several advantages, including the ability to simultaneously detect numerous pathogens when clinical signs are ambiguous in a fast turnaround time that may be crucial in determining hospital admission and infection control.

The Allplex™ multiplex RT-PCR applied here allowed the detection of 79 *D. fragilis* positive samples, with a percentage of infection of 9.1%. Overall, prevalence data in humans presented in other studies from Italy showed inconsistent values ranging from 1.68% [21] up to 21.4% [20]. Interestingly, the only study conducted on animals in Italy showed high prevalence values in pigs of 46.7%. About the age distribution, an unambiguous trend does not seem to be supported yet, since various studies reported different data. A higher detection of *D. fragilis* is often observed in children and young people, e.g. as reported by Fletcher et al. [28] in Sydney, Australia, similarly to our data (median age 21 years) and by Aykur et al. [29] in a comprehensive study in patients with

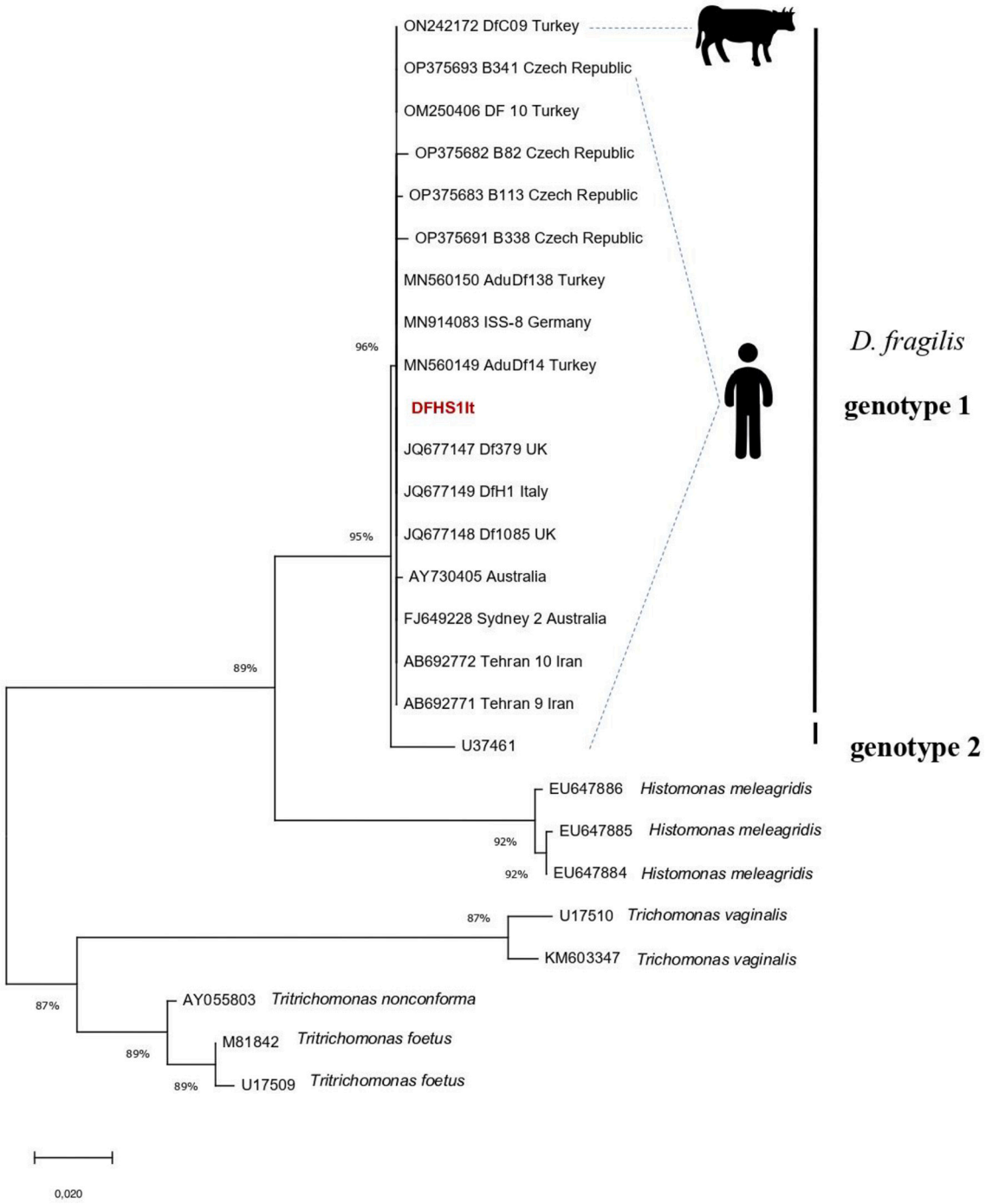

Fig. 1. Phylogenetic tree based on *D. fragilis SSU rRNA* gene partial sequences generated in the present study (represented in red and in bold) and selected reference sequences retrieved from GenBank. Analysis was conducted by a maximum likelihood method (ML). Genetic distances were calculated using the Tamura 3-parameter model + G. Numbers on the tree nodes indicate bootstrap values >80%. Accession numbers of sequences retrieved from GenBank are indicated together with isolate code and country. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
D. fragilis presence and genotype from different regions of Italy (2010–2023).

Region	Host	Diagnostic Tool	Infection rate % (Positive/N sample)	Genotype	References
Latium (Central Italy)	Human	Real-time PCR	9.1 (79/864)	genotype 1	Present Study
Emilia Romagna (Northeastern Italy)	Human	Microscopy, culture, real-time PCR	21.4 (105/491)	n.d.	[20]
Umbria, Marche (Central Italy)	Pig Human	Microscopy, real-time PCR	46.7 (71/152) 19.0 (4/21)	genotype 1	[10]
Emilia Romagna (Northeastern Italy)	Human	Microscopy, real-time PCR	1.68 (149/8886)	n.d.	[21]
Friuli-Venezia Giulia (Northeastern Italy)	Human	Microscopy, real-time PCR	14.8 (85/575)	n.d.	[23]
Emilia Romagna (Northeastern Italy)	Human	Microscopy, culture, real-time PCR	3.7 (606/16,275)	n.d.	[18]

gastrointestinal complaints in Turkey where *D. fragilis* positivity was found more frequently especially in primary school/secondary school and high school graduates. However, these results are in contrast with other studies, [17] also from Italy, where the highest prevalences of *D. fragilis* in adults were observed [23,30].

It is possible to presume that, as mentioned above, the differences between the diagnostic methodologies used, such as the diverse characteristics of the patients analyzed in the studies conducted to date in Italy, do not yet allow to have a true picture of the real diffusion of *D. fragilis* in our country.

