ST VINCENT'S HOSPITAL
SYDNEY

On the Importance of Validating *Dientamoeba fragilis* Real-Time PCR Assays

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Dientamoeba fragilis

- *D. fragilis* is a protozoan parasite
- worldwide distribution
- first discovered in 1909, described in 1918

Dobell C. Researches on the intestinal protozoa of monkeys and man. X. The life history of *Dientamoeba fragilis*: observations, experiments and speculations. *Parasitology* 1940;32:417-461.

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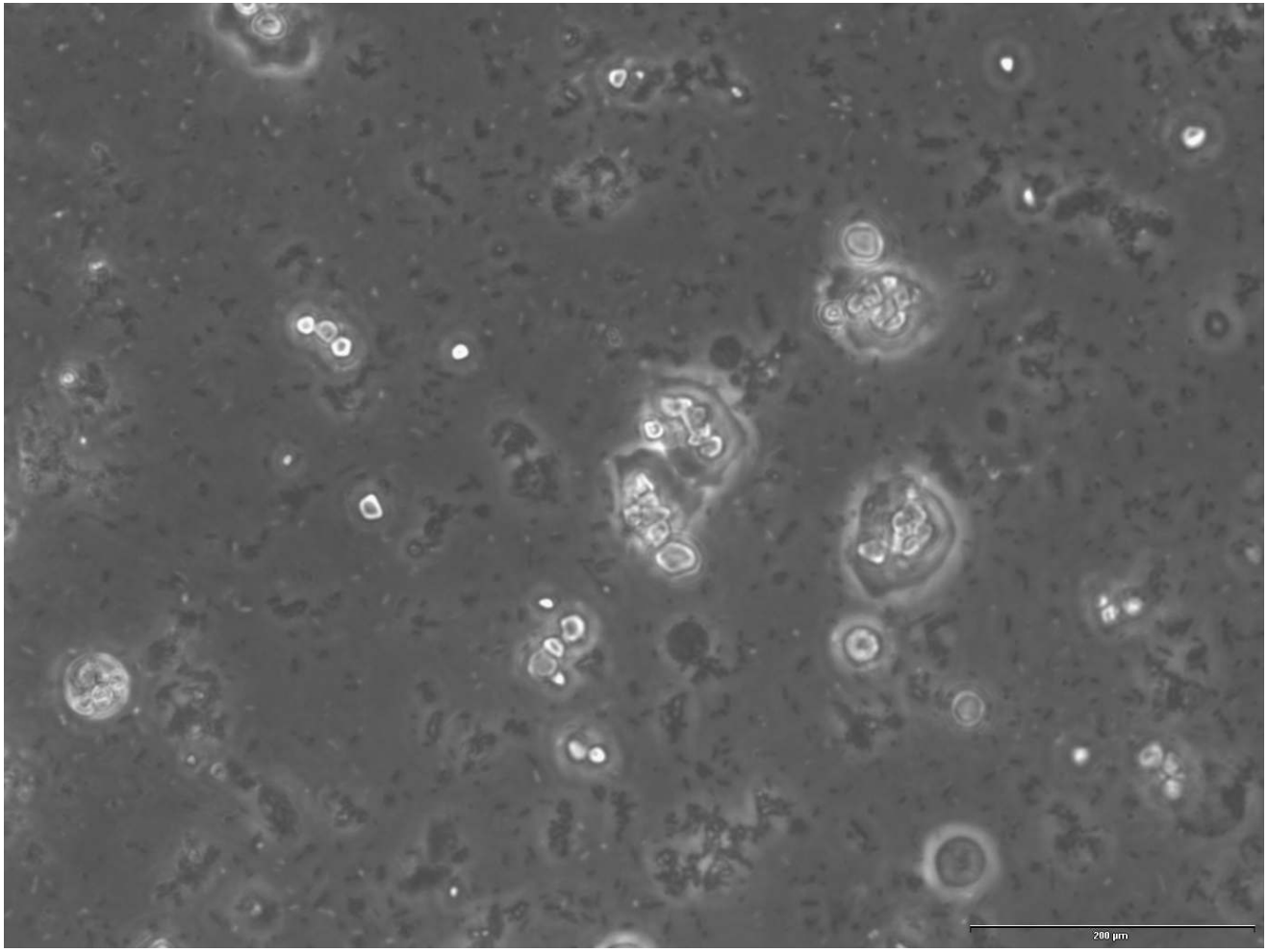
Dientamoeba



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The history of *D. fragilis*

- 1918 initial description by Jepps and Dobell ‘ a harmless commensal’
  – although several patients had GIT symptoms with no other cause found
- 1920’s - implicated as a cause of GIT disease
- sporadic ‘for’ and ‘against’ publications over the next 100 years
- pathogenicity still debated

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Dientamoeba a neglected parasite?

Parasite name	# DNA/cDNA Sequences in Genbank	# References in Pubmed
Entamoeba histolytica	35,543	7,626
Cryptosporidium hominis	38,226	8,510
Giardia intestinalis	26,278	8,180
Dientamoeba fragilis	336 (only 3 protein coding genes)	342

This is in spite of the fact that:

- *Dientamoeba* is more common than all of these bowel protozoa

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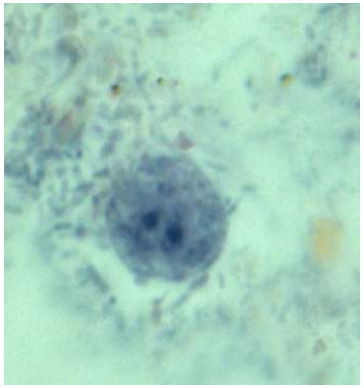
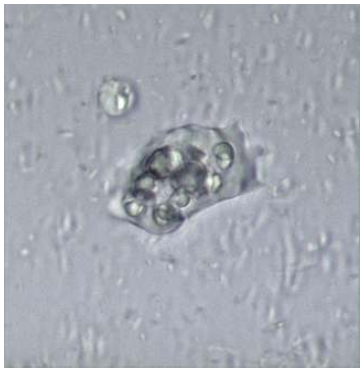
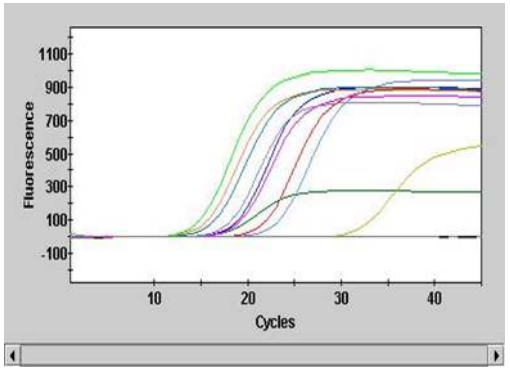
Laboratory Diagnosis





- Microscopy
- Culture
- PCR
  - Conventional
  - Nested
  - RT-PCR



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Problem???

