Pinning down the role of common luminal intestinal parasitic protists in human health and disease – status and challenges

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Abstract

While some single-celled intestinal parasites are direct causes of diarrhoea and other types of intestinal pathology, the impact of other gut micro-eukaryotes on human health remains elusive. The fact that some common luminal intestinal parasitic protists (CLIPPs) have lately been found more often in healthy than in diseased individuals has fuelled the hypothesis that some parasites might in fact be protective against disease. To this end, the use of new DNA technologies has helped us investigate trans-kingdom relationships in the gut. However, research into these relationships is currently hampered by the limited data available on the genetic diversity within the CLIPPs genera, which results in limited efficacy of publicly available DNA sequence databases for taxonomic annotation of sequences belonging to the eukaryotic component of the gut microbiota. In this paper, I give a brief overview of the status on CLIPPs in human health and disease and challenges related to the mapping of intestinal eukaryotic diversity of the human gut.

Introduction

Recent research into the human gut microbiome has vastly increased our understanding of the relationship between gut microbes and human health and disease. For instance, we now know that in adults, a low-diversity microbiota with increases in proportions of facultative anaerobes is linked to acute diarrhoea, inflammatory bowel disease, *Clostridium difficile* infection, metabolic syndrome and liver disease, just to mention a couple of conditions (Cani, 2018; Kriss *et al.*, 2018). Although most gut microbiome research has focussed on prokaryotic diversity, we have also gained significant insight into the micro-eukaryotic diversity of the human gut. DNA-based methods have been instrumental to this advancement. Three important points have emerged: (1) For some common luminal intestinal parasitic protists (CLIPPs), genetic diversity is surprisingly high; still, DNA sequence data available in publicly available databases such as the NCBI database is rudimentary, hence not reflecting this amount of diversity (Stensvold *et al.*, 2011*c*, 2012, 2018; Royer *et al.*, 2012; Poulsen and Stensvold, 2016). (2) We have come to realize that some CLIPPs are very common and often more common in gut-healthy individuals than in those with functional and inflammatory bowel diseases, contrary to previous general belief (Petersen *et al.*, 2013; Andersen *et al.*, 2015; Krogsgaard *et al.*, 2015, 2018; Rossen *et al.*, 2015; Beghini *et al.*, 2017; Jokelainen *et al.*, 2017; Mirjalali *et al.*, 2017). (3) Robust links between CLIPPs and gut bacteria have been identified by several research teams (Stensvold and van der Giezen, 2018).

These three points currently stimulate interdisciplinary research across the fields of parasitology, clinical microbiology, gastroenterology and ecology. Nevertheless, compared with advances within e.g. bacteriology and virology, progress in the research into CLIPPs and their role in human health and disease is still reflected mostly in simple stool microscopy-based surveys of parasites in selected populations, and is therefore still facing some major challenges. In the following, I will try to detail the status of the three above-mentioned points, and highlight some of the limitations and challenges to the work ahead that aims to identify the significance of CLIPPs in human health and disease.

Which are the most common luminal intestinal parasitic protists?

Intestinal eukaryotes that need a host to complete their life cycles (i.e. organisms that are referred to as 'parasites') include both helminths and protists. More typically, the distinction is made between 'helminths' and 'protozoa', but from a taxonomical point of view, the group of organisms referred to as protozoa does not include one of the most common micro-eukaryotes, namely *Blastocystis*, and so, the term 'protists' appears more relevant and applicable than 'protozoa' in this context. Moreover, while some parasitic intestinal protists are invasive (e.g. sporozoa) or adhere to the mucosal lining (e.g. *Giardia*), quite a few genera appear to be confined mainly to the gut lumen. These include *Blastocystis*, *Dientamoeba*, *Endolimax*, *Iodamoeba* and most species of *Entamoeba*, and given their prevalence, they could be referred to as CLIPPs.

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Contrary to the situation in developing countries, the number of carriers of helminth infestations other than those attributable to pinworm (*Enterobius vermicularis*) appears to be rapidly plummeting in human populations in the Western world (Verweij and van Lieshout, 2011; Verweij, 2014), and also in some parts of the developing world, which would probably reflect improved hygienic standards. Still, and for incompletely known reasons, a substantial proportion of the population is colonized by CLIPPs, especially *Blastocystis* and *Dientamoeba* (Verweij and van Lieshout, 2011; Roser *et al.*, 2013; Krogsgaard *et al.*, 2015, 2018; Jokelainen *et al.*, 2017) and, to a lesser extent, by one or more of the Amoebozoa, e.g. *Entamoeba coli* (Bruijnesteijn van Coppenraet *et al.*, 2009; Krogsgaard *et al.*, 2018; Stensvold and Nielsen, 2012; ten Hove *et al.*, 2007); these organisms will be introduced briefly below.

Blastocystis

Blastocystis is a genus comprising a perplexing variety of ribosomal lineages that are arguably separate species, judging from the amount of genetic diversity across complete nuclear ribosomal genes. So far, at least 17 ribosomal lineages, the so-called 'subtypes', have been acknowledged in humans, non-human primates, other mammals and birds (Alfellani *et al.*, 2013b; Clark *et al.*, 2013; Stensvold and Clark, 2016). Also reptiles, amphibia and insects have been identified as hosts for various species of *Blastocystis* (Yoshikawa *et al.*, 2016). While this parasite appears to be a rare or at least not so common finding in strict or moderately strict carnivores such as cats, dogs and hyenas (Ruaux and Stang, 2014; Wang *et al.*, 2014; Heitlinger *et al.*, 2017; Cociancic *et al.*, 2018; Moura *et al.*, 2018; Udonsom *et al.*, 2018), it may be more common in omni- and herbivores, including pigs, cows and sheep (Pakandl, 1991; Navarro *et al.*, 2008; Ramirez *et al.*, 2014; Masuda *et al.*, 2018; Moura *et al.*, 2018; Udonsom *et al.*, 2018). Nine distinct ribosomal lineages, the so-called 'subtypes', have been isolated from humans, with subtypes 1–4 predominating. Some subtypes even exhibit extensive within-subtype diversity that to some degree is host-specific; e.g. ST3 (Alfellani *et al.*, 2013a). Colonization is common in older children and adults than in infants and young children (El Safadi *et al.*, 2014; Scanlan *et al.*, 2014; Poulsen *et al.*, 2016; Salehi *et al.*, 2017; Scanlan *et al.*, 2018), with prevalence rates reaching 100% in developing countries (El Safadi *et al.*, 2014). Moreover, *Blastocystis* may colonize the human gut for several years (Scanlan *et al.*, 2014).

