First detection and molecular characterization of *Dientamoeba fragilis* in cattle

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Abstract

*Dientamoeba fragilis* is a flagellated protozoan with amoeba-like morphology that inhabits the human gastrointestinal tract. It is endemic in a vast geography around the world, including developed countries. There are limited studies on non-human hosts of the parasite, and suitable hosts have not been clarified. The parasite has been detected in non-human primates, pigs, cats, dogs and rats. There is no study in the literature investigating and detecting the presence of this parasite in cattle. In this study, stool samples taken from 163 different cattle and calves from 11 different farms between March 2017 and May 2022 were examined for the detection of *D. fragilis* via PCR. Trichrome staining was performed on all PCR-positive samples. The isolates with the expected amplicon size were sequenced using the 18S ribosomal RNA region, and their genotypes were determined by BLAST analysis. Sequences were analysed with the most similar and reference sequences in the literature, forming a phylogenetic tree. We detected *D. fragilis* in 31 (19.01%) of the 163 stool samples. *D. fragilis* cysts/trophozoites were detected by trichrome staining method in six of 31 samples. All PCR products selected for molecular analysis from positive samples had the same nucleotide sequence. As a result of BLAST analysis, all sequences were determined to belong to *D. fragilis* genotype 1. This study determined for the first time that cattle are suitable hosts for *D. fragilis*. Furthermore, the parasite subtype we detected belongs to genotype 1, which is the most common type in humans, suggesting that the parasite may have a zoonotic character. Our result is important in terms of the epidemiology of the parasite, as the mode of transmission is controversial, and available data on its suitable hosts are limited.

**KEYWORDS**
cattle, *Dientamoeba fragilis*, epidemiology, genotyping, parasitology, PCR

1 | INTRODUCTION

*Dientamoeba fragilis* is a flagellate protozoan 5-15 μm in size, localized in the intestine and detected in stool samples frequently as the trophozoite form (Silberman et al., 1996; Stark et al., 2016; Windsor & Johnson, 1999). However, as the cyst form could not be detected for many years, the transmission route of *D. fragilis* was not clearly understood until the last decade. During this period, it was thought that the parasite was transmitted by trophozoites carried in nematodes eggs, such as *Enterobius vermicularis*, that can only settle in humans (Girginkardeşler et al., 2008). In addition, due to the failure of different animal models, *D. fragilis* was thought to be identifiable only in humans (Stark et al., 2005).

Many studies from different countries provide information about the infection caused by *D. fragilis* in humans. These studies also provide different information about the clinical picture of the parasite in humans. Although *D. fragilis* infection is generally asymptomatic in most of the cases, it is stated that non-specific gastrointestinal
findings such as loss of appetite, diarrhoea, constipation and nausea-vomiting can be seen when symptomatic (Clemente et al., 2021; Girginkardeşler et al., 2003; Kurt et al., 2008; Sarzhanov et al., 2021; Sivcan et al., 2018; Stark et al., 2016; Yildiz et al., 2021). However, with the recent detection of the cyst form, it was thought that D. fragilis could also settle in non-human hosts, and studies aiming to detect the parasite in animals gained momentum (Cacciò et al., 2012; Chan et al., 2016; Munasinghe et al., 2013; Oggunniyi et al., 2014; Yetismis et al., 2022). Unfortunately, although a few previous studies report the occurrence of D. fragilis in animals, there are almost no studies in the literature in which molecular methods were used for confirmation (Chan et al., 2016; Yetismis et al., 2022).

In genotyping studies using different gene regions such as 18S rRNA, actin and elongation factor 1α gene, it has been reported that there are two different subtypes of the parasite and genotype 1 is more common than genotype 2 (Stensvold et al., 2013). However, there is a paucity of data in the literature concerning the genetic diversity in animals, except for a few studies (Cacciò et al., 2012; Yetismis et al., 2022). In the limited number of studies reporting genotyping of D. fragilis isolates in animals, genotype 1 was detected more frequently, similar to the findings in humans (Cacciò et al., 2012; Yetismis et al., 2022).

