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Impact of next-generation technologies on exploring socio-economically important parasites and developing new interventions

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Summary

High-throughput molecular and computer technologies have become instrumental for systems biological explorations of pathogens, including parasites. For instance, investigations of the transcriptomes of different developmental stages of parasitic nematodes give insights into gene expression, regulation and function in a parasite, which is a significant step to understanding their biology, as well as interactions with their host(s) and disease. This chapter gives (i) a background on some key parasitic nematodes of socio-economic importance, (ii) describes sequencing and bioinformatic technologies for large-scale studies of the transcriptomes and genomes of these parasites, (iii) provides some recent examples of applications, and (iv~~ii~~) emphasizes the prospects of fundamental biological explorations of parasites using these technologies for the development of new interventions to combat parasitic diseases.

Key words*:* Parasitic nematodes; genomics; transcriptomics; bioinformatics; next-generation sequencing; post-genomics; anthelminthic resistance; drug targets; diagnostic markers.

Running head: Next-generation technologies to understand parasites and develop new interventions

1. Introduction

Parasitic nematodes (roundworms) of humans and other animals are of particular significance as pathogens (***1-5***). For example, the soil-transmitted helminths (STHs) *Ascaris* spp. (giant roundworm), *Ancylostoma duodenale*, *Necator americanus* (hookworms) and *Trichuris trichiura* (whipworm) are estimated to infect almost one sixth of all humans (***6***, ***7***), and parasitic nematodes of livestock, including species of *Haemonchus*, *Ostertagia* and *Trichostrongylus* cause substantial losses estimated at billions of dollars per annum, due to poor productivity, failure to thrive, the costs of anthelmintic treatment and deaths (***8-10***). In addition to their socioeconomic impact, anthelmintic resistance in nematodes of livestock (***11-13***) has stimulated research towards developing alternative intervention and control strategies against these parasites. In spite of some knowledge of parasites and the diseases that they cause (***14, 15***), little is known about essential molecular processes and mechanisms in parasitic nematodes. Gaining an improved understanding of the molecular biology of these organisms offers a possible pathway for discovering new methods of diagnosis, treatment and control of parasitic diseases. Advances in genomic and bioinformatic technologies provide exciting opportunities to explore, for example, basic developmental and reproductive processes in nematodes. In particular, studies of the transcriptomes of parasites have become instrumental in various areas, such as gene discovery and characterization, and for gaining insights into aspects of gene expression, regulation and function (***16-19***). The purpose of this chapter is to give (i) a background on some socio-economically important parasitic nematodes of animals, (ii) describe sequencing and bioinformatic technologies for large-scale studies of the transcriptomes and genomes of these parasites, (iii) provide some recent examples of applications, and (iv~~ii~~) emphasize the prospects of fundamental biological explorations of parasites using these technologies for the development of new interventions to combat parasitic diseases.

2. Brief background on parasitic nematodes

As one of the most diverse phyla in the animal kingdom, the phylum Nematoda includes > 28,000 species, of which > 16,000 are parasites of animals or plants (***14, 20***). This phylum consists of two main classes, the Adenophorea and the Secernentea (***21***). Within the Secernentea, species within the orders Ascaridida, Oxyurida, Spirurida and Strongylida are parasites of humans and other vertebrates (***14***). Within the latter order, the superfamily Strongyloidea includes, amongst others, some intestinal parasites of pigs, ruminants (Chabertiidae) and equids (family Strongylidae) (***14***). Members of this superfamily are characterized by complex buccal capsules, often with a series of leaf-like structures on the border of the labial region (= *corona radiata*) (***22***). In contrast, the buccal capsule, lips and *corona radiata* of species of parasitic nematodes of the superfamily Trichostrongyloidea are greatly reduced or absent (***23-25***). Members of the~~e~~is latter superfamily ~~Trichostrongyloidea~~ ('trichostrongyles') are common parasites of mammals, particularly ruminants (***14, 26***). The superfamily Ancylostomatoidea ('hookworms') includes blood-feeding nematodes, characterized by large, globular buccal capsules, which enable them to attach to the intestinal wall to feed on blood (***14***). According to a molecular classification proposed by Blaxter et al. (***27***), members of the Strongylida, such as trichostrongyles and hookworms, as well as the free-living nematodes of the sub-order Rhabditina (e.g., *Caenorhabditis elegans*) and order Diplogasterida (e.g., *Pristionchus pacificus*), belong to 'clade V' of the Nematoda.