Dientamoeba fragilis

DNA-based methods helped overcome the diagnostic challenges related to the detection of *Dientamoeba fragilis*, a non-flagellated flagellate for which a cyst stage was reported only very recently (Munasinghe *et al.*, 2013; Stark *et al.*, 2014). *Dientamoeba fragilis* is the only known species in the genus. The first DNA-based detection methods for *D. fragilis* appeared in the mid-00s (Peek *et al.*, 2004; Stark *et al.*, 2005a, 2005b, 2006; Verweij *et al.*, 2007). Since then, such methods have helped us to realize that this parasite is very common in some populations, especially in Northern Europe (Röser *et al.*, 2013; de Jong *et al.*, 2014; Ögren *et al.*, 2015; Holtman *et al.*, 2017; Jokelainen *et al.*, 2017). In Denmark, *D. fragilis* is almost an obligate finding in children (Röser *et al.*, 2013; Jokelainen *et al.*, 2017). In other regions where methods of high sensitivity are also used, such as Australia, the parasite appears to be a lot less common (Stark *et al.*, 2016); however, studies involving screening of asymptomatic individuals for *D. fragilis* are very rare, and so the prevalence of the parasite in individuals without symptoms in most parts of the world remains largely unknown. Apart from humans, *D. fragilis* has been found in non-human primates and pigs (Stark *et al.*,

2008; Cacciò *et al.*, 2012). The diversity within the species appears very limited, and most cases of *D. fragilis* colonization are attributable to one of only two acknowledged genotypes (Genotype 1), no matter where sampling is performed (Stark *et al.*, 2005a, 2005b; Stensvold *et al.*, 2013; Cacciò *et al.*, 2016; Greigert *et al.*, 2018).

Entamoeba

A number of *Entamoeba* species can colonize the human intestine. Infections due to the potentially highly pathogenic *Entamoeba histolytica* are relatively rare compared with colonization by *Entamoeba dispar*, *Entamoeba hartmanni*, and, especially, *Entamoeba coli*, which has been found to colonise between 20 and 30% of individuals in surveyed populations in Brazil (Aguiar *et al.*, 2007; Neres-Norberg *et al.*, 2014; Higa *et al.*, 2017; Jeske *et al.*, 2018). Substantial genetic variation has been detected within *E. coli*, with *E. coli* subtype 1 and subtype 2 differing by 13% (Stensvold *et al.*, 2011c). Overall, the genetic diversity within octonucleated Entamoebas appears vast and still largely unaccounted for (Jacob *et al.*, 2016; Elsheikha *et al.*, 2018). *Entamoeba polecki* rarely infects humans; nevertheless, four subtypes have been detected with quite varying geographical distribution and host reservoirs (Stensvold *et al.*, 2011c, 2018); all four subtypes have been found in humans (Verweij *et al.*, 2001; Stensvold *et al.*, 2018).

It is currently unclear to which extent non-histolytica Entamoebas contribute to the development of intestinal symptoms.

Some other protists show up in stool every now and then (often accompanied by other CLIPPs) and these include parasites belonging to ciliates and the Amoebozoa. Although the amount of documentation is scarce, it is clear that for some of these parasites, especially *Iodamoeba* and *Endolimax*, the intra-generic diversity is vast, with a maximum genetic divergence of at least 31% (Stensvold *et al.*, 2012; Constenla *et al.*, 2014; Poulsen and Stensvold, 2016). *Endolimax nana* was recently shown to colonize 28.8% of 3245 individuals attending the Evandro Chagas National Institute of Infectious Diseases, Rio de Janeiro, Brazil (Faria *et al.*, 2017). To date, no complete, annotated nuclear genome sequences have been published for CLIPPs other than *Blastocystis* and *Entamoeba dispar*.

The extensive genetic diversity documented so far within these CLIPPs has informed the taxonomic terminology, and so, depending on the availability of morphology data and genetic diversity and SSU rDNA sequence coverage, sequences are annotated to species, subtypes, ribosomal lineage, or conditional lineage (Jacob *et al.*, 2016). Importantly, it appears that specific taxonomic terminologies are being developed for individual genera; these are based first and foremost on a pragmatic basis.

CLIPPs in a gut ecology setting

In addition to exploring parasite diversity in the gut, it could be important to try and look to gut microbial and ecological relationships in non-human hosts and in the environment, respectively, to better understand the role of CLIPPs in human health and disease. In the field of ecology, protists have been identified as important components of terrestrial and aquatic environments where they are integral constituents of trophic chains and nutrient cycles (Bates *et al.*, 2013; Maritz *et al.*, 2017). In geothermal springs, protist diversity appears to rely on pH and temperature (Oliverio *et al.*, 2018). The introduction of *Acanthamoeba* into the rhizosphere of *Arabidopsis thaliana* leads to rapid changes in associated bacterial communities due to the grazing of the amoeba (Rosenberg *et al.*, 2009). Gut flagellates and ciliates assist termites and ruminants in metabolizing/fermenting carbohydrates (Veira, 1986; Ohkuma, 2008; Moon-van der Staay *et al.*, 2014); examples are endless. The presence of protists in various

niches therefore appears to be driven by a variety of host- and environment-derived factors and may in turn have a number of vital or less vital consequences for the associated microbiome, be it the host-associated gut microbiome, plant rhizosphere or terrestrial and aquatic biomes. This understanding has to a large extent failed to resonate with professionals in clinical microbiology and related medical fields, where CLIPPs are generally seen as 'intruders' and (potential) pathogens, despite the fact that most of these are most probably non-invasive and may have unknown functions of potential benefit (Parfrey *et al.*, 2011; Lukeš *et al.*, 2015; Andersen and Stensvold, 2016).

Nevertheless, the concept of certain gut parasitic protists as 'ecosystem engineers' also in humans is sinking in, and studies on trans-kingdom relationships are emerging. For instance, Laforest-Lapointe and Arriet (2018) recently proposed a model for the ecological role of *Blastocystis* in the human gut microbiota. They suggested that *Blastocystis* by predation on abundant bacterial taxa lowers the competition for nutrients and space, leading to an increase in bacterial richness and community evenness. And indeed, carriers of *Blastocysts* and other CLIPPs have been shown to have gut bacterial microbiomes that differ significantly from those who do not carry these parasites in several recent studies, the findings of which were recently summarized by Stensvold and van der Giezen (2018). In fact, higher diversity and higher richness are typically observed in CLIPPs-positive individuals than in those who are negative. What is more is the fact that observations from a recent meta-analysis of metagenomics data indicated that *Blastocystis* carriage is linked to low body mass index (Andersen *et al.*, 2015; Beghini *et al.*, 2017), which again lends support to specific links to gut bacterial diversity. However, it remains to be identified, to which extent *Blastocystis* is actively driving this difference as proposed by Laforest-Lapointe and Arrieta, or whether *Blastocystis* is merely an indicator or specific bacterial community patterns. Stensvold and van der Giezen (2018) recently hypothesized that the increased intestinal oxygen concentrations observed during gut dysbiosis may prevent *Blastocystis* from establishing in the gut, which would suggest a role for *Blastocystis* as an indicator organism.