- Increased reporting of *D. fragilis* since transitioning to molecular testing
- Artefact or real
- High levels reported in Denmark/Europe
  - higher levels asymptomatic control groups
  - ?pathogenicity

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Prevalence

- Australia/NZ - 0.4% - 16.8%
- Northern Europe – up to 82%
- Prevalence varies widely - dependent of diagnostic testing
- More common than *Giardia*

SVH Sydney (2014/15/16/17)

Protozoa	Prevalence (%)
Blastocystis spp.	15.6%
Dientamoeba	**9.2%**
Giardia	2.6%
Cryptosporidium	1.5%
E. histolytica	0.3%

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Prevalence

STUDY	COUNTRY	YEAR	FINDINGS
Roser et al	Denmark	2013	*D. fragilis* incidence 43% (n=29,484)
Engsbro et al	Denmark	2014	Prevalence 35-41%
Bruijestein et al	Denmark	2015	Symptomatic patients 37.3% Asymptomatic control group 25.7%
de Jong et al	Netherlands	2015	Healthy controls 50.6% Paediatric patients presenting with abdominal pain 43.2%
Holtman et al	Netherlands	2017	*D. fragilis* prevalence in children 55%

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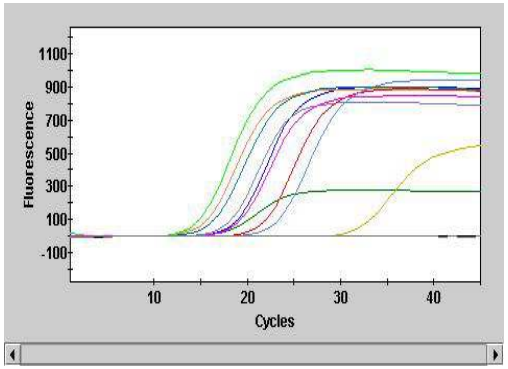
Evaluate RT-PCR

In House



- Verweij et al., 2007
  - 5.8S rRNA gene target
  - MGB probe

TIB MOLBIOL

LightMix® Modular Assays

Commercially Available

- EasyScreen Enteric Protozoan Detection kit (Genetic Signatures)

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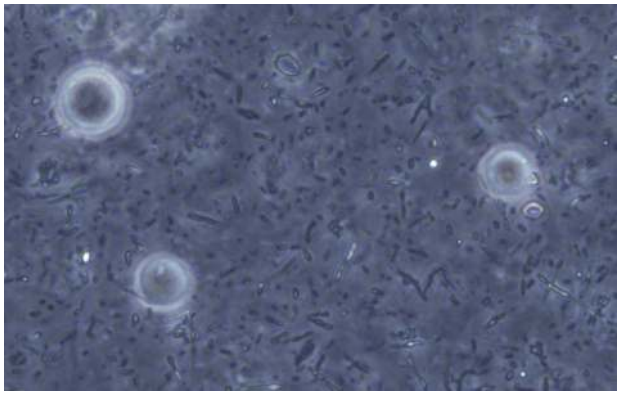
Limit of Detection

- *D. fragilis* trophozoites cells counts
- Serial dilutions
- Spiked into faecal sample
- DNA extracted
- Limit of Detection Assays



Live *Dientamoeba fragilis* trophozoites from a Loeffler slope with PBS overlay supplemented with rice starch.

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Limit of Detection

D. fragilis Trophozoites	GS	Verweij
500	30.33	23.73
50	33.02	27.52
5	34.48	30.64
0.5	Negative	Negative
0.05	Negative	Negative

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Specificity

- To assess the specificity of each PCR assay

1. *Dientamoeba fragilis*
2. *Tritrichomonas foetus (Pig)*
3. *Tritrichomonas foetus (Cat)*
4. *Trichomonas vaginalis*
5. *Pentatrichomonas hominis*
6. *Histomonas meleagridis*
7. *Hypotrichomonas acosta*
8. *Trichomonas mobilensis*
9. *Trichomonas muris*
10. *Enteromonas hominis*
11. *Retortamonas intestinalis*
12. *Chilomastix mesnili*

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Pilot study - Patient samples

- 10 fresh patient samples
  - Screened by microscopy and culture (X3)
  - 1/10 samples positive for *D. fragilis* by microscopy
  - Subsequently grew in modified culture media
- Ran on both assays