The trophozoite form of the parasite, which is frequently detected, is highly vulnerable to environmental conditions. Of note, it is not possible to identify the parasite in fresh stool preparations without utilizing permanent staining. Moreover, the specificity and sensitivity of staining methods in diagnosing D. fragilis are very low compared with molecular methods. For these reasons, molecular methods such as PCR and real-time PCR (RT-PCR) are preferred for the detection of D. fragilis in recent years and are accepted as the gold standard (Stark et al., 2008; Yildiz et al., 2021). Until now, D. fragilis has only been detected by molecular methods in cats, dogs, pet budgerigars, rodents and pigs (Cacciò et al., 2012; Chan et al., 2016; Oggunniyi et al., 2014; Yetismis et al., 2022). There is no study investigating the presence of D. fragilis in cattle. Therefore, this study aimed to investigate D. fragilis in cattle for the first time and genotype the detected parasites.

2 | MATERIALS AND METHODS

2.1 | Stool samples

Stool samples were obtained from 163 cattle and calves (102 were holstein and 61 were simmental) from 11 different farms located in the Aydin province of Turkey. Animals older than 6 months were considered as cattle and smaller ones as calf. Aydin is a city located on the south-western Mediterranean coast of Turkey. The samples were collected between March 2017 and May 2022 and taken only once from each animal under veterinary control. The identification numbers of the sampled animals were recorded to prevent more than one sample from being taken from an animal. The ages of the animals varied between 4 weeks and 4 years. Stool samples of cattle were taken from the floor immediately after defection, and there was no direct contact with the animals, and therefore, ethics committee approval was not required. Each stool sample was divided into two container. The first portion was taken directly into sterile stool containers for DNA isolation. The second part was taken in the sodium acetate–acetic acid-formalin (SAF) fixative (1:3 ratio) and reserved for the staining method. All samples were transported with cold chain shipment and reached our laboratory in perfect condition.

2.2 | Microscopic examination

The part of stool samples in which D. fragilis DNA was detected, taken into SAF fixative, was prepared to investigate the presence of trophozoites/cysts of the parasite. The preparations were stained according to the Wheatley’s trichrome staining method (Aldeen & Hale, 1992). The smears were examined with an immersion objective lens (1000x) for the presence of D. fragilis trophozoites or cysts.

2.3 | DNA extraction and PCR

Genomic DNA extraction was performed from 200mg stool sample using the Qiagen Stool Mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s recommendations. D. fragilis 18S rRNA gene was amplified with the following primers: DF400 (Forward: 5’-TATCAGGAGGGTAATGACC-3’) and DF1250 (Reverse: 5’-CATCTTCTCCTGCTTAGACG-3’) in a single PCR. Reaction’s setting was as follows: three minutes of denaturation held at 94°C, followed by 30 cycles (1 min at 94°C, 1.5 min at 57°C, 2 min at 72°C) and final elongation at 72°C for 10 min (Stark et al., 2005). PCR products were analysed by 1.5% agarose gel electrophoresis and then visualized with a UV gel imaging system (Vilber Lourmat Infinity-1000/20M).

2.4 | Sequencing and molecular characterization

Some positive PCR products were sent to Medsantek Co. (Istanbul, Turkey) for purification and sequence analysis. D. fragilis sequences obtained using the Basic local alignment search tool (BLAST) were

| Impacts |
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| - For the first time, cattle and calves have been shown to be suitable hosts for D. fragilis. |
| - As a result of the phylogenetic analysis, it was determined that the D. fragilis gene sequence that we detected in cattle was the same as that detected in humans. This indicates the potential for zoonotic transmission of D. fragilis. |
compared with existing sequences in Genbank (NIH genetic sequence database, http://www.ncbi.nlm.nih.gov/BLAST). As a result of the analysis, the subtypes of the sequences with total or highest similarity were determined. Genetic distances between isolates were examined according to the 18S rRNA gene region. With the obtained sequences, a phylogenetic tree based on genetic distance was drawn by using neighbour-joining method and bootstrap tests (1000 replicates) with use of the Molecular Evolutionary Genetics Analysis version 11.0 (MEGA) application. The evolutionary distances of the sequences were determined by the maximum composite likelihood method (Felsenstein, 1985; Tamura et al., 2004, 2021).

