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PARASITOLOGY

Dientamoeba fragilis, a Commensal in Children in Danish Day Care Centers

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ABSTRACT *Dientamoeba fragilis* is an intestinal protozoan of debated clinical significance. Here, we present cross-sectional and longitudinal observations on *D. fragilis* in children aged 0 to 6 years from a 1-year multi-day-care-center cohort study set in Copenhagen, Denmark. The inclusion period for the cohort was 2009 through 2012. Stool samples collected from the children were accompanied by questionnaires completed by the parents or guardians of the children. Using real-time PCR, *D. fragilis* was detected in the first stool sample from 97 of 142 (68.3%) children. We evaluated the associations between seven plausible risk factors (age, sex, having siblings, having domestic animals at home, having had infant colic, recent history of intake of antibiotics, and recent history of travel abroad) as well as six reported symptoms (lack of appetite, nausea, vomiting, abdominal pain, weight loss, and diarrhea) and testing positive for *D. fragilis*. The final multivariable model identified being >3 years old and having a history of recent travel abroad as risk factors for testing positive for *D. fragilis*. Moreover, univariable analyses indicated that having siblings was a risk factor. There was no statistical association between a recent history of gastrointestinal symptoms and testing positive for *D. fragilis*. Among the 108 children who were represented by ≥2 samples and thus included in the longitudinal analysis, 32 tested negative on the first sample and positive later, and the last sample from each of the 108 children was positive. The results are in support of *D. fragilis* being a common enteric commensal in this population.

KEYWORDS Denmark, dientamoebiasis, infants, preschoolers, risk factors, symptoms, toddlers

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Dientamoeba fragilis is an intestinal protozoan of debated clinical significance (1–7). In Denmark, *D. fragilis* appears to be common in the apparently healthy adult population (8); meanwhile, baseline data on the prevalence in apparently healthy children are lacking. *Dientamoeba fragilis* is commonly detected in stools from children tested for gastrointestinal pathogens, with a peak (ca. 70% of samples positive) in 7-year-olds (9). A randomized placebo-controlled clinical trial failed to show improved clinical outcome in *D. fragilis*-positive children treated with metronidazole compared with those treated with a placebo, and the observed eradicative effect of metronidazole was moderate and transient, and a spontaneous decrease in the proportion of children testing positive was observed in the placebo group (10).

We recently established an open cohort of children aged 0 to 6 years attending municipal day care centers in Copenhagen, Denmark, with a primary research focus on investigating enteroaggregative *Escherichia coli* over time (11–13). Using real-time PCR (14), DNA of *D. fragilis* was detected in the majority (516/688 [75.0%]) of the stool

TABLE 1 Prevalence of *Dientamoeba fragilis* in children attending day care centers in Copenhagen, Denmark, by selected plausible risk factors

Description	Total no.[a]	Positive		95% CI[b]	*P* value
		No.	%		
>3 years old	66	53	80.3	69.4–88.6	<0.01[c]
≤3 years old	72	41	56.9	45.3–68.0	
Male	78	51	65.4	54.4–75.3	0.42
Female	64	46	71.9	60.0–81.8	
At least one sibling	97	71	73.2	63.7–81.3	0.03[c]
No siblings	38	20	52.6	36.9–68.0	
Domestic animals at home	33	20	60.6	43.4–76.1	0.33
No domestic animals at home	103	72	69.9	60.5–78.2	
History of infant colic	14	9	64.3	37.6–85.6	0.76
No history of infant colic	126	86	68.3	59.8–75.9	
History of antibiotic intake or treatment[d]	28	19	67.9	49.1–83.1	0.99
No history of antibiotic intake or treatment[d]	109	74	67.9	58.7–76.1	
History of travel abroad[d]	27	22	81.5	63.6–92.9	0.09
No history of travel abroad[d]	112	72	64.3	55.1–72.8	
Total	142	97	68.3	60.3–75.6	

[a]Data for all risk factors were not available for all of the children.
[b]CI, confidence interval (MidP Exact) of the prevalence.
[c]Statistically significant difference (MidP Exact, two-tailed $P < 0.05$).
[d]History from the previous 2 months.

samples collected during the study (11). The present study was designed to investigate this observation in greater detail.

We aimed to estimate the prevalence of *D. fragilis* in children aged 0 to 6 years attending day care centers in Copenhagen, Denmark. In addition, we report on longitudinal observations on repeated *D. fragilis* testing at the individual child level during the study period. Finally, we evaluated the associations between possible risk factors as well as reported symptoms and testing positive for *D. fragilis*.

