Human Trichuriasis: Whipworm Genetics, Phylogeny, Transmission and Future Research Directions

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Abstract Human trichuriasis is a neglected tropical disease which affects hundreds of millions of people worldwide and is particularly prevalent among children living in areas where sanitation is poor. This review examines the current knowledge on the taxonomy, genetics and phylogeography of human *Trichuris* and its relationship to whipworm parasites in other host species. The evidence for zoonotic transmission of *Trichuris* and the emergence of anthelmintic resistance is assessed. In addition, the implications of the recent publication of the genomes and transcriptomes of multiple *Trichuris* species are discussed. Finally, priorities for future research in *Trichuris* genetics are proposed.

Keywords *Trichuris* · Whipworm · Phylogenetics · Genomics · Ancient DNA · Soil-transmitted helminth · Zoonosis · Anthelmintic resistance

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Introduction

Human trichuriasis is a neglected tropical disease caused by the whipworm parasites of the genus *Trichuris*. *Trichuris* is a highly prevalent soil-transmitted helminth (STH) infection, with an estimated 465 million people infected worldwide [1]. Infections are particularly prevalent among children living in communities where hygiene and sanitation are poor. Medium to heavy infections are generally associated with chronic morbidities including dysentery, abdominal pathology, stunting and wasting and reduced cognitive capacity, all of which can significantly perturb child development [1, 2]. It has been estimated that trichuriasis contributes 0.64 million disability-adjusted life years (DALYs) to the global burden of disease [3]. Despite the prevalence and undoubted public health importance of human trichuriasis, the number of studies published on the genetics and phylogeography of the *Trichuris* parasite is limited, especially when compared to the giant roundworm *Ascaris* which has similar epidemiological characteristics [4]. One possible explanation for this paucity of publications could be the difficulty in obtaining parasite material. Adult *Ascaris* worms are large and can be readily collected from infected individuals through chemoexpulsion. In contrast, adult *Trichuris* are much smaller and are embedded in the mucosa of the large intestine, which can make chemoexpulsion less efficient and expelled worms more difficult to spot [5].

Taxonomy and Phylogenetics

The genus *Trichuris* belongs to the class Enoplea and the order Trichinellida together with *Trichinella spiralis*. In the NCBI Taxonomy Browser, there are 16 designated species in the genus *Trichuris* that are found in different mammalian hosts.

Trichuris suis in pigs is the most closely related species to *Trichuris trichiura* but whether *Trichuris* in dogs and rodents (*Trichuris vulpis*, *Trichuris arvicolae*, *Trichuris muris*) or ruminants (*Trichuris ovis*, *Trichuris discolor*, *Trichuris skrjabini*) diverged before or after *Trichuris trichiura* is not yet settled [6, 7, 8•, 9•].

Whipworms in humans are traditionally designated *Trichuris trichiura*. However, recent publications have begun to address whether several species circulate in the human population and whether non-human primates (NHPs) share whipworms with humans. As the *Trichuris* genus has a wide geographical distribution and infects multiple host species, it is a likely candidate to contain cryptic species, meaning those that cannot be identified to species using traditional methods, including morphological examination [10].

Sampling of *Trichuris* from a range of wild and captive NHP host species, followed by sequencing of nuclear and mitochondrial markers, has allowed a more careful interrogation of the taxonomic relationship of *Trichuris* parasites in humans and NHP and the potential for cross-species transmission. Ravasi et al. sampled *Trichuris* from wild baboons on the Cape Peninsula in South Africa and compared internal transcribed spacer (ITS) sequences with those from human and pig *Trichuris* [11]. They found that the human *Trichuris* fell into two distinct clades (DG and CP-COB) both shared with *Trichuris* from baboons. Similar results were obtained by full mitochondrial DNA (mtDNA) genome analysis of worms from humans in China and Uganda and from baboons held in captivity in USA and Denmark [12]. Strikingly, the genetic distance between these worms was up to 20 %, and these studies suggest that *Trichuris* in humans comprises several species and that there is potential for parasite transmission between humans and baboons. *Trichuris* collected from captive baboons in Denmark and the USA were all found to fall in one phylogenetic group together with human *Trichuris* from Uganda based on sequence analysis of the conserved beta-tubulin gene, which appears to correspond to clade DG [13]. Analysis of *Trichuris* collected from humans and a range of NHP species living in and around Kibale National Park in Uganda revealed a similar separation of primate *Trichuris* into distinct clades to that described by Ravasi et al. [11]. Further substructuring of clade CP-GOB into two groups was suggested, one group comprising *Trichuris* from colobus and yellow-cheeked gibbon, and the other containing *Trichuris* from eight species of primate hosts including humans [14]. Sequencing of mitochondrial and nuclear markers of *Trichuris* collected from the endangered François' leaf-monkey indicated that this might represent a separate *Trichuris* species [15]. A very recent comprehensive study [8•] has integrated new and published data into the phylogeny proposed by Callejon et al. [16]. The authors proposed that clade 2 contains two subclades, DG (baboon and human *Trichuris*) and MF (macaque *Trichuris*), and clade 1 has at least two subclades, CA