Experimental models, such as that recently proposed by Pomajbikova and colleagues (Růžková *et al.*, 2018), could be used to develop longitudinal studies on bacterial community changes after the establishment of *Blastocystis* colonization. *Blastocystis* is one the few parasites that is readily established in culture (Clark and Stensvold, 2016), and cysts induced in cultures or obtained from donor material, isolated from stool by gradient centrifugation, can be used for inoculation in order not to co-introduce bacteria that would lead to experimental bias (Rene *et al.*, 2009). Here, the use of both eubiotic and dysbiotic animals could be used to study potential differences in colonization success rate.

The fact that some hosts (e.g. cats and dogs) are not so prone to harbouring a parasite such like *Blastocystis* while others (e.g. humans and artiodactyls) in the same habitat are much more likely hosts, should also be explored in detail, to identify whether this boils down to diet, behaviour (exposure), and/or other factors. If all the subtypes of the parasite are globally pervasive and the overall colonization pressure of *Blastocystis* strong, differences in intestinal colonization between hosts may rely – at least in part – on differences in gut microbiota composition.

It is intriguing that not only *Blastocystis*, but also other CLIPPs have been shown to be linked to specific microbiota patterns (Stensvold and van der Giezen, 2018). Studying gut microbiomes of rural Africans, Morton *et al.* (2015) could predict the presence/absence of *Entamoeba* by 79% accuracy, based on the composition of any individual's gut microbiota. To this end, Xiong *et al.* (2018) identified that shrimp health status could

be predicted with 92.4% accuracy based on eukaryotic taxon profiling.

Nucleated life within the human intestine also include fungi. Common genera found in stool include *Candida*, *Saccharomyces*, *Malassezia*, *Pichia* and *Aspergillus* (Laforest-Lapointe and Arrieta, 2018); however, our understanding of the extent to which these genera in fact colonize the human intestinal tract or merely reflect dietary components is incomplete, and recent evidence appears to suggest that fungal colonization of the intestinal tract of healthy individuals is minimal (Auchtung *et al.*, 2018).

The faecal eukaryome – mapping of eukaryotic diversity in vertebrate stool

As observed by e.g. Hamad *et al.* (2018), differences in observed microbiome profiles may reflect differences in DNA extraction protocols, DNA amplification and sequencing technologies, plus queried databases (SILVA, Greengenes, RDP, NCBI, self-curated databases, etc.). So far, mapping of eukaryotic diversity in human and non-human stool samples has used mainly one of two approaches: Shotgun sequencing or amplicon-based sequencing of genomic DNA extracted from stool (Cristescu, 2014). The applicability of shotgun sequencing in terms of detecting and differentiating CLIPPs is hampered by the fact that relatively few CLIPPs genomes are available for reference. Amplicon-based sequencing has typically used nuclear small subunit ribosomal DNA (18S) as the target. However, some variation in amplicon-based approaches is seen, mostly in terms of the choice of target(s) and DNA sequence data processing. The most informative regions of the 18S appear to be the V3, V4, V5 and the V9 regions (Maritz *et al.*, 2017; Krogsgaard *et al.*, 2018). As an example, Krogsgaard and colleagues used three different primer sets for eukaryotic DNA (G3F1/G3R1 and G6F1/G6R1 targeting the V3–V4 region of the 18S rRNA gene and G4F1/G4R1 targeting the V3–V5 region) and one set of primers for prokaryotic DNA [341F/806R (Yu *et al.*, 2005)] (Krogsgaard *et al.*, 2018). Sequences were mapped using BION (http://box.com/bion), a newly developed k-mer-based analytical semi-commercial open-source package which allows annotation to species level. Prokaryotic DNA sequences were mapped against the RDP 11.04 reference database, while eukaryotic DNA sequences were mapped using SILVA version 123 reference database with an improved in-house seven-tier taxonomy for eukaryotes, similar to the tiers defined for prokaryotes (phylum, class, order, family, genus, species and sequence levels).

Published data on differences in the eukaryome across vertebrate populations and links between bactierial and eukaryotic signatures are still scarce.

Krogsgaard *et al.* (2015) found that CLIPPs diversity was higher in healthy individuals compared with patients with irritable bowel syndrome and also observed that individuals colonized by CLIPPs typically had a higher bacterial richness and diversity than those without (Krogsgaard *et al.*, 2018).

Heitlinger *et al.* (2017) used 4 16S and 44 18S primers in a Fluidigm-based approach, followed by taxonomic analysis using dada2 to map eukaryotic diversity in spotted hyenas. While no differences were found in eukaryome richness, diversity, evenness or genus abundance across age groups in a population of spotted hyenas, a more diverse eukaryome was identified in high-ranking than in low-ranking animals (Heitlinger *et al.*, 2017).

Maritz *et al.* (2017) recently developed and evaluated an 18S rRNA assay employing ILLUMINA-based sequencing and annotation of sequence data using locally curated as well as QIIME formatted SILVA databases with a view to detecting and differentiating protists in sewage with special emphasis on trichomonads. The team used vertebrate blocking primers to increase

protist data yield (Maritz et al., 2017). Choice of primers is critical too as evidenced by the differing outcomes in terms of e.g. Amoebozoan data obtained by Moreno et al., 2010 and Matsunaga et al. (2014), who both aimed at mapping eukaryotic diversity in wastewater/sludge.

The extent to which primate gut eukaryotic diversity is only rudimentarily reflected in reference databases can be exemplified by the following: In a metabarcoding study of non-human primate gut eukaryomes, only 0.01% of all SSU rDNA reads matched sequences in the Silva 123 database at a 100% threshold (Wilcox and Hollocher, 2018). In that study, de novo operational taxonomic unit (OTU) assignment revealed 4293 eukaryotic OTUs at a 97%-identity level, and reference-based taxonomy assignment matched sequences to 2021 unique eukaryotic genera. Investigating the sewage eukaryome of sludge digesters in Japan, Matsubayashi et al. (2017) found that 85% of the clones obtained by 18S rRNA gene clone library construction showed less than 97.0% sequence identity to what they termed as 'described eukaryotes', indicating most of the eukaryotes in anaerobic sludge digesters are largely unknown.