2.1. Selected examples of nematodes (order Strongylida) of major socio-economic importance

2.1.1. Trichostrongyles

Within the superfamily Trichostrongyloidea, *Haemonchus contortus* (barber's pole worm) and *Trichostrongylus* spp., for example, are responsible for substantial production losses in the

livestock industries worldwide (***10, 28***). *H. contortus* is the most important nematode of small ruminants in subtropical and tropical (summer rainfall) areas, whereas some *Trichostrongylus* spp. are often dominant in winter rainfall areas due to their ability to develop and survive at lower temperatures than *H. contortus* does (***29***). The life cycles of *H. contortus* and *T. colubriformis* are similar and direct, with eggs (***26, 30, 31***) being laid by females in the abomasum (*H. contortus*) or small intestine (*Trichostrongylu*s) of the host (***30, 31***). Under suitable environmental conditions (***30, 32***), first-stage larvae (L1s) hatch from eggs to develop, *via* the second-stage larvae (L2s), to infective, third-stage larve (L3s). The cuticle of the L2 is retained as a sheath around the L3 and protects it from desiccation (***14, 30, 32***). Small ruminants acquire the infection by ingesting L3s from contaminated pastures. The L3s pass through the forestomachs and undergo an exsheathment process to then establish, *via* the parasitic fourth-stage larvae (L4s), as adult males and females in the abomasum (*H. contortus*) or small intestine (*Trichostrongylus*) within ~3 weeks (***14, 26, 30, 32***). The exsheathment process is triggered by stimuli within the host and may include (depending on the species of nematode) dissolved gaseous CO_2 and undissociated carbonic acid (*H. contortus*) or hydrochloric acid and pepsin (*T. colubriformis*) in the abomasum.; Tthe L3s respond to these stimuli by producing an exsheathment fluid which determines the detachment of the sheath from the bodies of the larvae (***5, 33-35***).

The adults of *H. contortus* feed on blood from vessels in the gastric wall. Consequently, the main clinical signs of acute haemonchosis are anaemia, variable degrees of oedema, as well as lethargy, decreased live-weight gain, impaired wool/milk production and decreased reproductive performance, often leading to death in severely affected animals (***36, 37***). Trichostrongylosis is triggered by the presence of adult parasites in mucus-covered tunnels in the epithelial surface of the small intestine (***38***), usually associated with extensive villous atrophy, combined with hyperplasia of the sub-mucosal glands, mucosal thickening and erosion as well as infiltration of lymphocytes and neutrophils into affected areas (***38-42***). Clinical signs of trichostrongylosis

include malabsorption, weight loss, progressive emaciation and diarrhoea (= scouring or 'black scour').