(*Trichuris* from range of primate species including humans) and SUIS (*Trichuris* from pigs and occasionally humans), with *Trichuris* from black-and-white colobus and gibbons falling into subclade CA or a separate grouping depending on the method used for phylogenetic tree building. *Trichuris* from black-and-white colobus has recently been described as a new *Trichuris* species based on morphology and named *Trichuris colobae* [17]. Our own analysis of the data used by Cavallero et al. but also including ITS sequences from François' leaf-monkey *Trichuris* identified the same clades and subclades and suggested that François' leaf-monkey *Trichuris* comprises its own subclade (see Fig. 1).

Whipworms in humans may therefore represent multiple *Trichuris* species, some of which are shared with NHPs or alternately comprise a species complex [8•, 12]. More species or subspecies may be identified in the future as current studies have been hampered by small numbers of samples from humans in only a few localities and by different researchers using different genetic markers. Only recently, multiple *Trichuris* from humans from two geographically distinct areas, namely, China ($n=7$) and Ecuador ($n=15$), were genetically compared by sequencing the *rrnL* gene of the mtDNA [19•]. A phylogeographic pattern was observed as worms from the same region clustered together, but whether they represent a subspecies remains unclear. There is an urgent need to sample additional worms from humans and NHPs from locations worldwide and apply multiple genetic markers to further explore the taxonomic relationship between worms. Previous studies have mainly relied on sequence analysis of the ITS regions [8•, 11, 14, 20, 21•], and as this region contains multiple repeats, the alignment of sequences even between closely related worms will include a number of gaps. This makes inference of the phylogenetic relationship between worms problematic, and it is therefore highly recommended to supplement such analysis with other genetic markers such as mitochondrial DNA genes [16].

Zoonotic Transmission

Although it is generally considered that most cases of human trichuriasis are attributable to the species *Trichuris trichiura*, there is some evidence of human infection with other *Trichuris* species and of zoonotic transmission of *Trichuris* parasites. Based on identification of its characteristically large eggs in human faecal samples, it has been suggested that *Trichuris vulpis*, which generally infects dogs and wolves, can be transmitted to humans [22]. However, there are natural variations in the size of *Trichuris trichiura* eggs, and females may produce large eggs which are similar in size to *Trichuris vulpis* eggs [22, 23], so conclusions based on egg size alone should be treated with caution. Using molecular markers, 11 % of *Trichuris*-positive children in Thailand were shown

Fig. 1 Phylogenetic tree based on sequences of the entire ITS region (ITS1–5.8S–ITS2) of *Trichuris* from humans, non-human primates and other mammalian hosts [8•] and including ITS sequences from *Trichuris* from François' leaf-monkey [14]. Sequences were aligned using MUSCLE, and a maximum likelihood tree was constructed in MEGA v6 [18], using the Tamura-Nei model with gamma distributed rates and including invariant sites. *Ascaris lumbricoides* was used as an outgroup and branch support is provided by bootstrapping (1000 replicates). Host species which have been associated with each subclade, either in this analysis or in published studies discussed in the main text, are indicated in brackets. NHP indicates a range of species including baboon, grivet, red-tailed monkey, L'Hoest's monkey, blue monkey, eastern red colobus, grey-cheeked mangabey and chimpanzee. Clades and subclades are labelled as in [8•]. Triangles indicate multiple sequences and the width of the triangle is proportional to the number of sequences. A new subclade (SUBCLADE-TF for *Trachypithecus francoisi* or François' leaf monkey) is proposed. A tree constructed using the neighbour joining method showed a similar topology, although in this case the proposed SUBCLADE-TF fell clearly within clade 2

to have *Trichuris vulpis* eggs in their faeces, highlighting the possible zoonotic potential of this parasite in this region [24].