Advancing the mapping of intestinal eukaryotic diversity: Wastewater and new sequencing technologies–the way forward?

In summary, the characterization of nuclear small subunit (SSU) ribosomal RNA genes has been the backbone of DNA-mapping the tree of life. In the field of clinical microbiology, taxon-specific genetic variation across nuclear SSU ribosomal RNA genes has been instrumental to the development of a vast variety of targeted DNA-based diagnostic methods over the past few decades (Verweij and Stensvold, 2014); however, the development and use of such diagnostics are limited by the DNA sequence data available in NCBI (Stensvold et al., 2011b).

The SSU rRNA gene has proved useful for the detection and differentiation of several species of parasites. For helminths, however, this gene generally appears very conserved, and mitochondrial genes or ITS data are taxonomically more informative. Likewise, ITS data appear more relevant for differentiating between non-parasitic eukaryotic organisms often found in the gut, such as yeasts and molds, and so the genes providing most taxonomic resolution differ and depend on the type of organism.

The presence of large intra-generic diversity in some parasites has spurred hypotheses on differences in pathogenicity being associated with species/subtype/genotype, and so our ability to detect and differentiate not only genera and species but also subtypes, ribosomal lineages, etc., is important. Again, while the 18S has proved particularly useful in differentiating between Blastocystis subtypes and even subtype alleles (Stensvold et al., 2011a), this marker provides very little resolution within the species of for instance D. fragilis. For other parasites, such as a couple of genera belonging to the Amoebozoa, namely Entamoeba, Endolimax and Iodamoeba, we are only beginning to appreciate the vast extent of genetic diversity (Silberman et al., 1999; Clark, 2000; Verweij et al., 2001; Stensvold et al., 2010, 2011c; Royer et al., 2012; Jacob et al., 2016; Elsheikha et al., 2018). The work and methodological limitations involved in mapping the intra-generic diversity in these organisms have led to issues related to resolving the phylogeny among this group of organisms and left some 'dark holes' in publicly available databases. Briefly, the largest limitations here are as follows: although hypervariable regions within 18S, ITS or 28S may prove useful for studies into eukaryotic diversity, robust analysis of phylogenetic relationships, including the very delineation of novel ribosomal lineages, and optimal yield of analysis of sequence data from metagenomics or other amplicon-based sequencing studies requires sequencing

of complete, or near-complete ribosomal genes. When genomic DNA extracted directly from e.g. stool is used, the application of general primers with a view to amplifying near-complete ribosomal genes often results in preferential amplification of some organisms over other. As an example, individuals colonized by Iodamoeba and/or Endolimax are typically co-colonised with Blastocystis, and because the length of the SSU rRNA gene is only 1.8 kbp in Blastocystis while 2.5 kbp or more in Iodamoeba and Endolimax, Blastocystis ribosomal genes are more likely to be amplified from faecal genomic DNA due to the shorter DNA sequence. Another limitation is related to intra-cellular variation (hypervariable regions), which makes Sanger sequencing of polymerase chain reaction (PCR) products of some sequence stretches unsuitable, e.g. due to the presence of sequence variation within homo-polymers. TA cloning of PCR products has been tried with some success, but this is relatively expensive, time-consuming and laborious (Stensvold et al., 2012). Even next-generation sequencing methods such as ILLUMINA do not provide much better solutions to overcoming this issue. Clearly, alternative ways to effectively obtain data are needed.

Meanwhile, Pacific Biosciences (PacBio) RS II, considered a third-generation sequencer, uses single-molecule real-time technology and can be used for sequencing of single DNA molecules in real-time without prior amplification steps, enabling direct observation of DNA synthesis by DNA polymerase (Nakano et al., 2017). Importantly, this technology enables the production of long reads (typically >20 kbp with a maximum of 60 kbp) at relatively low costs (Nakano et al., 2017). Orr and colleagues used culturing and targeted PacBio RS II amplicon sequencing to expand on data on the diversity within the class of Diphyllatea, a group of protists that may represent one of the earliest diverging eukaryotic lineages (Orr et al., 2018). By obtaining near full-length 18S rRNA sequences in addition to mining publicly available databases, they were able to resolve the phylogeny within the class and better map the distribution of members of the class. The technology was also recently used for characterizing and quantifying protistan sequences from environmental samples (Jones and Kustka, 2017), and in terms of gut microbial diversity, one of the few studies using it so far is that by Myer et al. (2016) to generate data for phylogenetic analysis of rumen bacterial communities.

A limit to this technology is the relatively high rate of sequencing-related introduced errors; however, there are several ways to reduce or completely eliminate these errors using software tools and by decreasing the time the machine is used. Moreover, PacBio appears to be better at overcoming the issues related to the sequencing of hypervariable regions that e.g. ILLUMINA sequencing may have problems with. Critics of PacBio might argue that the use of this technology should rather be seen as an adjunctive, supportive and possibly exploratory tool that may provide a scaffold that could inform and guide more sophisticated and precise analyses. Such analyses could include Illumina-based sequencing of overlapping 300–400-bp amplicons using sequence-specific primers. Nevertheless, complete and accurate de novo assemblies of Escherichia coli strains could be accomplished using data generated solely from the PacBio RS II (Powers et al., 2013). The team found that addition of other sequencing technology data obtained by Ion Torrent and MiSeq offered no improvements over the use of PacBio data alone (Powers et al., 2013).

Apart from identifying the best possible technological and data processing pipelines, it is also worthwhile considering types of material for studying diversity. For instance, untreated sewage may be particularly useful in terms of detecting and mapping micro-eukaryotic diversity, since this material reflects pooled faecal samples from a large population of humans with some spillover of material from non-human sources.

Chouari *et al.* (2017) investigated eukaryotic diversity in wastewater using 18S sequencing, and of 1519 analysed sequences, 160 operational taxonomic units were identified. No less than 56.9% of the phylotypes were assigned to novel phylogenetic molecular species, showing <97% sequence similarity with their nearest affiliated representative within public databases. Similarly, Matsunaga *et al.* (2014) observed that 60% of their 18S rRNA gene clones obtained from DNA extracted from municipal wastewater had <97% sequence identity to described eukaryotes. In both studies, data on *Blastocystis* and Amoebozoa were observed. These studies highlight not only the vast DNA data gap in the eukaryotic tree of life, but also the relevance of using sewage as study material for investigations into eukaryotic diversity.