RESULTS

Children and samples. A total of 142 children aged 0.9 to 6.6 (median, 2.8) years at the time of completing the first questionnaire were included in this study. Most questionnaires were completed on the day the stool sample was taken (range, 11 days before to 24 days after). The longitudinal analysis included *D. fragilis* results from testing a total of 449 stool samples from 108 of the children (2 to 7 *D. fragilis* test results per child). The advice to sample every second month for 1 year was not followed for all of the children.

Prevalence of *D. fragilis*. At the first observational point, 97 of the 142 children tested positive for *D. fragilis*, yielding an apparent prevalence estimate of 68.3% (95% confidence interval [CI], 60.3 to 75.6). Twenty-nine of the 142 children (20.4%; 95% CI, 14.39 to 27.65) tested highly positive.

The prevalence estimate (68.3%) did not differ statistically significantly ($P = 0.1$) from the proportion of positive samples among all the samples collected during the cohort study (75.0%) (10). In the subset included in the longitudinal analysis, the proportion of positive samples was 70.4% (76/108) among the first samples and 92.4% (415/449) among all samples (comparisons with the prevalence estimate 68.3%, $P = 0.7$ and $P < 0.001$, respectively).

Risk factors. The prevalence of *D. fragilis* was significantly higher in children >3 years of age than in children ≤3 years of age ($P < 0.01$) and in children who had at least one sibling than in children who had no siblings ($P < 0.05$) (Table 1).

The univariable analyses identified older age and siblings as statistically significant risk factors for testing positive for *D. fragilis* and for testing highly positive for *D. fragilis*. Children >3 years of age had 3.08 (95% CI, 1.43 to 6.63) times higher odds of testing positive and 10.37 (95% CI, 3.37 to 31.91) times higher odds of testing highly positive than did children ≤3 years of age. The odds of testing positive increased with age by

TABLE 2 Prevalence of *Dientamoeba fragilis* in children attending day care centers in Copenhagen, Denmark, by reported symptoms during the previous 2 months

Description	Total no.[a]	Positive		95% CI[b]	*P* value
		No.	%		
At least one symptom	74	47	63.5	52.1–73.9	0.25
No symptoms	66	48	72.7	61.1–82.4	
Lack of appetite	42	27	64.3	49.1–77.6	0.63
No lack of appetite	92	63	68.5	58.5–77.3	
Nausea	21	14	66.7	44.9–84.1	0.53
No nausea	87	64	73.6	63.6–82.0	
Vomiting	35	21	60.0	43.3–75.1	0.23
No vomiting	104	74	71.2	61.9–79.2	
Abdominal pain	24	16	66.7	46.4–83.2	0.77
No abdominal pain	76	53	69.7	58.7–79.3	
Weight loss	13	9	69.2	41.3–89.4	0.97
No weight loss	114	79	69.3	60.4–77.2	
Diarrhea	34	19	55.9	39.1–71.8	0.08
No diarrhea	102	74	72.5	63.3–80.5	
Total	142	97	68.3	60.3–75.6	

[a]Data for all symptoms were not available for all of the children.
[b]CI, confidence interval (MidP Exact) of the prevalence.

a factor of 1.66 (95% CI, 1.23 to 2.25) for each year of age, and the odds of testing highly positive more than doubled (2.37; 95% CI, 1.67 to 3.37) for each year of age. A child who had at least one sibling had 2.46 (95% CI, 1.13 to 5.36) times higher odds of testing positive and 3.84 (95% CI, 1.08 to 13.60) times higher odds of testing highly positive than an only child. The univariable model using the number of siblings as a continuous variable suggested that the odds of testing positive would double (2.02; 95% CI, 1.14 to 3.58) for each additional sibling and that the odds of testing highly positive would increase by a factor of 1.87 (95% CI, 1.06 to 3.31) for each additional sibling.

Age was a significant factor and acted as a confounder in both final multivariable models. The final multivariable logistic regression model for testing positive for *D. fragilis* had two risk factors: being >3 years old and having a history of traveling abroad during the previous 2 months, with odds ratios of 3.49 (95% CI, 1.59 to 7.70) and 3.15 (95% CI, 1.06 to 9.36), respectively. The area under the receiver operating characteristic (ROC) curve was 0.67, indicating moderate prediction. The final multivariable logistic regression model for testing highly positive for *D. fragilis* had the same two risk factors: being >3 years old and having a history of traveling abroad during the previous 2 months, with odds ratios of 16.00 (95% CI, 4.37 to 58.60) and 5.69 (95% CI, 1.62 to 19.96), respectively. The area under the ROC curve was 0.79, suggesting that the model had good predictive power.