It is anticipated that *Trichuris suis*, which naturally infects pigs and can cause substantial economic losses in pig production, is a more obvious candidate for zoonotic transmission than *Trichuris vulpis*, as it is more closely related to *Trichuris trichiura* (see Fig. 1). However, morphological examination alone does not allow eggs, larvae and adult worms to be distinguished between *Trichuris trichiura* and *Trichuris suis* (reviewed in [25]). This means that potential cases of cross-

transmission between host species cannot be identified using standard parasitological methods. Recently, there have been some efforts to apply molecular approaches to investigate the zoonotic potential of *Trichuris suis*. Nissen et al. (2012) demonstrated that 3 of 29 worms from humans living sympatrically with pigs in Uganda were in fact *Trichuris suis* [21•]. They used sequencing and PCR-RFLP analysis of the ITS-2 region, supplemented by sequencing of the beta-tubulin gene. In contrast, analysis of nuclear and mitochondrial markers in *Trichuris* sampled from pigs and humans in rural Ecuador

did not find any evidence of zoonotic transmission of *Trichuris suis* in this setting [19•]. However, two pig *Trichuris* demonstrated a “heterozygous-type” PCR-RFLP pattern, suggesting that genetic exchange had taken place between *Trichuris trichiura* and *Trichuris suis*. Sequencing of the ITS region and the *rrnL* gene of *Trichuris* derived from pigs and humans in China did not provide any evidence of cross-transmission between species; however, the number of samples ($n=16$) examined was very limited [9•, 26]. Further sympatric sampling of *Trichuris* from humans and dogs or pigs in different geographical locations combined with molecular analysis is warranted and required to determine the extent and importance of transmission of *Trichuris* between domestic animals and humans.

Trichuris infections have been found in a range of NHP species living in natural habitats including colobus monkeys, macaques, baboons and chimpanzees [27–29]. Based on molecular studies as outlined above, some *Trichuris* species seem to be specific to particular NHPs whereas others most likely have the potential to circulate between humans and NHPs as they are genetically identical. This is particularly important when humans and NHPs are living in close proximity, as is becoming increasingly common with human encroachment into pristine habitats and NHPs accessing gardens and farms in search of food, and has significant implications for both human health and wildlife conservation.

Anthelmintic Resistance

Regular mass distribution of benzimidazole anthelmintic drugs, i.e. single-dose albendazole or mebendazole, to school-aged children is the mainstay of control of human trichuriasis [30], and there is currently a scale-up of deworming programmes to reach the WHO target of providing preventive chemotherapy to 75 % of all preschool- and school-age children at risk by 2020 [4, 31]. However, when used as single doses, albendazole and mebendazole are not very effective for treatment of *Trichuris*, with cure rates in the order of 30–70 %. Concerns about the development of drug resistance have also been raised [2, 5, 32, 33]. The frequent use of anthelmintics for treatment of nematodes of veterinary importance, such as gastrointestinal nematodes of ruminants and horses, has led to the widespread development of anthelmintic resistance [34–37].

Resistance to benzimidazole drugs is commonly associated with a single SNP in the parasite beta-tubulin gene leading to substitution of phenylalanine by tyrosine at codon 200 [38]. Non-synonymous SNPs at codons 167 and 198 are also occasionally associated with resistance [39, 40]. Initial work investigating sequence variation in the beta-tubulin gene of 72 *Trichuris trichiura* isolates from seven countries did not identify any mutations indicative of benzimidazole resistance [41].