In conclusion, DNA mapping of nucleated life within the intestine and exploring it in ecological contexts are critical to further our understanding of gut microbial diversity and its role in health and disease. Application of more detailed reference data will allow for subtle and robust trans-kingdom analyses of gut microbes and will moreover expand our knowledge on host specificity, transmission patterns and links to clinical phenotypes. The use of genomic DNA from the pooled stool, as e.g. represented by sewage and amplicon-based third-generation sequencing may be a way to ensure the acquisition of quick and robust data to uncover the missing branches of the gut microbial eukaryotic tree.

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References

Aguiar JI, Gonçalves AQ, Sodré FC, Pereira SOR, Bóia MN, de Lemos ER and Daher RR (2007) Intestinal protozoa and helminths among Terena Indians in the State of Mato Grosso do Sul: high prevalence of *Blastocystis hominis*. *Revista da Sociedade Brasileira de Medicina Tropical* **40**, 631–634.

Alfellani MA, Jacob AS, Perea NO, Krecek RC, Taner-Mulla D, Verweij JJ, Levecke B, Tannich E, Clark CG and Stensvold CR (2013a) Diversity and distribution of Blastocystis sp. subtypes in non-human primates. *Parasitology* **140**, 966–971.

Alfellani MA, Taner-Mulla D, Jacob AS, Imeede CA, Yoshikawa H, Stensvold CR and Clark CG (2013b) Genetic diversity of Blastocystis in livestock and zoo animals. *Protist* **164**, 497–509.

Andersen LO and Stensvold CR (2016) Blastocystis in health and disease: are we moving from a clinical to a public health perspective? *Journal of Clinical Microbiology* **54**, 524–528.

Andersen LO, Bonde I, Nielsen HB and Stensvold CR (2015) A retrospective metagenomics approach to studying Blastocystis. *FEMS Microbiology Ecology* **91**, PubMed PMID: 26130823. doi: 10.1093/femsec/fiv072.

Auchtung TA, Fofanova TY, Stewart CJ, Nash AK, Wong MC, Gesell JR, Auchtung JM, Ajami NJ and Petrosino JF (2018) Investigating colonization of the healthy adult gastrointestinal tract by fungi. *mSphere* **3**, pii: e00092–18. doi: 10.1128/mSphere.00092-18.

Bates ST, Clemente JC, Flores GE, Walters WA, Parfrey LW, Knight R and Fierer N (2013) Global biogeography of highly diverse protistan communities in soil. *The ISME Journal* **7**, 652–659.

Beghini F, Pasolli E, Truong TD, Putignani L, Cacciò SM and Segata N (2017) Large-scale comparative metagenomics of Blastocystis, a common member of the human gut microbiome. *The ISME Journal* **11**, 2848–2863.

Bruijnesteijn van Coppenraet LE, Wallinga JA, Ruijs GJ, Bruins MJ and Verweij JJ (2009) Parasitological diagnosis combining an internally controlled real-time PCR assay for the detection of four protozoa in stool samples with a testing algorithm for microscopy. *Clinical Microbiology and Infection* **15**, 869–874.

Cacciò SM, Sannella AR, Manuali E, Tosini F, Sensi M, Crotti D and Pozio E (2012) Pigs as natural hosts of *Dientamoeba fragilis* genotypes found in humans. *Emerging Infectious Diseases* **18**, 838–841.

Cacciò SM, Sannella AR, Bruno A, Stensvold CR, David EB, Guimarães S, Manuali E, Magistrali C, Mahdad K, Beaman M, Maserati R, Tosini F and Pozio E (2016) Multilocus sequence typing of *Dientamoeba fragilis* identified a major clone with widespread geographical distribution. *International Journal for Parasitology* **46**, 793–798.

Cani PD (2018) Human gut microbiome: hopes, threats and promises. *Gut* **67**, 1716–1725.

Chouari R, Leonard M, Bouali M, Guermazi S, Rahli N, Zrafi I, Morin L and Sghir A (2017) Eukaryotic molecular diversity at different steps of the wastewater treatment plant process reveals more phylogenetic novel lineages. *World Journal of Microbiology and Biotechnology* **33**, 44.

Clark CG (2000) Cryptic genetic variation in parasitic protozoa. *Journal of Medical Microbiology* **49**, 489–491.

Clark CG and Stensvold CR (2016) Blastocystis: isolation, xenic cultivation, and cryopreservation. *Current Protocols in Microbiology* **43**, 20A.21.21–20A.21.28.

Clark CG, van der Giezen M, Alfellani MA and Stensvold CR (2013) Recent developments in blastocystis research. *Advances in Parasitology* **82**, 1–32.

Cociancic P, Zonta ML and Navone GT (2018) A cross-sectional study of intestinal parasitoses in dogs and children of the periurban area of La Plata (Buenos Aires, Argentina): zoonotic importance and implications in public health. *Zoonoses and Public Health* **65**, e44–e53.

Constenla M, Padrós F and Palenzuela O (2014) *Endolimax piscium* sp. nov. (Amoebozoa), causative agent of systemic granulomatous disease of cultured sole, Solea senegalensis Kaup. *Journal of Fish Diseases* **37**, 229–240.

Cristescu ME (2014) From barcoding single individuals to metabarcoding biological communities: towards an integrative approach to the study of global biodiversity. *Trends in Ecology & Evolution* **29**, 566–571.

de Jong MJ, Korterink JJ, Benninga MA, Hilbink M, Widdershoven J and Deckers-Kocken JM (2014) *Dientamoeba fragilis* and chronic abdominal pain in children: a case-control study. *Archives of Disease in Childhood* **99**, 1109–1113.

El Safadi D, Gaayeb L, Meloni D, Cian A, Poirier P, Wawrzyniak I, Delbac F, Dabboussi F, Delhaes L, Seck M, Hamze M, Riveau G and Viscogliosi E (2014) Children of Senegal River Basin show the highest prevalence of Blastocystis sp. ever observed worldwide. *BMC Infectious Diseases* **14**, 164.

Elsheikha HM, Regan CS and Clark CG (2018) Novel entamoeba findings in nonhuman primates. *Trends in Parasitology* **34**, 283–294.

Faria CP, Zanini GM, Dias GS, da Silva S, de Freitas MB, Almendra R, Santana P and Sousa MD (2017) Geospatial distribution of intestinal parasitic infections in Rio de Janeiro (Brazil) and its association with social determinants. *PLoS Neglected Tropical Diseases* **11**, e0005445.