Travel destinations were reported for 26 children: 19 children had traveled within Europe (Austria, Bulgaria, Germany, Greece, Norway, Spain, Sweden, and the United Kingdom), while seven had traveled outside Europe (Egypt, Israel, Tanzania, Thailand, and the United Arab Emirates). The most common destination was the neighboring country Sweden: 11 children had visited Sweden, and 10 of them tested positive. However, the *ad hoc* plausible risk factor "Sweden" did not prove statistically significant in any of the analyses.

Symptoms. The prevalence of *D. fragilis* at the first observational point did not differ statistically ($P = 0.25$) in reportedly symptomatic and asymptomatic children (Table 2). The proportions testing highly positive for *D. fragilis* were 17.6% in children who had had at least one symptom within the previous 2 months and 22.7% in reportedly asymptomatic children ($P = 0.46$).

None of the symptoms were associated with testing positive for *D. fragilis* or with testing highly positive for *D. fragilis* in the univariable analyses. For testing highly positive for *D. fragilis*, weight loss and age yielded an acceptable model; age was a confounder. The odds of testing highly positive for *D. fragilis* were higher if a child had

lost weight, with an odds ratio of 4.65 (95% CI, 1.06 to 20.36) for weight loss and an odds ratio of 10.74 (95% CI, 3.18 to 36.31) for the older age group, with an area under the ROC curve of 0.76. However, when considered for the final model, weight loss was omitted as nonsignificant.

Observations over time. Of the 449 stool samples from the 108 children represented by ≥2 samples each, 415 were positive for *D. fragilis* (92.4%; 95% CI, 89.7 to 94.6). The first sample was positive for 76 of the 108 children (70.4%; 95% CI, 61.3 to 78.4). Only positive results were recorded for 75 of the 108 children.

All 32 children who tested negative at the first observational point tested positive later. The ages of these 32 children ranged from 1.3 to 5.5 (median, 2.7) years at the time of testing positive. From one child, the two first samples, which—against the advice—had been taken within a single month, were both negative, and the third and fourth samples were positive. For all the others apparently acquiring *D. fragilis* during the study, the first samples were negative and subsequent samples were positive. Upon a positive result, all children, except for one, tested consistently *D. fragilis* positive on all consecutive samples. For that one child, the first and second samples were positive, the 3rd was negative, and the 4th and 5th samples were again positive. The last samples from all of the 108 children were positive.

DISCUSSION

To our knowledge, this is the first study to present not only cross-sectional but also longitudinal observations on *D. fragilis* in children at the individual child level. The results of this study support a commensal nature for *D. fragilis* in this population.

The prevalence of *D. fragilis* in the children, who were all attending day care centers, was 68.3%. The result supports the previous observations of *D. fragilis* being common in Denmark (8, 9) but might not represent the situation for children who do not attend day care centers or for children who live outside the urban environment of the capital city. In the Netherlands, *D. fragilis* was a common finding in children attending day care centers (3); however, attending a day care center was not associated with testing positive for *D. fragilis*, while living in rural areas was a risk factor (5).

Among the children aged 0 to 6 years, all the analyses supported older age as a significant risk factor for testing positive, and highly positive, for *D. fragilis*. Moreover, this study illustrates that *D. fragilis* is commonly acquired at an early age. These results are in line with the results of the recent Dutch studies (3, 5) and with the observed peak in the positive proportion in clinical samples at the age of seven in Denmark (9).

The final multivariable models identified having a history of recent travel abroad as a risk factor for testing positive for *D. fragilis* as well as for testing highly positive for *D. fragilis*. The reasons for this remain unknown. Four of five children with histories of traveling abroad tested positive, and 10 of 11 children who had been to Sweden tested positive, but it should be noted that the prevalence was >60% in children with no travel history and that these differences were not statistically significant.

Univariable analyses indicated that having siblings was a risk factor for testing positive for *D. fragilis* and for testing highly positive for *D. fragilis*. Because few children had several siblings, the increase in odds of testing positive and highly positive with number of siblings should be interpreted cautiously. However, similar results were obtained in the recent Dutch study (5). The epidemiological role of siblings remains unknown, as the transmission routes of *D. fragilis* need clarification (15). In particular, the transmission has been proposed to be linked to the pinworm (*Enterobius vermicularis*) (15). The stool samples collected during the cohort study were investigated for a selection of parasites, bacteria, and viruses (11). No pinworm eggs were detected, but the method used, microscopy of stool samples, lacks sensitivity with regard to the detection of pinworm eggs, and thus the negative result should be interpreted with caution. Moreover, information on possible pinworm infections among the family members of the study subjects and other children attending the day care centers was not available.