It was suggested that anthelmintic resistance was likely to arise less rapidly in *Trichuris trichiura* than in trichostrongyles of farmed animals due its lower genetic diversity and smaller effective population size. More recently, pyrosequencing assays have been developed to detect non-synonymous SNPs at codons 167, 198 and 200 in *Trichuris trichiura* and other soil-transmitted helminths [42, 43]. Among *Trichuris* sampled from Kenyan children who were believed not to have received benzimidazole treatment, 2.6 % were homozygous for the resistance mutation at codon 200 suggesting that these SNPs are naturally occurring in the whipworm populations. In addition, five out of eight *Trichuris trichiura* egg pools collected from children treated with a single dose of albendazole contained only the codon 200 mutation associated with resistance [42]. In *Trichuris trichiura* samples collected before and after treatment with albendazole in Kenya and Haiti, there was a significant increase of the homozygous resistance genotype at codon 200 of beta-tubulin following treatment, and this was associated with poor egg reduction rates in both locations [44•]. Polymorphisms were also detected at codons 198 and 167 in *Trichuris* from Panama. In contrast, Hansen et al. did not detect any SNPs at beta-tubulin codons 167, 198 and 200 in *Trichuris* from humans in Uganda, domestic animals, wildlife and captive baboons [13, 45]. However, 41 % of the human *Trichuris* samples were collected by chemoexpulsion using mebendazole, which may partly explain the absence of resistance SNPs. It is likely that when using DNA extracted from expelled worms, the resistance markers are less frequently observed as only worms without the resistance marker will be expelled. In contrast, screening of eggs from faeces will provide insights into the beta-tubulin genotypes of resistant and non-resistant female worms which produced the eggs.

In conclusion, although the data to date on whipworms do demonstrate the presence of SNPs in the beta-tubulin gene which are associated with anthelmintic resistance in other nematodes, more studies are required to definitively demonstrate that benzimidazole treatment is exerting selective pressure to increase the frequency of these mutations and that these SNPs are responsible for reduced efficacy of albendazole and mebendazole in treatment of human trichuriasis. Other, not mutually exclusive, explanations for poor performance of anthelmintics could include the location of adult *Trichuris trichiura* in the large intestine as the drug has to pass through most of the gastrointestinal system before it reaches the parasite. Another factor influencing poor treatment outcomes may be the fact that the anterior part of the *Trichuris trichiura* worm is embedded in the intestinal mucosa. Because of this, although worms may be paralysed by the anthelmintic, they are less likely to be expelled as they are attached to the host and can recover once the treatment has passed through the system. Also, while it is thought that the drug enters the worms through passive diffusion [46],

Trichuris may be able to excrete the drug through P-glycoprotein-mediated transport [47].

Ancient DNA

Paleoparasitological studies have provided evidence for human infection with *Trichuris* for thousands of years even in places where infections are no longer prevalent, such as Europe and North America. This is based on the recovery of *Trichuris* eggs in archeological contexts such as human intestinal content, coprolites, latrine or environmental samples [48–52]. Traditionally, paleoparasitological studies rely on morphological assessments of parasite eggs, which limit identifications to genus level as discussed briefly above, in the section “Zoonotic Transmission.” The major hurdle with ancient samples is degradation of DNA into small fragments that are not amplifiable using traditional PCR. Recently, typing of ancient DNA from parasite eggs has become feasible through advances in DNA extraction methods and the amplification and sequencing of short species-specific amplicons [53, 54]. In this regard, targeting small overlapping sequences of *Trichuris* 18S SSUrRNA has made it possible to reconstruct longer sequences from excavations in Denmark (1030 AD) and Korea (1755 AD) homologous to *Trichuris trichiura* [54, 55•]. Continuing technological advances should make it possible to gain more detailed molecular insights into ancient parasites shedding light on prehistorical distributions and dispersal patterns.

Genomics and Transcriptomics

With the advent of advanced and high-throughput genomic and transcriptomic tools, we are now moving into a new era where a fundamentally novel approach can be used to obtain unprecedented insight into the molecular biology of parasites [56]. The genomes of *Trichuris trichiura*, *Trichuris muris*, and *Trichuris suis* have recently been published and represent a hall mark within *Trichuris* research [57•, 58•]. In addition, for *Trichuris suis* and *Trichuris muris*, the genomes were supported by transcriptome analysis thereby providing deep insights into the molecules and genes these parasites express as they invade, live in and interact with their hosts.