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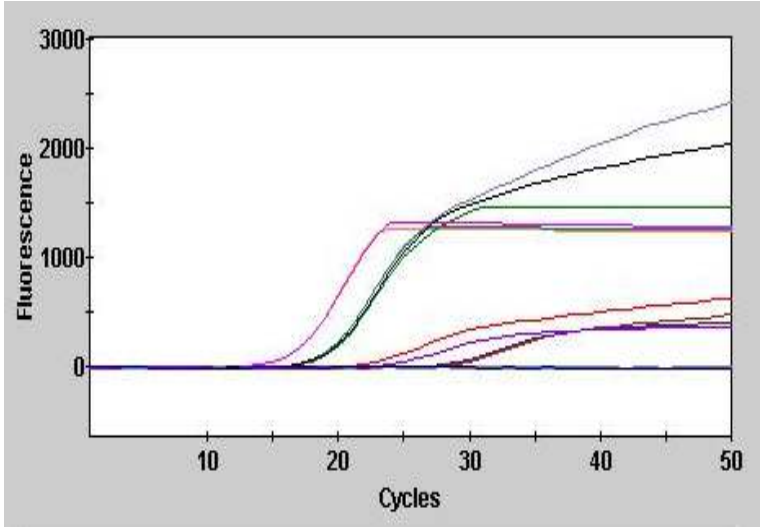
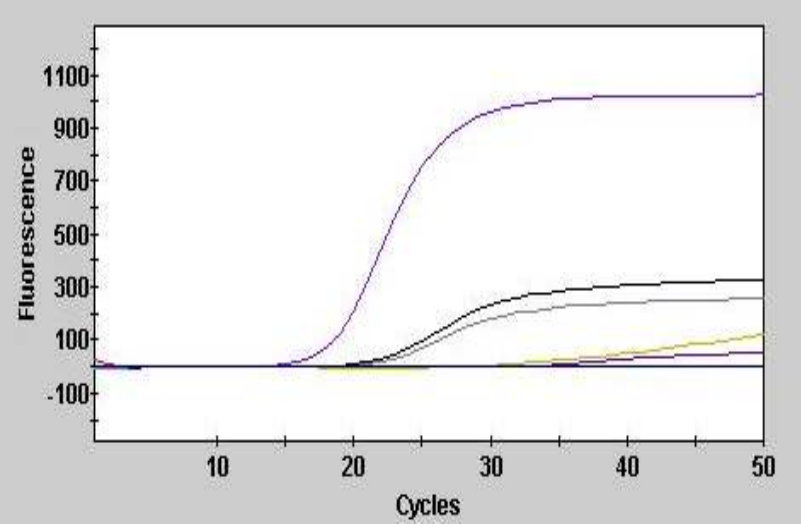
Verweij et al, 2007





Specificity Assay

Samples were found to cross react with *T. foetus*

Patient Samples

D. fragilis detected in 4/10 patient samples – 3 false positives?

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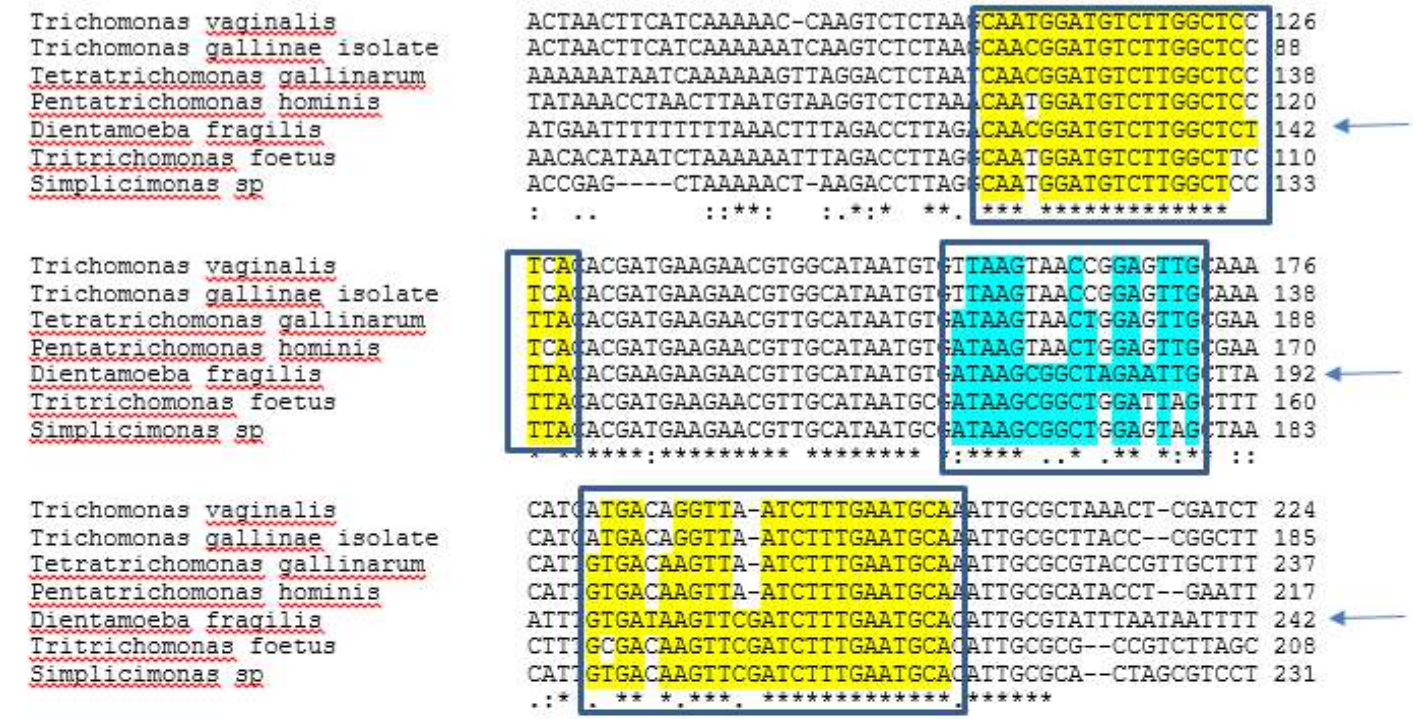
Verweij et al, 2007