Dientamoeba fragilis may have zoonotic potential (16), and we included the ques-

tion about having domestic animals at home to the risk factor analyses. In this study, having domestic animals at home did not appear as a risk factor for testing positive for *D. fragilis*. This result did not change when including only animal species suggested as potential hosts (data not shown).

The use of antibiotics appeared to be common in the children (Table 1), and associations between the intake of antimicrobials and testing positive for *D. fragilis* have been described (17). However, in this study, no association was evident between a recent history of reported intake of antibiotics and testing positive for *D. fragilis*. This study relied on questionnaire-derived information and recall bias was possible.

The clinical significance of *D. fragilis* has been both emphasized and questioned (1–7, 10). Our results support the view of commensal colonization. We detected no statistical association between having a history of infant colic or a recent history of gastrointestinal symptoms reported in the questionnaire and testing positive for *D. fragilis* (Tables 1 and 2). However, the odds of testing highly positive for *D. fragilis* appeared higher in children who had reportedly lost weight. The symptoms were reported by the parents or guardians of the children, and the task was understandably challenging. Especially for the very young children, it may have been difficult to evaluate whether a symptom was present. Moreover, recall bias was possible and interobserver variance was likely.

Detecting DNA of *D. fragilis* in stool samples using real-time PCR is regarded as the gold standard diagnostic test. A linear relationship is assumed to exist between *D. fragilis* load and the cycle threshold (C_T) value. However, differences in the amount and genetic diversity of competing template might influence the ability to amplify DNA from *D. fragilis*, and the C_T values should be interpreted with some caution.

The questions regarding symptoms, travel abroad, and antibiotics only pertained to the previous 2 months, whereas any preceding history of having those same risk factors remains unknown; some children regarded as not having a given risk factor may have had it earlier. Moreover, it is unknown when the children who tested *D. fragilis* positive at the first observational point had acquired it. The follow-up period in the cohort study was 1 year, with a substantial loss to follow-up (12), and the *D. fragilis* status of the children after the last sample remains unknown.

The odds of testing positive increased with age. Moreover, among the 108 children represented by at least two samples, each of the 32 children who tested negative at the first sample tested positive later, while only a single negative result was recorded for one child who tested positive earlier and later on. The prevalence of *D. fragilis* at the first observational point among these children was similar to that in all of the children, suggesting the subset was representative. However, in the whole set of 449 samples collected from this subset, the proportion of positive samples was higher. It is possible that children who acquired *D. fragilis* during the study period were for unknown reasons more likely to remain in the study; however, the children also became older during the study, and older age was associated with testing positive.

These data do not allow for ruling out the possibility of repeated new infections, but it appears that a large proportion of children acquire *D. fragilis* efficiently at an early age and, when acquired, it appears to be a stable colonizer. In contrast to the clinical trial (10), apparent spontaneous clearance was not observed in this study, except for in one child for whom one negative sample was observed; however, this sample was followed by positive samples. A high infection pressure might explain this finding. Moreover, how the observations of this study relate to the development and overall establishment of the gut microbiota, including both eukaryotic and prokaryotic microorganisms, remains to be revealed.

The results of this study add to the knowledge on *D. fragilis* from both epidemiological and clinical points of view. Importantly, our results do not support the suggested pathogenic nature of *D. fragilis*. *Dientamoeba fragilis* was detected in the majority of the children who were healthy enough to attend day care centers in Denmark, both in those reported to have symptoms and in those reportedly asymptomatic. The prevalence increased with age, and for each of the children represented

by ≥2 samples, the last sample was positive. These observations should be taken into account in decisions on whether and when to test for *D. fragilis* as well as in the clinical assessment, management, and follow-up of children who are tested; *primum non nocere*.

MATERIALS AND METHODS

Ethics statement. The present study was a nested study of a larger cohort study. The cohort study was approved by The National Committee on Health Research Ethics (protocol number H-A-2008-111). Written informed consent was obtained from the parents or guardians of the children, and participation was voluntary. Data were handled confidentially and the results presented so that individual children cannot be identified.

Setting and study design. The open cohort study (11–13) was a multi-day-care-center study with limited geographical coverage and voluntary participation. It was set in 36 municipal day care centers located in Copenhagen, Denmark. The inclusion period was 2009 through 2012, and the follow-up period was 1 year with an observational point (stool sample and questionnaire) every second month (i.e., 6 stool samples and questionnaires per child in total).