The genome of *Trichuris trichiura* is about 75.2 MB in size and encodes 9650 genes and is therefore smaller in size and gene number than *Trichuris suis* (male 83.6 MB, 14,781 genes, and female 87.2 MB, 14,470 genes) and *Trichuris muris* (85.0 MB and 11,004 genes), with the majority of gene families shared between *Trichuris muris* and *Trichuris trichiura* [57•]. The karyotype of *Trichuris muris* comprises two pairs of autosomes and one pair of X and Y sex chromosomes ($2n=6$), and it appears that this also is the case for

Trichuris trichiura. The genomes encode a high number of genes likely to be involved in parasite-host interactions and in immuno-regulation (or immuno-modulation) of the infected host. For example, 618 excretory-secretory proteins were predicted for *Trichuris suis*, and while secretory proteins make up about 4 % of the gene set, they represent about 10 % of the transcribed genes [58•]. Also, the anterior end of the worms which is embedded in host mucosal tissue produces proteases and protease inhibitors which were overexpressed in both *Trichuris muris* and *Trichuris suis* suggesting a central role of these molecules in host-tissue degradation and in regulating protein functions associated with immunomodulation [57•, 58•].

Genomic and transcriptomic data also offer possibilities to discover novel drugs by targeting molecules that are vital and unique to the parasite, which is referred to as genome-guided drug discovery [59]. For both *Trichuris suis* and *Trichuris trichiura*, hundreds of potential drug targets were identified, and in Foth et al., this list was further narrowed down to contain 29 top protein candidates all representing homologues to targets for existing approved drugs [57•]. The function of small non-coding RNAs in gene regulation was investigated by Jex et al. [58•], and due to their central role as gene regulators, these RNAs species may also serve as novel drug targets [60].

Future Research Directions

Novel insights into *Trichuris* biology are expected in the coming years due to lower assay cost and easier implementation of “-omic” based approaches, including genomic, transcriptomic, epigenomic and proteomic studies. Advances have opened the way for comparative studies of whole-genome sequences at a population level [61, 62], which promise to revolutionize evolutionary and phylogeographic studies of *Trichuris*. This will lead to identification of new subspecies and hybridization events between existing species and enable a much clearer and more detailed understanding of dispersal patterns.

The major obstacle to genetic research on *Trichuris* will likely continue to be the availability of worms as anthelmintic treatments are not very effective. However, whole-genome sequencing of *Trichuris trichiura* [57•] has already identified a number of alternate drug candidates, which could make worm sampling easier in the future. Nonetheless, it is likely that readily available faecal samples containing *Trichuris* eggs will become integral in large-scale comparative studies as the required input sample is decreasing rapidly. Whole-genome sequences and even transcriptomes have been generated from single cells [63, 64], which should allow for similar studies on embryonated eggs that contain hundreds of cells in the near future. A first step could be micromanipulation that has

allowed researchers to select individual helminth eggs for subsequent PCR-based amplification [45, 65].

Despite the fact that there seems to be a pattern of infection with different *Trichuris* species infecting particular host species (Fig. 1), there are several issues that need to be further explored. Are humans infected with a single or multiple *Trichuris* spp. and, if so, can they be found in the same area and do they differ with respect to pathology, epidemiology and drug susceptibility? Is there natural *Trichuris* transmission between NHP and humans and, if so, to what extent? Are the different *Trichuris* species in primates host specific, i.e. can *Trichuris* in one clade infect hosts of the other clades? We have just started to unravel some of these questions, and there is a need for further studies applying multiple genetic marker makers to *Trichuris* collected from humans and NHPs from sympatric areas and worldwide locations. This will illuminate parasite transmission routes between these primates and allow implementation of appropriate control and prevention measures.

Unravelling the mechanisms underlying host-parasite interactions is another key future research area, which is currently being pursued on a broader scale by ‘the 50 Helminth Genomes project’ [66] and in the ‘959 Nematode Genomes’ initiative (<http://www.nematodes.org/nematodegenomes/index.php>). These genomes will provide researchers with deep and unprecedented insight into the molecular biology and evolution of helminth parasites. One poorly understood mechanism in *Trichuris* development is that of host stimulus-induced egg hatching during passage through the small intestine. This has been shown to be highly host-parasite specific as *Trichuris suis* eggs require different stimuli to *Trichuris muris* for hatching [67–69]. Other mechanisms relate to larval establishment and evasion of host-immune responses, which are better understood today due to the *Trichuris suis* and *Trichuris muris* genome and transcriptome data [57•, 58•]. *Trichuris trichiura* transcriptome data will undoubtedly also provide new insights but are not yet published.