3 | RESULTS

Dientamoeba fragilis was detected in seven of 11 farms and not in four of them. In 31 of 163 isolates examined in our study, amplification was observed at the expected size (~863 bp), demonstrating D. fragilis positivity (Figure 1). D. fragilis trophozoites were detected in six of 31 PCR-positive samples by trichrome staining (Figure 2). Of the D. fragilis-positive samples, 26 consisted of calves less than 6 months old. The mean age of positive calves was 49 ± 11 days. The remaining five positives were obtained from adult cattle, and the mean age of the cattle was calculated separately and was determined as 26 ± 7.5 months. When evaluated according to their breeds, 22 of the positive cattle belonged to the Holstein and 9 belonged to the Simmental. Of the 31 positive PCR products, five were selected by simple random sampling (paying attention to the fact that each sample was obtained from different farms), and sequence analysis was performed. All five sequences analysed were 100% similar, and the obtained sequence was registered in GenBank (Reference code: Dfc09, Genbank Acc. No. ON242172). There is a single base difference between our sequence and the genotype 1 reference sequence (Genbank Acc. No. AY730405.1). As a result of the BLAST analysis, the two sequences showed 99.75% similarity. In addition, the strain we obtained was determined to be 100% similar to the D. fragilis strain obtained from pigs in Italy (Genbank Acc. No. JQ677148.1) and the D. fragilis strain obtained from human faeces in Iran (Genbank Acc. No. AB692772.1). There was a 95.04% similarity between the D. fragilis genotype 2 reference sequence (Genbank Acc. No. DFU37461) and ours. The alignment of the D. fragilis reference sequences with the sequences we detected using the MEGA program is shown in Figure 3.

It was determined that all five D. fragilis strains sequenced in line with our data belonged to genotype 1. The reference sequences, the most similar sequences with ours, and the D. fragilis isolate we obtained were evaluated together to form a phylogenetic tree (Figure 4).

4 | DISCUSSION

Dientamoeba fragilis is a protozoan that has been neglected in human and animal studies, even though it has been more than 100 years since its description. It is almost impossible to detect the parasite in direct microscopic examinations. Furthermore, detection in stained preparations is also not easy even when assessed by experienced laboratory workers (McHardy et al., 2014; Röser et al., 2013; Verweij et al., 2007). In studies comparing the specificities and sensitivities of different diagnostic methods in detecting D. fragilis, molecular methods seem much more sensitive than direct microscopy, stained preparation examination and culture methods (David et al., 2015; Sarafraz et al., 2013; Yıldız et al., 2021). In the present study, samples of which parasite DNA was detected by PCR were stained with trichrome and evaluated by microscopy in terms of the presence of D. fragilis cysts/trophozoites. D. fragilis trophozoites were detected in only six of 31 stool samples by microscopy. Similar to other studies comparing D. fragilis diagnostic methods, microscopy sensitivity was found to be low in our study (19.35%). However, molecular methods are still not widely used in diagnosing this parasite as D. fragilis is frequently overlooked and molecular methods have a high cost than microscopic examinations. Hence, the reported D. fragilis detection rates often do not reflect the real-world status.
Almost all molecular data regarding the detection rates and characteristics of *D. fragilis* are obtained from scientific studies in humans (Brujinesteijn van Coppenraet et al., 2009; Menéndez et al., 2019; Stark et al., 2014). Studies in animals are exceedingly rare even in comparison with human studies which are also insufficient. Thus, the zoonotic features of the parasite have not been clarified, and epidemiological data contain serious deficiencies. In addition, available data on the natural hosts of the parasite are minimal. Especially, after the initial imaging of the cyst form of the parasite in 2013, a consensus emerged that the transmission route of *D. fragilis* was faecal-oral. This issue has made it more important to investigate the possible natural hosts and zoonotic characteristics of the parasite (Chan et al., 2016; Munasinghe et al., 2013; Yetismis et al., 2022). In this context, although there are few studies investigating *D. fragilis* in animals, this issue has become one of the topics that have interested researchers in recent years. In the first quarter of the 21st century, several studies were conducted that examined the role of different animals in the transmission of the parasite, possible natural hosts and the elucidation of the zoonotic features of *D. fragilis*. In pioneering studies, various wild bird species and some domestic animals were examined, but the presence of *D. fragilis* was not detected (Ogunniyi et al., 2014; Stark et al., 2008). However, as the scopes of the studies have been expanded, successful results are obtained. In a large-scale study conducted in Australia, 420 faecal samples were collected.
from 37 different animal species including cats, dogs, horses, lions, tigers and zebras, and all samples were examined for the presence of *D. fragilis*. In this comprehensive research, in terms of animal species, the method has also been strengthened by using three different diagnostic methods (conventional PCR, real-time PCR and nested PCR methods). As a result, *D. fragilis* DNA was detected in only two of 420 samples (a cat and a dog). There are no cattle among the 37 animal species included in the study (Chan et al., 2016).