The present study used the questionnaire data gathered from the first observational point (highest *n*, cross-sectional study design) and the *D. fragilis* real-time PCR results from the testing of stool samples collected at all observational points throughout the cohort study.

Inclusion criteria. We included the children for whom both questionnaire data and a *D. fragilis* real-time PCR result were available from the first observational point. Children for whom the questionnaire had been completed later than 1 month after stool sampling and children with mismatches between questionnaire-reported and laboratory-reported sampling dates were excluded.

Longitudinal data included subsequent *D. fragilis* real-time PCR results, C_T values of positive samples, and sampling dates for each observational point for the individual children. Data from samples with no sampling date and data from children represented by one sample only were omitted from the longitudinal analysis.

Samples. The stool samples were sent fresh and unpreserved by mail to the laboratory. Instructions were given not to send samples immediately prior to weekends or official holidays and that they could be stored in a refrigerator or freezer before sending (11). Upon arrival, the samples were frozen and stored at −80°C until processing. Since the cohort was dynamic in nature, the real-time PCR analyses were performed in several runs, and the duration of the storage varied, being up to 2 to 3 years.

DNA extraction and real-time PCR for detecting *D. fragilis*. From each sample, DNA was extracted from 200 mg of stool using the QIAmp DNA stool minikit (Qiagen, Hilden, Germany) as instructed by the manufacturer, with two modifications. After homogenization in stool lysis buffer, 200 mg sterile zirconia/silica beads (diameter 0.01 mm; BioSpec Products, Roth, Karlsruhe, Germany) were added for a mechanical disruption procedure of 6 min and 30 Hz in a TissueLyzer (Qiagen Retsch GmbH, Hannover, Germany), followed by lysis for 5 min at 95°C. Real-time PCR was used to detect *D. fragilis* (14), and the results are presented as positive or negative. For positive samples, C_T values were recorded. The controls included in each of the real-time PCR runs were a positive control (*D. fragilis* DNA), negative controls (one water sample from the DNA extraction step and another from the real-time PCR step), and inhibition controls.

Sample size. Using the open-source software OpenEpi (18), the sample size available was calculated to be acceptable for estimating the prevalence. The proportion of positive samples among all the samples collected during the cohort study, 75% (11), was used as the expected prevalence. With a confidence level of 80%, the minimum sample size was 124, but for a confidence level of 95%, 289 samples would be required. Possible clustering (by day care center or family) was ignored.

Estimation of *D. fragilis* prevalence. We calculated an estimate of the apparent prevalence of *D. fragilis* using the *D. fragilis* real-time PCR result of the first observational point of the cohort study for each child (cross-sectional study design). The confidence interval (95% CI) for the estimate was calculated using MidP Exact of OpenEpi (18).

Evaluation of associations with risk factors and reported symptoms. To evaluate the associations between potential risk factors as well as reported symptoms and *D. fragilis* real-time PCR results, we used the data from the questionnaires of the first observational point of the cohort study. The questionnaire answers "I do not know" and unanswered questions were regarded as "no data." Confidence intervals (MidP Exact) were calculated, and preliminary two-by-two-table comparisons were made using OpenEpi (18). We considered *P* values of < 0.05 as statistically significant. Multivariable logistic regression models were built using Stata 13.1 (StataCorp, College Station, TX, USA). All variables with a liberal *P* value of <0.2 in the univariable analysis were included in the model, followed by removal of the nonsignificant variables starting from the highest *P* value, provided they did not act as confounders. We also checked the variables for collinearity and interaction. The predictive power of the models was expressed as the area under the ROC curve.

The primary outcome was testing positive for *D. fragilis* at the first observational point. A secondary outcome was testing highly positive for *D. fragilis*, which was defined as testing positive for *D. fragilis* with a C_T value of ≤30. The lowest 25% of the recorded C_T values were below this selected threshold; hence, one quarter of the positive samples—reflecting those samples with the highest loads of *D. fragilis* DNA—were classified as highly positive.

We evaluated seven plausible dichotomous risk factor variables: age group (up to 3 years of age versus older age), sex (female versus male), having siblings (0 versus ≥1), having domestic animals at

home, having had infant colic, the intake of antibiotics during the previous 2 months, and having traveled abroad during the previous 2 months. Alternatively, age and having siblings were considered for the model as continuous variables (age in years and number of siblings). The symptoms reported for the previous 2 months that we evaluated were a lack of appetite, nausea, vomiting, abdominal pain, weight loss, and diarrhea. The symptoms were considered for the model both separately and as a combined variable; i.e., having no symptoms versus having at least one of the symptoms.

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The authors declare that they have no conflict of interest.

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