On the other hand, the association of *D. fragilis* with *Blastocystis,* one of the most commonly detected protists in human clinical samples [31], appears to be consistent, as previously described by several authors [18,23,32]. The use of the Allplex™ multiplex panel allowed to easily reveal co-infections in our cohort of patients, highlighting the highest combination with *Blastocystis* (27.8%). Recently, Jirků et al. [12] observed that 46% of the samples showed co-infection in gut-healthy volunteers. The present study was limited by the fact that no healthy individuals were tested, which precluded any speculation on the effects related to the simultaneous presence of *D. fragilis* and *Blastocystis* in human. However, identifying the co-occurrence between different parasitic species harboring the human intestine represents an intriguing challenge in order to better deciphering the relationship among microorganisms, health and disease.

From the molecular point of view, the 14 new isolates attributable to *D. fragilis* from Italian patients analyzed here were characterized as genotype 1 on the basis of phylogenetic analysis. These findings, along with those previously described by Cacciò et al. [10], are the only available data regarding the genetic diversity of *D. fragilis* in Italy.

As expected, a low level of polymorphism was observed among isolates from our patients and from other human samples with different geographical origin. A remarkably minimal variability among isolates identified as genotype 1 has been also observed by using a multilocus sequence typing of *D. fragilis* by Cacciò et al. [33] suggesting that the population structure of *D. fragilis* is clonal. However, the recent study conducted by Jirků et al. [12] allowed to identify three additional supported subgroups, including several human samples.

As for animals, *D. fragilis* isolates from cattle in Turkey by Yildiz et al. [14] showed 100% identity with our samples. The fact that the same genotype 1 was also collected from domestic pigs and farmers [10], suggests the idea of a putative zoonotic transmission of the parasite.

Lastly, in the phylogenetic analysis, *D. fragilis* is robustly differentiated from all the other species included in the comparison, confirming through the topology of the tree the closer relationship between *D. fragilis* and *H. meleagridis* with respect to other parabasalids, as proposed by the recent parabasalia taxonomic revision based on molecular phylogeny [34].

In this study some limitations are present. The first, as reported above, is that no asymptomatic patients were enrolled. The second is that care should be taken when comparing parasitological results related to samples tested during the COVID-19 pandemic period in 2020–2022, especially in country (such as Italy) where severe restrictions were applied. This might lead to a bias due to the reduction of subjects attending laboratories, and a possible change in the characteristics of the patient cohort analyzed.