Greigert V, Abou-Bacar A, Brunet J, Nourrisson C, Pfaff AW, Benarbia L, Pereira B, Randrianarivelojosia M, Razafindrakoto JL, Solotiana Rakotomalala R, Morel E, Candolfi E and Poirier P (2018) Human intestinal parasites in Mahajanga, Madagascar: the kingdom of the protozoa. *PLoS ONE* **13**, e0204576.

Hamad I, Abou Abdallah R, Ravaux I, Mokhtari S, Tissot-Dupont H, Michelle C, Stein A, Lagier JC, Raoult D and Bittar F (2018) Metabarcoding analysis of eukaryotic microbiota in the gut of HIV-infected patients. *PLoS ONE* **13**, e0191913.

Heitlinger E, Ferreira SCM, Thierer D, Hofer H and East ML (2017) The intestinal eukaryotic and bacterial biome of spotted hyenas: the impact of social status and age on diversity and composition. *Frontiers in Cellular and Infection Microbiology* **7**, 262.

Higa MG, Cardoso WM, Weis SMDS, França AO, Pontes ERJC, Silva PVD, Oliveira MP and Dorval MEMC (2017) Intestinal parasitism among waste pickers in Mato Grosso do Sul, Midwest Brazil. *Revista do Instituto de Medicina Tropical de Sao Paulo* **59**, e87.

Holtman GA, Kranenberg JJ, Blanker MH, Ott A, Lisman-van Leeuwen Y and Berger MY (2017) *Dientamoeba fragilis* colonization is not associated with gastrointestinal symptoms in children at primary care level. *Family Practice* **34**, 25–29.

Jacob AS, Busby EJ, Levy AD, Komm N and Clark CG (2016) Expanding the entamoeba universe: new hosts yield novel ribosomal lineages. *Journal of Eukaryotic Microbiology* **63**, 69–78.

Jeske S, Bianchi TF, Moura MQ, Baccega B, Pinto NB, Berne MEA and Villela MM (2018) Intestinal parasites in cancer patients in the South of Brazil. *Brazilian Journal of Biology* **78**, 574–578.

Jokelainen P, Hebbelstrup Jensen B, Andreassen BU, Petersen AM, Röser D, Krogfelt KA, Nielsen HV and Stensvold CR (2017) *Dientamoeba fragilis* – a commensal in children in Danish day care centers. *Journal of Clinical Microbiology* 55, 1707–1713.

Jones BM and Kustka AB (2017) A quantitative SMRT cell sequencing method for ribosomal amplicons. *Journal of Microbiological Methods* 135, 77–84.

Kriss M, Hazleton KZ, Nusbacher NM, Martin CG and Lozupone CA (2018) Low diversity gut microbiota dysbiosis: drivers, functional implications and recovery. *Current Opinion in Microbiology* 44, 34–40.

Krogsgaard LR, Engsbro AL, Stensvold CR, Nielsen HV and Bytzer P (2015) The prevalence of intestinal parasites is not greater among individuals with irritable bowel syndrome: a population-based case-control study. *Clinical Gastroenterology and Hepatology* 13, 507–513.e502

Krogsgaard LR, Andersen LO, Johannesen TB, Engsbro AL, Stensvold CR, Nielsen HV and Bytzer P (2018) Characteristics of the bacterial microbiome in association with common intestinal parasites in irritable bowel syndrome. *Clinical and Translational Gastroenterology* 9, 161.

Laforest-Lapointe I and Arrieta MC (2018) Microbial eukaryotes: a missing link in gut microbiome studies. *mSystems* 3, pii: e00201–17.

Lukeš J, Stensvold CR, Jirků-Pomajbíková K and Wegener Parfrey L (2015) Are human intestinal eukaryotes beneficial or commensals? *PLoS Pathogens* 11, e1005039.

Maritz JM, Rogers KH, Rock TM, Liu N, Joseph S, Land KM and Carlton JM (2017) An 18S rRNA workflow for characterizing protists in sewage, with a focus on zoonotic trichomonads. *Microbial Ecology* 74, 923–936.

Masuda A, Sumiyoshi T, Ohtaki T and Matsumoto J (2018) Prevalence and molecular subtyping of Blastocystis from dairy cattle in Kanagawa, Japan. *Parasitology International* 67, 702–705.

Matsubayashi M, Shimada Y, Li YY, Harada H and Kubota K (2017) Phylogenetic diversity and *in situ* detection of eukaryotes in anaerobic sludge digesters. *PLoS ONE* 12, e0172888.

Matsunaga K, Kubota K and Harada H (2014) Molecular diversity of eukaryotes in municipal wastewater treatment processes as revealed by 18S rRNA gene analysis. *Microbes and Environments* 29, 401–407.

Mirjalali H, Abbasi MR, Naderi N, Hasani Z, Mirsamadi ES, Stensvold CR, Balaii H, Asadzadeh Aghdaei H and Zali MR (2017) Distribution and phylogenetic analysis of Blastocystis sp. subtypes isolated from IBD patients and healthy individuals in Iran. *European Journal of Clinical Microbiology & Infectious Diseases* 36, 2335–2342.

Moon-van der Staay SY, van der Staay GW, Michalowski T, Jouany JP, Pristas P, Javorský P, Kišidayová S, Varadyova Z, McEwan NR, Newbold CJ, van Alen T, de Graaf R, Schmid M, Huynen MA and Hackstein JH (2014) The symbiotic intestinal ciliates and the evolution of their hosts. *European Journal of Protistology* 50, 166–173.

Moreno AM, Matz C, Kjelleberg S and Manefield M (2010) Identification of ciliate grazers of autotrophic bacteria in ammonia-oxidizing activated sludge by RNA stable isotope probing. *Appl Environ Microbiol* 76, 2203–2211.

Morton ER, Lynch J, Froment A, Lafosse S, Heyer E, Przeworski M, Blekhman R and Ségurel L (2015) Variation in Rural African gut microbiota is strongly correlated with colonization by entamoeba and subsistence. *PLoS Genetics* 11, e1005658.

Moura RGF, Oliveira-Silva MB, Pedrosa AL, Nascentes GAN and Cabrine-Santos M (2018) Occurrence of Blastocystis spp. in domestic animals in Triângulo Mineiro area of Brazil. *Revista da Sociedade Brasileira de Medicina Tropical* 51, 240–243.

Munasinghe VS, Vella NG, Ellis JT, Windsor PA and Stark D (2013) Cyst formation and faecal-oral transmission of *Dientamoeba fragilis* – the missing link of the life cycle of an emering pathogen. *International Journal for Parasitology* 43, 879–883.

Myer PR, Kim M, Freetly HC and Smith TP (2016) Metagenomic and near full-length 16S rRNA sequence data in support of the phylogenetic analysis of the rumen bacterial community in steers. *Data in Brief* 8, 1048–1053.