- 5.8s target



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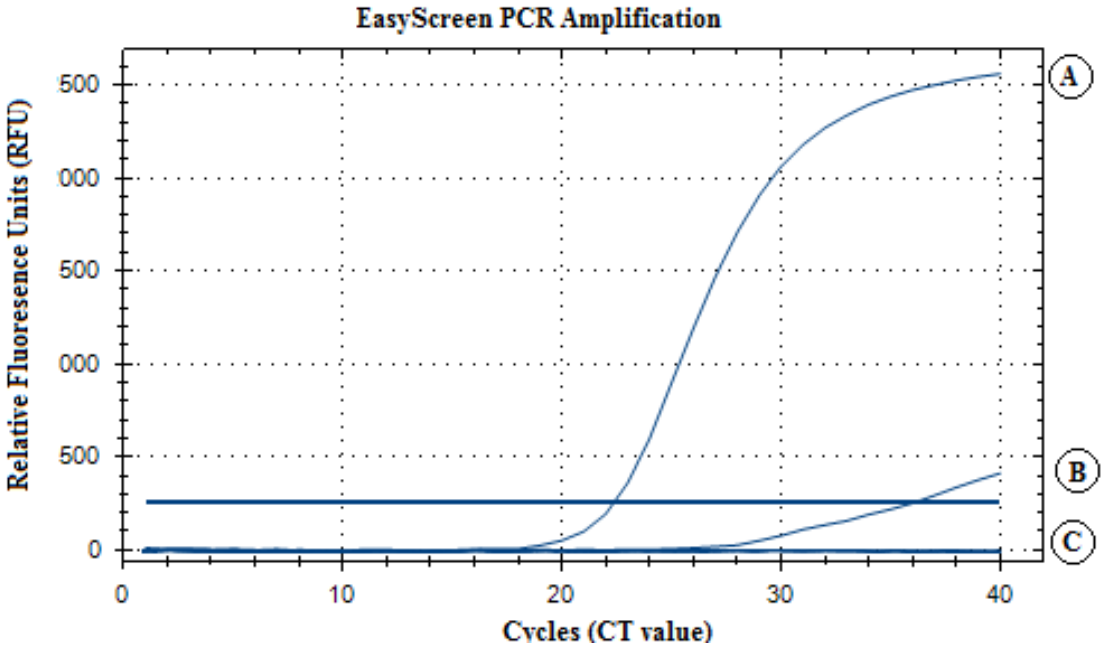
Genetic Signatures

- 3 base technology
- Bisulphate conversion
- Primers/probe targeting “new” sequence



Specificity Assay

Cross reacted with *P. hominis* (ONLY AT 100,000 copies)

However melt curve analysis differentiates *D. fragilis* and *P. hominis*

Patient Samples

D. fragilis detected in 1/10 patient samples

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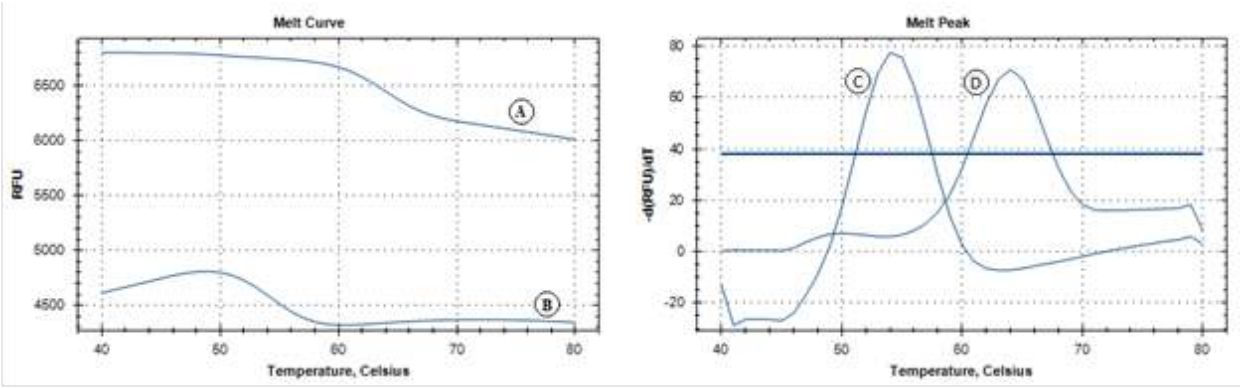
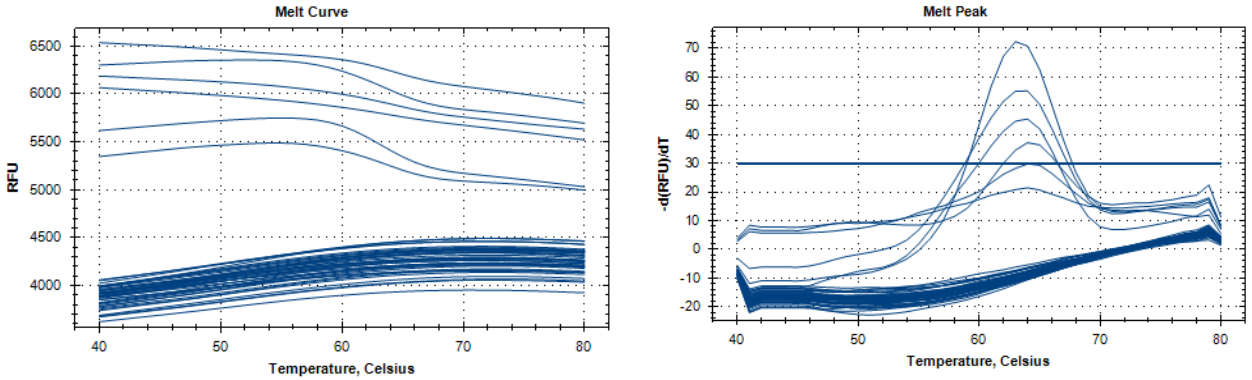
Melt curve/peaks



Melt curve analysis of *D. fragilis* (A) compared to *P. hominis* (B),
P. hominis had a melting peak at 54ºC (C), compared to *D. fragilis* at 64ºC (D)



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“Patient” Samples

Sample	Microscopy (DF +)	GS	Verweij et al
1	-	-	+
2	-	-	-
3	-	-	-
4	-	-	-
5	+	+	+
6	-	-	-
7	-	-	-
8	-	-	-
9	-	-	+
10	-	-	+