5. Conclusions and perspectives

The present study adds new data on the presence and molecular characterization of *D. fragilis* from Italian symptomatic patients. Some crucial aspects can be highlighted through the analysis of the results. First, the need to use more reliable molecular tools to better describe the real occurrence of this parasite. The use of PCR techniques enables a rapid identification of *D. fragilis* and represents a valid diagnostic alternative to traditional methods such as microscopy. Moreover, they could help in overcoming the issues related to the controversial prevalence rates and the epidemiological knowledge of dientamoebiasis. Secondly, the use of multiplex PCR panels is necessary to uncover possible co-infections with other intestinal pathogens and therefore to evaluate their role in the human gut health. Finally, the importance of genotyping *D. fragilis* isolates is strongly emphasized. There is a clear need for large-scale human and animal studies and for additional informative markers to better explore the genetic diversity of *D. fragilis* at population level. This represents an essential requisite to improve our understanding on host range and zoonotic transmission of this little-known gastrointestinal protozoan.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

The authors declare that no conflict of interest exists.

Data availability

All data are within the paper and its Supporting Information files. All DNA sequences reported here are deposited in the GenBank.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2023.102816.

References

[1] R.S. van Gestel, J.G. Kusters, J.F. Monkelbaan, A clinical guideline on *Dientamoeba fragilis* infections, Parasitology. 146 (2019) 1131–1139, https://doi.org/10.1017/S0031182018001385.
[2] L.S. Garcia, *Dientamoeba fragilis*, one of the neglected intestinal protozoa, J. Clin. Microbiol. 54 (2016) 2243–2250, https://doi.org/10.1128/JCM.00400-16.
[3] J.L. Barratt, J. Harkness, D. Marriott, J.T. Ellis, D. Stark, A review of *Dientamoeba fragilis* carriage in humans: several reasons why this organism should be considered in the diagnosis of gastrointestinal illness, Gut Microbes 2 (2011) 3–12, https://doi.org/10.4161/gmic.2.1.14755.
[4] J. Ögren, O. Dienus, S. Löfgren, P. Iveroth, A. Matussek, *Dientamoeba fragilis* DNA detection in *Enterobius vermicularis* eggs, Pathog. Dis. 69 (2013) 57–58, https://doi.org/10.1111/2049-632X.12071.
[5] C.G. Clark, D. Röser, C.R. Stensvold, Transmission of *Dientamoeba fragilis*: pinworm or cysts? Trends Parasitol. 30 (2014) 136–140, https://doi.org/10.1016/j.pt.2014.01.005.
[6] D. Stark, J. Barratt, T. Roberts, D. Marriott, J. Harkness, J. Ellis, Comparison of microscopy, two xenic culture techniques, conventional and real-time PCR for the detection of *Dientamoeba fragilis* in clinical stool samples, Eur. J. Clin. Microbiol. Infect. Dis. 29 (2010) 411–416, https://doi.org/10.1007/s10096-010-0876-4.
[7] V.S. Munasinghe, N.G. Vella, J.T. Ellis, P.A. Windsor, D. Stark, Cyst formation and faecal-oral transmission of *Dientamoeba fragilis*-the missing link in the life cycle of an emerging pathogen, Int. J. Parasitol. 4 (2013) 879–883, https://doi.org/10.1016/j.ijpara.2013.06.003.
[8] D. Stark, L.S. Garcia, J.L. Barratt, O. Phillips, T. Roberts, D. Marriott, J. Harkness, J. T. Ellis, Description of *Dientamoeba fragilis* cyst and precystic forms from human samples, J. Clin. Microbiol. 52 (2014) 2680–2683, https://doi.org/10.1128/JCM.00813-14.
[9] D. Stark, J. Barratt, D. Chan, J.T. Ellis, *Dientamoeba fragilis*, the neglected trichomonad of the human bowel, Clin. Microbiol. Rev. 29 (2016) 553–580, https://doi.org/10.1128/CMR.00076-15.
[10] S.M. Cacciò, A.R. Sannella, E. Manuali, F. Tosini, M. Sensi, D. Crotti, E. Pozio, Pigs as natural hosts of *Dientamoeba fragilis* genotypes found in humans, Emerg. Infect. Dis. 18 (2012) 838–841, https://doi.org/10.3201/eid1805.111093.
[11] D. Chan, J. Barratt, T. Roberts, O. Phillips, J. Šlapeta, U. Ryan, D. Marriott, J. Harkness, J. Ellis, D. Stark, Detection of *Dientamoeba fragilis* in animal faeces using species specific real time PCR assay, Vet. Parasitol. 227 (2016) 42–47, https://doi.org/10.1016/j.vetpar.2016.07.025.
[12] M. Jirků, A. Kašparová, Z. Lhotská, M. Oborník, K. Brožová, K.J. Petrželková, P. Samaš, O. Kadlecová, C.R. Stensvold, K. Jirků, A cross-sectional study on the occurrence of the intestinal protist, *Dientamoeba fragilis*, in the gut-healthy volunteers and their animals, Int. J. Mol. Sci. 23 (2022) 15407, https://doi.org/10.3390/ijms232315407.
[13] G. Yetismis, A. Yildirim, D. Pekmezci, O. Duzlu, A. Ciloglu, Z. Onder, E. Simsek, N. Ercan, G.Z. Pekmezci, A. Inci, First report and genotyping of *Dientamoeba fragilis*