Exosomes are small (30–100 nm) microvesicles used by complex organisms for cell-cell communication. Intriguingly, a few recent studies have shown that parasites also produce and excrete exosomes containing microRNA which can be internalized by host cells [70–72]. Interestingly, Buck et al. (2014) have shown that exosomes secreted by the gastrointestinal nematode *Heligmosomoides polygyrus* suppress the type 2 innate response in mice and activate specific immune genes in vitro [73]. It was also shown that *Trichuris suis* also secretes vesicles in the size of exosomes and that these contain RNA, although their specific function remains unknown [74]. Future studies should therefore further characterize the contents of these exosomes and explore their function in the host-parasite interplay, e.g. whether *Trichuris* use exosomes for manipulating the host’s immune response in order to dampen inflammation and optimize its survival.

In paleoparasitology, genotyping of ancient *Trichuris* eggs is likely to be carried out with increasing frequency. As *Trichuris* spp. have a narrow host range, genotyping a given egg finding has been suggested as a good way to identify the host of origin [55•], which provides paleoparasitologists with a tool to address prehistoric interactions between hosts. Furthermore, genotyping *Trichuris* eggs will provide solid anchor points in phylogeographic studies of prehistoric dispersal patterns. In this regard, *Trichuris trichiura* dispersal into the Americas is still very much debated [75]. The conundrum is that *Trichuris trichiura*, like other STH infections, can most likely not be maintained in Arctic climates which the human migration across the Beringian Land Bridge into the Americas would have necessitated [76, 77].

Finally, molecular diagnostics at the point of care is likely to become available for human trichuriasis, possibly in conjunction with other STH infections. It is imperative that testing can be performed in developing world settings with limited infrastructure and at low cost. Initial studies have identified loop-mediated isothermal amplification (LAMP) [78], or variants thereof [79, 80], as suitable technologies. Detection of DNA from *Strongyloides stercoralis* [81] and *Opisthorchis viverrini* [82] extracted from human stool samples has been achieved using LAMP. At present, the major hurdle lies in sample preparation and its integration with DNA extraction and detection [83]. This has been successfully accomplished in one device for testing of gastrointestinal pathogens, but so far not for any STHs [18, 84]. Large-scale point-of-care testing of human stool samples for STHs with molecular-based methods also raises the possibility of addressing the zoonotic potential of *Trichuris* spp. and revealing to what extent and with which species humans are actually infected.

Conclusion

Over the past few years, research has shed new light on human trichuriasis and the genetics of the *Trichuris* parasite. Significant results include publication of the *Trichuris trichiura* genome, as well as the *Trichuris suis* and *Trichuris muris* genomes and transcriptomes. This has identified important potential mechanisms of host-parasite interaction and immunomodulation, and possible novel drug targets. Alleles in the beta-tubulin gene associated with resistance in nematodes of veterinary importance have only been identified in a handful of studies on human *Trichuris trichiura* isolates. Recent progress has been made in understanding the phylogeny of the *Trichuris* genus. There seem to be host-specific patterns in infection with particular *Trichuris* species or subspecies. However, there is evidence of zoonotic transmission, especially regarding *Trichuris trichiura* infections in NHPs and possibly also for *Trichuris suis* in pigs and *Trichuris vulpis* in dogs. Future research priorities should include multiple

genetic marker analysis of *Trichuris* sampled from humans, NHPs, pigs and dogs in sympatric and diverse geographical locations to provide insights into parasite transmission within and between host species. This will also open up new possibilities for monitoring the emergence of anthelmintic resistance and will aid in the development of point-of-care molecular tests for sensitive diagnosis. Progress in these areas will greatly enhance control of this widespread parasitic disease.

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Compliance with Ethics Guidelines

Conflict of Interest Martha Betson, Martin Jensen Søre and Peter Nejsum declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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