Sample 5 positive by microscopy and culture

Sample 1, 2, 3, 4, 6, 7, 8, 9, 10 all negative by culture and microscopy

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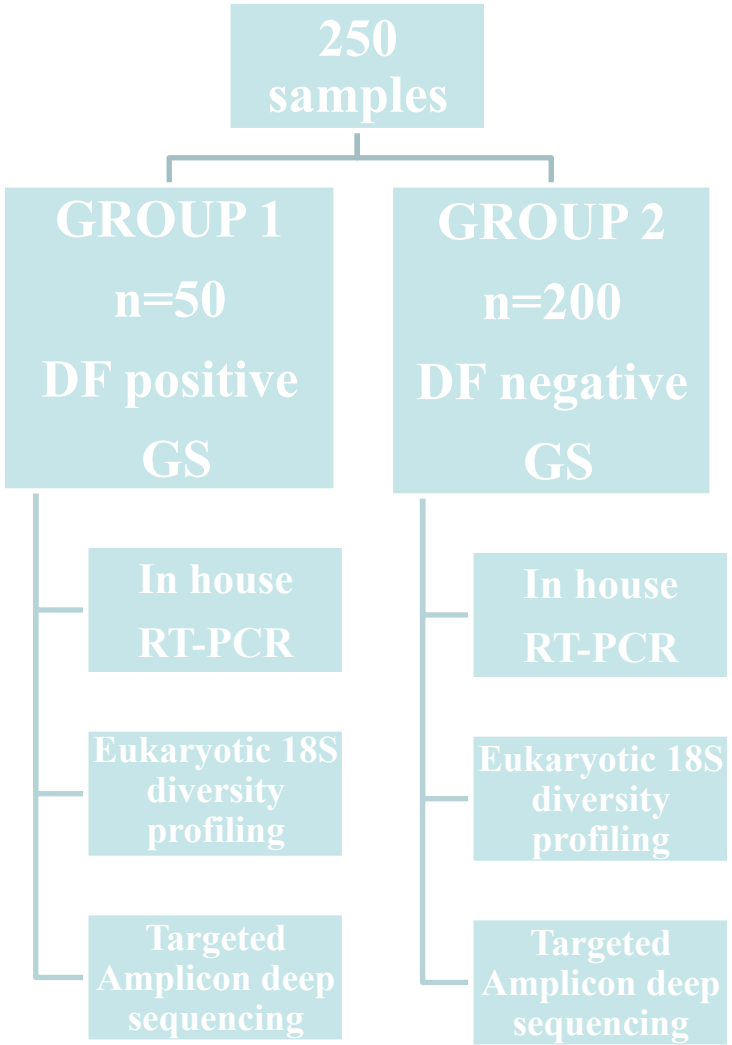
Evaluation



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Evaluation

- The samples were screened using the in-house RT-PCR on four real time PCR platforms.

- Eukaryotic 18S diversity profiling in order to identify the presence or absence of *D. fragilis* DNA.
  - This approach also allowed for the detection of additional protozoan species in samples that may be responsible for cross reactivity in these samples.

- Polymerase Chain Reaction (PCR) targeting the 18S Euk1391F – EukBR target of the 18S ribosomal subunit DNA using the forward primer sequence 5'- GTACACACCGCCCGTC-3' and the reverse primer sequence 5'- TGATCCTTCTGCAGGTTCACCTAC-3'.
  - The amplicons from each sample were then sequenced in multiplex, on the Illumina MiSeq platform, utilizing Illumina's Nextera XT v2 indices and paired end sequencing chemistry.

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Group 1
Dientamoeba "positive" samples

Assay	Platform	No. positive	No. negative	% positive	Ct range	% Ct >35
EasyScreen (Stark *et al.*, 2014)	**Bio-Rad CFX384**	50	0	100%	21.47 – 38.37	16%
In house real time PCR assay (*Verweij et al.*, 2007)	**Cepheid SmartCycler II**	50	0	100%	17.36 - 36.83	6%
	Roche LightCycler 480	42	8	84%	23.40 - 43.75	48%
	Bio-Rad CFX96	43	7	86%	23.34 - 48.91	62%
	ABI 7500	49	1	98%	17.94 - 38.07	12%

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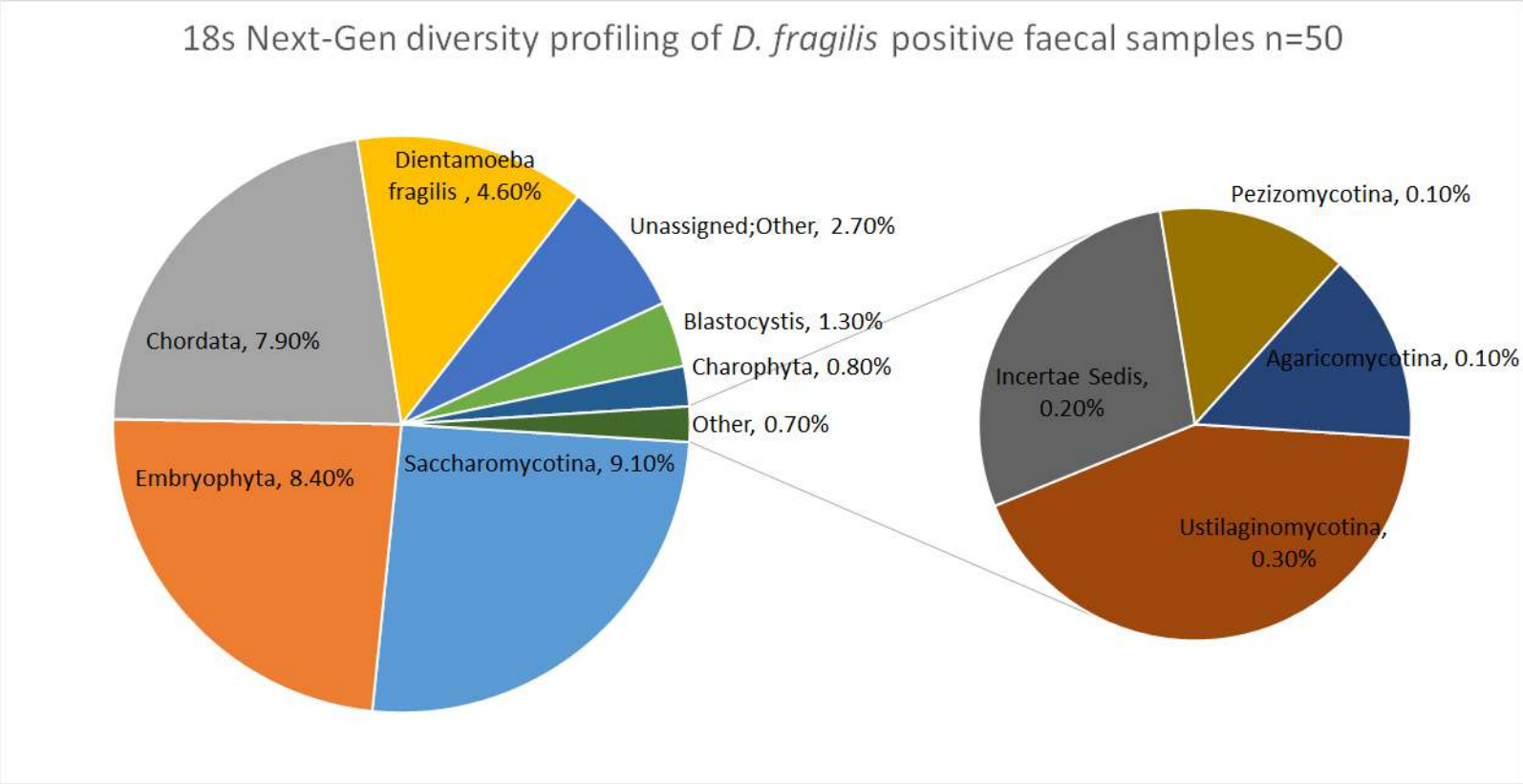
18s NGS diversity profiling