in pet budgerigars (*Melopsittacus undulatus*), with zoonotic importance, Zoonoses Public Health 69 (2022) 572–578, https://doi.org/10.1111/zph.12949.
[14] I. Yildiz, Z.E. Aynur, First detection and molecular characterization of *Dientamoeba fragilis* in cattle, Zoonoses Public Health 69 (2022) 897–903, https://doi.org/10.1111/zph.12986.
[15] J.A. Johnson, C.G. Clark, Cryptic genetic diversity in *Dientamoeba fragilis*, J. Clin. Microbiol. 38 (2000) 4653–4654, https://doi.org/10.1128/jcm.38.12.4653-4654.2000.
[16] J.J. Windsor, C.G. Clark, L. Macfarlane, Molecular typing of *Dientamoeba fragilis*, Br. J. Biomed. Sci. 6 (2004) 153–155, https://doi.org/10.1080/09674845.2004.11978138.
[17] D. Stark, N. Beebe, D. Marriott, J. Ellis, J. Harkness, Prospective study of the prevalence, genotyping, and clinical relevance *of Dientamoeba fragilis* infections in an Australian population, J. Clin. Microbiol. 43 (2005) 2718–2723, https://doi.org/10.1128/jcm.43.6.2718-2723.2005.
[18] A. Calderaro, M. Buttrini, S. Montecchini, S. Rossi, B. Farina, M.C. Arcangeletti, F. De Conto, C. Chezzi, Prevalence of intestinal Parasitoses in a non-endemic setting during a 10-year period (2011−2020): a focus on *Dientamoeba fragilis*, Microorganisms. 10 (2022) 426, https://doi.org/10.3390/microorganisms10020426.
[19] J.J. Verweij, B. Mulder, B. Poell, D. van Middelkoop, E.A. Brienen, L. van Lieshout, Real-time PCR for the detection of *Dientamoeba fragilis* in fecal samples, Mol. Cell. Probes 21 (2007) 400–404, https://doi.org/10.1016/j.mcp.2007.05.006.
[20] A. Calderaro, C. Gorrini, S. Montecchini, S. Peruzzi, G. Piccolo, S. Rossi, F. Gargiulo, N. Manca, G. Dettori, C. Chezzi, Evaluation of a real-time polymerase chain reaction assay for the detection of *Dientamoeba fragilis*, Diagn. Microbiol. Infect. Dis. 67 (2010) 239–245, https://doi.org/10.1016/j.diagmicrobio.2010.02.016.
[21] A. Calderaro, S. Montecchini, S. Rossi, C. Gorrini, F. De Conto, M.C. Medici, C. Chezzi, M.C. Arcangeletti, Intestinal parasitoses in a tertiary-care hospital located in a non-endemic setting during 2006–2010, BMC Infect. Dis. 16 (14) (2014) 264, https://doi.org/10.1186/1471-2334-14-264.
[22] C. Guidetti, L. Ricci, L. Vecchia, Prevalenza delle parassitosi intestinali a Reggio Emilia e provincia nel corso del 2009 [Prevalence of intestinal parasitosis in Reggio Emilia (Italy) during 2009], Infez. Med. 18 (2010) 154–161 (Italian).
[23] L. Clemente, M. Pasut, R. Carlet, M. Ruscio, F. Fontana, *Dientamoeba fragilis* in the north-east of Italy: prevalence study and treatment, Parasitol. Int. 80 (2021), 102227, https://doi.org/10.1016/j.parint.2020.102227.
[24] D. Stark, N. Beebe, D. Marriott, J.T. Ellis, J. Harkness, Detection of *Dientamoeba fragilis* in fresh stool specimens using PCR, Int. J. Parasitol. 35 (2005) 57–62, https://doi.org/10.1016/j.ijpara.2004.09.003.
[25] S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura, Molecular evolutionary genetics analysis across computing platforms, Mol. Biol. Evol. 1 (2018) 1547–1549, https://doi.org/10.1093/molbev/msy096.