Nakano K, Shiroma A, Shimoji M, Tamotsu H, Ashimine N, Ohki S, Shinzato M, Minami M, Nakanishi T, Teruya K, Satou K and Hirano T (2017) Advantages of genome sequencing by long-read sequencer using SMRT technology in medical area. *Human Cell* 30, 149–161.

Navarro C, Domínguez-Márquez MV, Garijo-Toledo MM, Vega-García S, Fernández-Barredo S, Pérez-Gracia MT, García A, Borrás R and Gómez-Muñoz MT (2008) High prevalence of Blastocystis sp. in pigs reared under intensive growing systems: frequency of ribotypes and associated risk factors. *Veterinary Parasitology* 153, 347–358.

Neres-Norberg A, Guerra-Sanches F, Blanco Moreira-Norberg PR, Madeira-Oliveira JT, Santa-Helena AA and Serra-Freire NM (2014) [Intestinal Parasitism in Terena Indigenous People of the Province of Mato Grosso do Sul, Brazil]. *Revista de Salud Publica (Bogota)* 16, 859–870.

Ögren J, Dienus O, Löfgren S, Einemo IM, Iveroth P and Matussek A (2015) *Dientamoeba fragilis* prevalence coincides with gastrointestinal symptoms in children less than 11 years old in Sweden. *European Journal of Clinical Microbiology & Infectious Diseases* 34, 1995–1998.

Ohkuma M (2008) Symbioses of flagellates and prokaryotes in the gut of lower termites. *Trends in Microbiology* 16, 345–352.

Oliverio AM, Power JF, Washburne A, Cary SC, Stott MB and Fierer N (2018) The ecology and diversity of microbial eukaryotes in geothermal springs. *The ISME Journal* 12, 1918–1928.

Orr RJS, Zhao S, Klaveness D, Yabuki A, Ikeda K, Makoto WM and Shalchian-Tabrizi K (2018) Enigmatic Diphyllatea eukaryotes: culturing and targeted PacBio RS amplicon sequencing reveals a higher order taxonomic diversity and global distribution. *BMC Evolutionary Biology* 18, 115.

Pakandl M (1991) Occurrence of Blastocystis sp. in pigs. *Folia Parasitologica (Praha)* 38, 297–301.

Parfrey LW, Walters WA and Knight R (2011) Microbial eukaryotes in the human microbiome: ecology, evolution, and future directions. *Frontiers in Microbiology* 2, 153.

Peek R, Reedeker FR and van Gool T (2004) Direct amplification and genotyping of *Dientamoeba fragilis* from human stool specimens. *Journal of Clinical Microbiology* 42, 631–635.

Petersen AM, Stensvold CR, Mirsepasi H, Engberg J, Friis-Møller A, Porsbo LJ, Hammerum AM, Nordgaard-Lassen I, Nielsen HV and Krogfelt KA (2013) Active ulcerative colitis associated with low prevalence of Blastocystis and *Dientamoeba fragilis* infection. *Scandinavian Journal of Gastroenterology* 48, 638–639.

Poulsen CS and Stensvold CR (2016) Systematic review on *Endolimax nana*: a less well studied intestinal ameba. *Tropical Parasitology* 6, 8–29.

Poulsen CS, Efunshile AM, Nelson JA and Stensvold CR (2016) Epidemiological aspects of Blastocystis colonization in children in Ilero, Nigeria. *American Journal of Tropical Medicine and Hygiene* 95, 175–179.

Powers JG, Weigman VJ, Shu J, Pufky JM, Cox D and Hurban P (2013) Efficient and accurate whole genome assembly and methylome profiling of E. coli. *BMC Genomics* 14, 675.

Ramirez JD, Sanchez LV, Bautista DC, Corredor AF, Florez AC and Stensvold CR (2014) Blastocystis subtypes detected in humans and animals from Colombia. *Infection Genetics and Evolution* 22, 223–228.

Rene BA, Stensvold CR, Badsberg JH and Nielsen HV (2009) Subtype analysis of Blastocystis isolates from Blastocystis cyst excreting patients. *American Journal of Tropical Medicine and Hygiene* 80, 588–592.

Rosenberg K, Bertaux J, Krome K, Hartmann A, Scheu S and Bonkowski M (2009) Soil amoebae rapidly change bacterial community composition in the rhizosphere of *Arabidopsis thaliana*. *The ISME Journal* 3, 675–684.

Roser D, Simonsen J, Nielsen HV, Stensvold CR and Molbak K (2013) *Dientamoeba fragilis* in Denmark: epidemiological experience derived from four years of routine real-time PCR. *European Journal of Clinical Microbiology & Infectious Diseases* 32, 1303–1310.

Rossen NG, Bart A, Verhaar N, van Nood E, Kootte R, de Groot PF, D'Haens GR, Ponsioen CY and van Gool T (2015) Low prevalence of Blastocystis sp. in active ulcerative colitis patients. *European Journal of Clinical Microbiology & Infectious Diseases* 34, 1039–1044.

Royer TL, Gilchrist C, Kabir M, Arju T, Ralston KS, Haque R, Clark CG and Petri WA (2012) *Entamoeba bangladeshi* nov. sp., Bangladesh. *Emerging Infectious Diseases* 18, 1543–1545.

Ruaux CG and Stang BV (2014) Prevalence of blastocystis in shelter-resident and client-owned companion animals in the US Pacific Northwest. *PLoS ONE* 9, e107496.

Růžková J, Květoňová D, Jirků M, Lhotská Z, Stensvold CR, Parfrey LW and Jirků Pomajbíková K (2018) Evaluating rodent experimental models for studies of Blastocystis ST1. *Experimental Parasitology* 191, 55–61.

Salehi R, Haghighi A, Stensvold CR, Kheirandish F, Azargashb E, Raeghi S, Kohansal C and Bahrami F (2017) Prevalence and subtype identification of. *Gastroenterology and Hepatology From Bed To Bench* 10, 235–241.

Scanlan PD, Stensvold CR, Rajilić-Stojanović M, Heilig HG, De Vos WM, O'Toole PW and Cotter PD (2014) The microbial eukaryote Blastocystis is a prevalent and diverse member of the healthy human gut microbiota. *FEMS Microbiology Ecology* 90, 326–330.

Scanlan PD, Hill CJ, Ross RP, Ryan CA, Stanton C and Cotter PD (2018) The intestinal protist Blastocystis is not a common member of the healthy infant gut microbiota in a Westernized country (Ireland). *Parasitology* **145**, 1274–1278.