In total, 18S diversity profiling on samples from group 1 yielded individual eukaryotic OTU's (Observational Taxonomical Units), consisting of *Dientamoeba fragilis* reads for all samples.

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Group 2
Dientamoeba "negative" samples

Assay	*Platform*	*No. positive*	*No. negative*	*% positive*	*Ct range*	*% Ct >35*
EasyScreen (Stark *et al.*, 2014)	**Bio-Rad CFX384**	0	200	0%	NA	NA
In house real time PCR assay (Verweij *et al.*, 2007)	**Cepheid SmartCycler II**	15	185	8.1%	32.00 - 45.16	73%
	Roche LightCycler 480	4	196	2.0%	39.59 - 45.00	100%
	Bio-Rad CFX96	14	186	7.5%	37.53 - 47.07	100%
	ABI 7500	6	194	3.0%	32.34 - 40.11	67%

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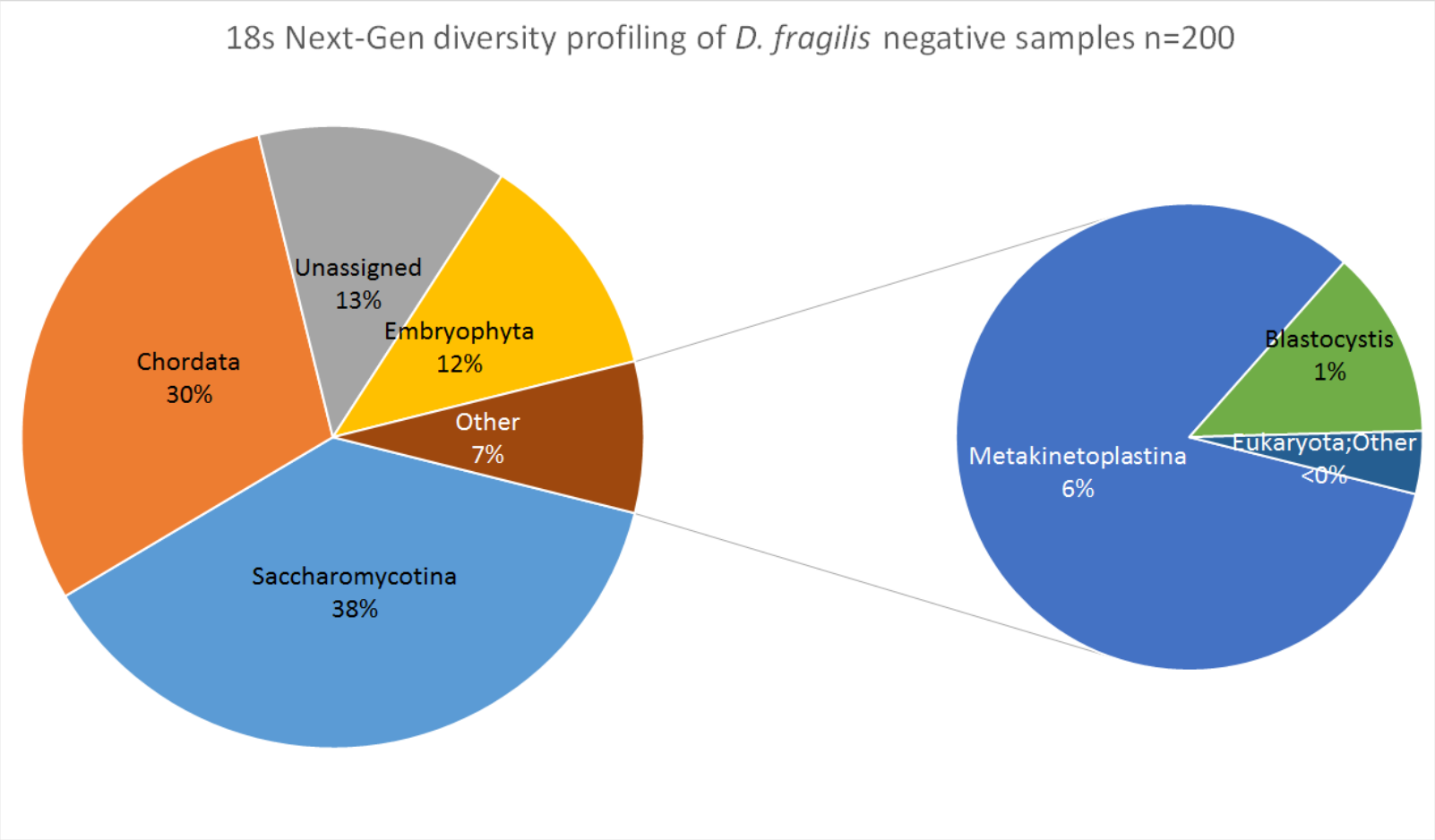
18s NGS diversity profiling



The samples sent from group 2 yielded saw zero abundance of *Trichomonad* related OTU's.