[26] S.M. Adl, D. Bass, C.E. Lane, J. Lukeš, C.L. Schoch, A. Smirnov, S. Agatha, C. Berney, M.W. Brown, F. Burki, P. Cárdenas, I. Čepička, L. Chistyakova, J. Del Campo, M. Dunthorn, B. Edvardsen, Y. Eglit, L. Guillou, V. Hampl, A.A. Heiss, M. Hoppenrath, T.Y. James, A. Karnkowska, S. Karpov, E. Kim, M. Kolisko, A. Kudryavtsev, D.J.G. Lahr, E. Lara, L. Le Gall, D.H. Lynn, D.G. Mann, R. Massana, E.A.D. Mitchell, C. Morrow, J.S. Park, J.W. Pawlowski, M.J. Powell, D.J. Richter, S. Rueckert, L. Shadwick, S. Shimano, F.W. Spiegel, G. Torruella, N. Youssef, V. Zlatogursky, Q. Zhang, Revisions to the classification, nomenclature, and diversity of eukaryotes, J. Eukaryot. Microbiol. 66 (2019) 4–119, https://doi.org/10.1111/jeu.12691.
[27] I. Cepicka, V. Hampl, J. Kulda, Critical taxonomic revision of parabasalids with description of one new genus and three new species, Protist. 161 (2010) 400–433, https://doi.org/10.1016/j.protis.2009.11.005.
[28] S. Fletcher, G. Caprarelli, J. Merif, D. Andresen, S.V. Hal, D. Stark, J. Ellis, Epidemiology and geographical distribution of enteric protozoan infections in Sydney, Australia, J. Public Health Res. 25 (2014) 298, https://doi.org/10.4081/jphr.2014.29.
[29] M. Aykur, C. Calıskan Kurt, D. Dirim Erdogan, C. Biray Avcı, R. Vardar, S. Aydemir, N. Girginkardeşler, C. Gündüz, H. Dagci, Investigation of *Dientamoeba fragilis* prevalence and evaluation of sociodemographic and clinical features in patients with gastrointestinal symptoms, Acta Parasitol. 64 (2019) 162–170, https://doi.org/10.2478/s11686-018-00017-5.
[30] D. Crotti, M.L. D'Annibale, G. Fonzo, M. Lalle, S.M. Cacciò, E. Pozio, *Dientamoeba fragilis* is more prevalent than *Giardia duodenalis* in children and adults attending a day care Centre in Central Italy, Parasite. 12 (2005) 165–170, https://doi.org/10.1051/parasite/2005122165.
[31] C.R. Stensvold, C.G. Clark, Current status of *Blastocystis*: a personal view, Parasitol. Int. 65 (2016) 763–771, https://doi.org/10.1016/j.parint.2016.05.015.
[32] F. Sarzhanov, F. Dogruman-Al, M. Santin, J.G. Maloney, A.S. Gureser, D. Karasartova, A. Taylan-Ozkan, Investigation of neglected protists *Blastocystis* sp. and *Dientamoeba fragilis* in immunocompetent and immunodeficient diarrheal patients using both conventional and molecular methods, PLoS Negl. Trop. Dis. 15 (10) (2021) e0009779, https://doi.org/10.1371/journal.pntd.0009779.
[33] S.M. Cacciò, A.R. Sannella, A. Bruno, C.R. Stensvold, E.B. David, S. Guimarães, E. Manuali, C. Magistrali, K. Mahdad, M. Beaman, R. Maserati, F. Tosini, E. Pozio, Multilocus sequence typing of *Dientamoeba fragilis* identified a major clone with widespread geographical distribution, Int. J. Parasitol. 46 (2016) 793–798, https://doi.org/10.1016/j.ijpara.2016.07.002.
[34] S. Noda, C. Mantini, D. Meloni, J. Inoue, O. Kitade, E. Viscogliosi, M. Ohkuma, Molecular phylogeny and evolution of parabasalia with improved taxon sampling and new protein markers of actin and elongation factor-1α, PLoS One 7 (2012), e29938 https://doi.org/10.1371/journal.pone.0029938.