Silberman JD, Clark CG, Diamond LS and Sogin ML (1999) Phylogeny of the genera Entamoeba and Endolimax as deduced from small-subunit ribosomal RNA sequences. *Molecular Biology and Evolution* **16**, 1740–1751.

Stark D, Beebe N, Marriott D, Ellis J and Harkness J (2005*a*) Detection of *Dientamoeba fragilis* in fresh stool specimens using PCR. *International Journal for Parasitology* **35**, 57–62.

Stark D, Beebe N, Marriott D, Ellis J and Harkness J (2005*b*) Prospective study of the prevalence, genotyping, and clinical relevance of *Dientamoeba fragilis* infections in an Australian population. *Journal of Clinical Microbiology* **43**, 2718–2723.

Stark D, Beebe N, Marriott D, Ellis J and Harkness J (2006) Evaluation of three diagnostic methods, including real-time PCR, for detection of *Dientamoeba fragilis* in stool specimens. *Journal of Clinical Microbiology* **44**, 232–235.

Stark D, Phillips O, Peckett D, Munro U, Marriott D, Harkness J and Ellis J (2008) Gorillas are a host for *Dientamoeba fragilis*: an update on the life cycle and host distribution. *Veterinary Parasitology* **151**, 21–26.

Stark D, Garcia LS, Barratt JL, Phillips O, Roberts T, Marriott D, Harkness J and Ellis JT (2014) Description of *Dientamoeba fragilis* cyst and precystic forms from human samples. *Journal of Clinical Microbiology* **52**, 2680–2683.

Stark D, Barratt J, Chan D and Ellis JT (2016) *Dientamoeba fragilis*, the neglected trichomonad of the human bowel. *Clinical Microbiology Reviews* **29**, 553–580.

Stensvold CR and Clark CG (2016) Current status of Blastocystis: a personal view. *Parasitology International* **65**, 763–771.

Stensvold CR and Nielsen HV (2012) Comparison of microscopy and PCR for detection of intestinal parasites in Danish patients supports an incentive for molecular screening platforms. *Journal of Clinical Microbiology* **50**, 540–541.

Stensvold CR and van der Giezen M (2018) Associations between Gut Microbiota and common luminal intestinal parasites. *Trends in Parasitology* **34**, 369–377.

Stensvold CR, Lebbad M and Clark CG (2010) Genetic characterisation of uninucleated cyst-producing *Entamoeba* spp. from ruminants. *International Journal for Parasitology* **40**, 775–778.

Stensvold CR, Alfellani M and Clark CG (2011a) Levels of genetic diversity vary dramatically between Blastocystis subtypes. *Infection Genetics and Evolution* **12**, 263–273.

Stensvold CR, Lebbad M and Verweij JJ (2011b) The impact of genetic diversity in protozoa on molecular diagnostics. *Trends in Parasitology* **27**, 53–58.

Stensvold CR, Lebbad M, Victory EL, Verweij JJ, Tannich E, Alfellani M, Legarraga P and Clark CG (2011c) Increased sampling reveals novel lineages of Entamoeba: consequences of genetic diversity and host specificity for taxonomy and molecular detection. *Protist* **162**, 525–541.

Stensvold CR, Lebbad M and Clark CG (2012) Last of the human protists: the phylogeny and genetic diversity of iodamoeba. *Molecular Biology and Evolution* **29**, 39–42.

Stensvold CR, Clark CG and Röser D (2013) Limited intra-genetic diversity in *Dientamoeba fragilis* housekeeping genes. *Infection Genetics and Evolution* **18**, 284–286.

Stensvold CR, Winiecka-Krusnell J, Lier T and Lebbad M (2018) Evaluation of a PCR method for detection of *Entamoeba polecki*, with an overview of Its molecular epidemiology. *Journal of Clinical Microbiology* **56**, pii: e00154–18.

ten Hove R, Schuurman T, Kooistra M, Möller L, van Lieshout L and Verweij JJ (2007) Detection of diarrhoea-causing protozoa in general practice patients in The Netherlands by multiplex real-time PCR. *Clinical Microbiology and Infection* **13**, 1001–1007.

Udonsom R, Prasertbun R, Mahittikorn A, Mori H, Changbunjong T, Komalamisra C, Pintong AR, Sukthana Y and Popruk S (2018) Blastocystis infection and subtype distribution in humans, cattle, goats, and pigs in central and western Thailand. *Infection Genetics and Evolution* **65**, 107–111.

Veira DM (1986) The role of ciliate protozoa in nutrition of the ruminant. *Journal of Animal Science* **63**, 1547–1560.

Verweij JJ (2014) Application of PCR-based methods for diagnosis of intestinal parasitic infections in the clinical laboratory. *Parasitology* **141**, 1863–1872.

Verweij JJ and Stensvold CR (2014) Molecular testing for clinical diagnosis and epidemiological investigations of intestinal parasitic infections. *Clinical Microbiology Reviews* **27**, 371–418.

Verweij JJ and van Lieshout L (2011) Intestinal parasitic infections in an industrialized country; a new focus on children with better DNA-based diagnostics. *Parasitology* **138**, 1492–1498.

Verweij JJ, Polderman AM and Clark CG (2001) Genetic variation among human isolates of uninucleated cyst-producing entamoeba species. *Journal of Clinical Microbiology* **39**, 1644–1646.

Verweij JJ, Mulder B, Poell B, van Middelkoop D, Brienen EA and van Lieshout L (2007) Real-time PCR for the detection of *Dientamoeba fragilis* in fecal samples. *Molecular and Cellular Probes* **21**, 400–404.

Wang W, Owen H, Traub RJ, Cuttell L, Inpankaew T and Bielefeldt-Ohmann H (2014) Molecular epidemiology of Blastocystis in pigs and their in-contact humans in Southeast Queensland, Australia, and Cambodia. *Veterinary Parasitology* **203**, 264–269.

Wilcox JJS and Hollocher H (2018) Unprecedented symbiont eukaryote diversity Is governed by internal trophic webs in a wild Non-human primate. *Protist* **169**, 307–320.

Xiong J, Yu W, Dai W, Zhang J, Qiu Q and Ou C (2018) Quantitative prediction of shrimp disease incidence *via* the profiles of gut eukaryotic microbiota. *Applied Microbiology and Biotechnology* **102**, 3315–3326.

Yoshikawa H, Koyama Y, Tsuchiya E and Takami K (2016) Blastocystis phylogeny among various isolates from humans to insects. *Parasitology International* **65**, 750–759.

Yu Y, Lee C, Kim J and Hwang S (2005) Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnology and Bioengineering* **89**, 670–679.