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Discrepant results

- Targeted Amplicon deep sequencing was performed
  - *Dientamoeba fragilis* 5.8s ribosomal DNA region

- **Group 1 samples -** 50/50 resulted in successful amplification with reads ranging from 5,610 and 523,933.

- **Group 2 samples -** 4/200 resulted in successful amplification, with the number of reads ranging between 8,322 and 87,192.

- BLAST analysis matched sequences from all samples that produced reads to *Dientamoeba fragilis* 5.8s ribosomal DNA with 99% identity.

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False positives?

Table 3. Individual in-house real time PCR results for positive group 2 samples using multiple PCR platforms.

Sample	*Cepheid SmartCycler II*	*Bio-Rad CFX96*	*Roche LightCycler 480*	*ABI 7500*	*NGS*
Sample 3	+	-	-	-	-
Sample 4	+	-	-	-	-
Sample 7	+	-	-	+	-
Sample 8	-	+	-	-	-
Sample 11	+	+	-	-	-
Sample 13	+	-	-	-	-
Sample 16	-	+	-	-	-
Sample 23	-	+	-	-	-
Sample 28	+	-	-	-	-
Sample 31	+	-	-	-	-
Sample 40	-	-	-	+	-
Sample 49	-	+	-	-	-
Sample 51	-	-	-	+	-
Sample 59	-	-	-	+	-
Sample 52	-	-	+	-	-
Sample 61	-	+	-	-	-
Sample 64	-	+	-	-	-
Sample 77	-	+	-	-	-
Sample 94	-	-	+	-	-
Sample 102	+	-	-	-	-
Sample 106	-	+	-	-	-
Sample 109	-	-	+	-	-
Sample 111	+	-	-	-	-
Sample 114	-	-	+	-	-
Sample 122	-	+	-	-	-
Sample 124	+	-	-	-	+
Sample 130	+	-	-	-	+
Sample 140	+	-	-	+	+
Sample 141	+	-	-	-	-
Sample 154	-	+	-	-	-
Sample 163	+	-	-	-	-
Sample 170	+	-	-	-	+
Sample 178	-	+	-	-	-
Sample 185	-	-	-	+	-
Sample 186	-	+	-	-	-
Sample 195	-	+	-	-	-

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Sensitivity/Specificity

- Using targeted amplicon deep NGS as the gold standard
  - These results conclude that, in total, 54 of the 250 samples tested did in fact contain *Dientamoeba fragilis* DNA (Group 1:50/50 | Group 2: 4/200).

	Genetic Signatures assay	In house RT-PCR
Sensitivity	93%	87%-100%
Specificity	100%	86%-94%
PPV	100%	53%-78%
NPV	98%	98%-100%

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Conclusion

- GS assay showed excellent sensitivity, specificity, NPV and PPV.
- This study highlights several problems regarding the sensitivity and specificity of the in-house RT-PCR used for the detection of *Dientamoeba.*
- Highlights the need for standardisation of detection assays.
- Proper validation protocols for diagnostic assays – even research assays.

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Conclusion

- Inaccurate detection can result in overrepresentation that can mislead researchers to conclude upon false assumptions regarding pathogenicity when basing decisions on prevalence alone.

- In the absence of full genomic sequencing, transcriptome data and animal models, rash declarations on the pathogenicity of *D. fragilis* must be resisted.

- More research is needed!

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Recommendation's

The Royal College of Pathologists of Australasia
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Durham Hall 207 Albion Street Surry Hills NSW 2010 Australia
Telephone 61 2 8356 5858 Facsimile 61 2 8356 5828

RCPA
The Royal College of Pathologists of Australasia

Guideline

Subject:	**Faecal pathogen testing by PCR and the detection of *Dientamoeba fragilis* and *Blastocystis* species**
Approval Date:	November 2015
Review Date:	November 2019
Review By:	Microbiology Advisory Committee
Number:	6/2015

Summary:

The role of both *Dientamoeba fragilis* (a trichomonad protozoon) and *Blastocystis* species (a steramanopile closely related to algae) as gastrointestinal pathogens is highly controversial. *Dientamoeba fragilis* in particular has been difficult to identify by microscopy in the laboratory. It is only due to the recent introduction of DNA-based molecular methods (nucleic acid amplification testing including PCR) that we now know that these organisms are much more common than previously thought. Two genotypes of *Dientamoeba fragilis* and 17 genotypes (9 in humans) of *Blastocystis* (possibly separate species) have now been documented[1]. Both these organisms are found in faeces of humans as well as in a number of animal species worldwide. Pathogenicity of *Blastocystis spp* and *Dientamoeba fragilis* has not been established in humans.

What has changed?

Since 2013, many laboratories in Australia have introduced a more sensitive and time saving technique (multiplex PCR), which detects *Entamaoeba histolytica, Giardia lamblia, Cryptosporidium spp., Blastocystis spp* and *Dientamoeba fragilis*, to screen for protozoa in faeces with potential to replace detection by microscopy, which is both subjective and time consuming [2]. Following the introduction of PCR, the number of positives for these parasites has increased markedly – up to 20% of all faeces received in the laboratory. The increase has predominantly (approximately 75% of total) been due to *Blastocystis spp* and *Dientamoeba fragilis*. Positive results are predominantly in children [3] and in formed or semi-formed faeces and rarely in loose faeces. Symptoms are often falsely attributed to the presence of these organisms leading to overtreatment. This can result in unnecessary anxiety for patients or their families and possible harm due to disruption of normal gut flora if antibiotics are prescribed. Laboratories are also being asked to 'test for clearance' in asymptomatic patients after treatment. This is clearly adding to the cost and time of pathology testing without evidence of clinical benefit.