In a study examining gastrointestinal parasites in the howler monkey species (*Alouatta palliata aequatorialis*), 96 stool samples were examined, and *D. fragilis* was detected in three samples (Helenbrook et al., 2015). In another study investigating the ectoparasites and endoparasites of rats in Nigeria, 50 house rats were examined for *D. fragilis*, and positivity was found in two of the animals (Ogunniyi et al., 2014).

In the literature, only two studies were found in which genotyping of *D. fragilis* isolates detected in animals was performed. In one of these studies, 150 pet budgerigar faeces were examined for the presence of *D. fragilis* using real-time PCR and iron–haematoxylin staining methods. Similar to our study, the rate of parasites detected by the molecular method (21.33%) was significantly higher than the rate determined by the staining method (8.66%). In addition, it has
been reported that all of the isolates are genotype 1, as in all the limited number of studies on this subject, including ours (Yetismis et al., 2022).

A study similar to ours (due to the examination of livestock) reported the genotypes of *D. fragilis* detected. In a total of 152 pig faeces samples, the presence of *D. fragilis* using microscopy and molecular methods was investigated. A total of 71 samples were positive for *D. fragilis*, and it was reported that pigs could be one of the natural hosts of *D. fragilis*. In addition, one of their sequences (Genbank Acc. No. JQ677148.1) showed 100% similarity with ours (Genbank Acc. No. ON242172), and it was also reported as genotype 1 (Cacciò et al., 2012). However, our study differs from others in terms of detecting the presence of *D. fragilis* in cattle for the first time and determining the genotypes of the isolates. In addition, the fact that the sequence we obtained is the same as genotype 1, which is the most frequently detected subtype in humans, supports the idea that the parasite may exhibit zoonotic transmission from cattle. It has also been thought that cattle may be one of the suitable hosts for *D. fragilis*. In geographies where animal husbandry is common, diseases seen in these animals cause economic losses and threaten public health.

There are some limitations in our study. The first of these is the risk of contamination of the sampled animal faeces with the faeces of other cattle, as the samples are taken from the floor after defecation. The second of these is the lack of clinical evaluation of animals with parasites. In further studies on this subject, it is important to investigate the clinical picture caused by the parasite in these animals.

5 | CONCLUSION

It has been demonstrated for the first time in our study that cattle, one of the essential farm animals, is a suitable host for *D. fragilis*. These data are crucial to clarify the epidemiology of the parasite, as cattle are one of the most widely raised livestock worldwide and human interaction/exposure through this route is unquestionable. In addition, our data suggest that cattle may represent an important factor of the zoonotic transmission dynamics of the parasite, leading to a possible risk to public health. Therefore, to investigate the natural host range of *D. fragilis* and to better understand the possible zoonotic transmission mechanism, we believe that larger-scale studies with other farm animals and different domestic animals should be conducted.

AUTHOR CONTRIBUTIONS

İ.Y. wrote the main manuscript text, İ.Y prepared Figures 2 and 3 and Z.E.A prepared Figure 1. Z.E.A contributed to study design. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The results/data/figures in this manuscript have not been published elsewhere, nor are they under consideration by another publisher.

ETHICAL APPROVAL

Stool samples of cattle were taken from the floor immediately after natural defecation, and there was no direct contact with the animals, and therefore, ethics committee approval was not required.

CONSENT FOR PUBLICATION

All of the material is owned by the authors and/or no permissions are required.

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