The issues:

1. PCR is very sensitive: validated cut offs (Ct values) have not been established.
2. The various genotypes of *Blastocystis* cannot be differentiated in the diagnostic laboratory; nor can potential pathogenic types be identified. *Dientamoeba* PCR may cross react with other animal trichomonads and the current test cannot differentiate these.
3. The pathogenic potential of both organisms is not known.

Australasian Society for Infectious Diseases (ASID) Inc
ABN: 20 108 151 093/ARBN: 155 632 698

From: Australian and New Zealand Paediatric Infectious Diseases Group (ANZPID)

ANZPID

Royal College of Pathologists of Australasia (RCPA)
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Surry Hills NSW 2010
Australia

Via email: president@rcpa.edu.au, debrag@rcpa.edu.au

9 June 2015

Dear Colleagues at the RCPA,

We write on behalf of the Australian and New Zealand Paediatric Infectious Diseases Group (ANZPID), which is a special interest group of the Australasian Society for Infectious Diseases (ASID). We have followed the recent adoption of molecular testing for gastrointestinal bacterial and parasitic pathogens with interest. We recognise the importance of this change from microscopy and culture to molecular diagnostics, which streamlines efficiency with attendant cost savings to the laboratory and the health care sector. The ability to detect multiple enteric bacterial and parasitic pathogens with a multiplex PCR and offer a turn-around-time of less than five hours is impressive.

However, we have significant concerns regarding the parasites in the multiplex PCR for children and their families. Of the five parasites included in the multiplex panels, only three are of clinical significance; *Giardia, Cryptosporidium* and *E. histolytica* and these have been detected by PCR with similar performance characteristics to previous methods. Our understanding is that both *Dientamoeba fragilis* and *Blastocystis hominis*, which are of uncertain clinical significance and may be colonising flora, have been detected at much higher rates using molecular methods, than occurred with routine microscopy. Prevalence rates of 17% for *D. fragilis*, and higher for *B. hominis*, have been reported. The median age of detection is 7 years, with a smaller peak in early adulthood – possibly the parents of these children. Hence, children and their families are the core group affected by the significant increase in detection of these two parasites. In our experience, this has caused increased numbers of consultations to medical practitioners, unnecessary use of antimicrobials and anxiety and uncertainty for families.

We write this open letter as a collective group of paediatric infectious diseases physicians who are fielding an increasing number of consultations, clinic visits and phone calls from concerned parents of children with *D. fragilis* or *B. hominis* detected by stool PCR and their GPs who are unsure how to interpret the results. We recognise that this will continue to be an issue as more laboratories around the country transition to the use of stool multiplex molecular testing.

As such, we would suggest that the molecular identification of these two parasites should not be routinely reported unless research can elaborate on the pathogenicity, natural history and response to treatment in children using these newer diagnostic techniques. To date the best evidence in children (a double-blind RCT) showed no difference between treatment and placebo for dientamoebiasis (Roser *et al. Clin Inf Dis* 2014).

We would welcome discussion of the potentially adverse consequences of reporting these results and whether a more nuanced reporting of the results is warranted until the clinical implications and response to treatment is better defined. There is a precedent for this with the rational reporting and cascading of bacterial sensitivity results for broad-spectrum antibiotics.

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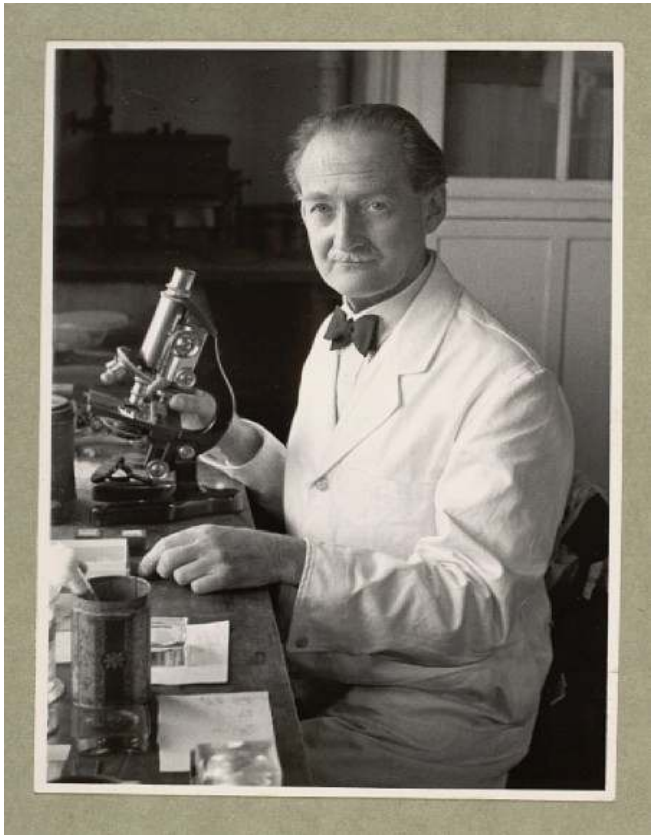
Conclusion

- *"to the protozoologist – if not the physician – D. fragilis is now, perhaps, the most interesting of all the intestinal amoebae of man : for we know less about it than any of the others...its life history and activities are still mysterious...yet after more than 20 years of work and cogitation, I am still baffled…"*



Cecil Clifford Dobell. 1886-1949

Dobell C. Researches on the intestinal protozoa of monkeys and man. X. The life history of *Dientamoeba fragilis*: observations, experiments and speculations. *Parasitology* 1940;32:417-461.

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Acknowledgements

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  - Dr Joel Barratt
  - Rory Gough

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