2.1.2. Hookworms

The hookworms *N. americanus* and *An. duodenale* of humans are estimated to infect ~1 billion people in rural regions of the subtropics and tropics (***1***), with the highest prevalence (~17%) recorded in areas of sub-Saharan Africa and China (***1, 43, 44***), and causing an estimated disease burden of 22 million disability-adjusted life years (DALYs) (***45***). Although *N. americanus* is the most widely distributed hookworm of humans globally (***1***), a related species, *An. caninum*, is a cosmopolitan hookworm of the small intestine of dogs and other canids (***14, 26***). The life cycle of these nematodes is direct, with female hookworms excreting thin-shelled eggs, which are passed in the faeces of the host (***26, 46***). Under suitable environmental conditions (i.e., 23-33 °C) the L1s hatch from the eggs (***26, 46***), feed on microbes and, within 2 days, moult to L2s, and then to L3s within 4-5 days. The L3 stage retains the cuticle of the L2 (i.e., sheath) and is called a 'filariform' larva (***46***). Infection occurs when the L3s penetrate the skin of the vertebrate host following cuticular shedding (***47***); then, larvae enter the subcutaneous tissues and migrate *via* the circulatory system to the heart and lungs, where they moult to fourth-stage larvae (L4s). From the lungs, the larvae migrate (*via* the airways and pharynx) to the small intestine, where they develop to adult males and females within 2-7 weeks, depending on species (***14, 26, 48, 49***). The adult stages attach by their buccal capsule to the intestinal mucosa, rupture capillaries and feed on blood (***50, 51***). Although skin penetration is considered the main route, ingestion of L3s might also lead to infection (***52***). L3s of *Ancylostoma* spp. can undergo hypobiosis (= developmental arrest) in the somatic tissues of the vertebrate host and, following activation during pregnancy, undergo transmammary transmission to the offspring (***53-55***). Hookworm disease relates mainly to the blood-feeding activity by the adult worms within the host (***50***). Focal lesions caused by the attachment of the worms are characterized by local haemorrhage, tissue cytolysis and neutrophilic immune response (***50***). The clinical expression of

disease relate mainly to iron-deficiency anaemia, which can cause physical and mental retardation and sometimes deaths in children as well as maternal mortality, impaired lactation, prematurity and low birth rates (***3, 56, 57***).

2.2. Host immune responses

Various studies have described molecules and cells implicated in host immune responses against parasitic nematodes (***2, 58-70***). The primary immunological responses induced by nematodes are dependent on the processes and mechanisms of invasion of and establishment in the host (***2***). For example, migrating hookworm L3s stimulate a marked peripheral blood eosinophilia in the mammalian host, both systemic and in the lungs (***71, 72***). Conversely, nematodes that do not undergo extensive tissue migration stimulate a mucosal immune response at the site of infection (***73***). For instance, the invasion of the abomasa of small ruminants by larvae of *H. contortus* and *T. axei* leads to a localised IgE-mediated immune response (***73***). However, it has been observed that the infection of pigs with L3s of *Oesophagostomum dentatum* is associated with a systemic production of IgG antibodies (***74-76***), followed by the formation of eosinophilic cysts containing the larvae (= nodules) within the intestinal mucosa (***77***).

In spite of variation in immune responses induced by larvae of strongylid nematodes, adult stages appear to stimulate similar immunological responses in their mammalian host(s). These responses include (i) increased production of mucus by the gastrointestinal epithelium of the host, (ii) eosinophilia and increased presence of mast cells and leucocytes at the infection site, and (iii) production of specific antibodies (***2, 60***). Responses against primary infections by gastrointestinal parasitic nematodes are reported to be linked to a T helper (Th) 2-type immune response which, in turn, relates to the secretion of multiple types of cytokines, including IL-4, IL-5, IL-9 and IL-13 (***2, 62, 68, 78-80***). In contrast, immunological responses in hosts with chronic infections appear to be regulated mainly by a Th1-type immune response, characterized by a production of IL-2, IL-18 and interferon-γ (***65, 66, 70***). In particular, individuals infected chronically by hookworms show a significant alteration of the immune response to helminth

infections, characterised by a dysfunction of the antigen-presenting ability of dentritic cells, which results in a 'hypo-responsiveness' of the antigen-induced proliferation of T-lymphocytes (***79***).

2.3. Drugs and vaccine research

The control of gastrointestinal nematodes relies heavily on the use of anthelmintic drugs (***81***). Such drugs include imidazothiazoles/tetrahydropyrimines (e.g., levamisole and pyrantel), benzimidazoles (e.g., albendazole and mebendazole) and macrocyclic lactones (e.g., ivermectin and moxidectin) (***81***). Levamisole and pyrantel act by binding to a subgroup of nematode acetylcholine receptor ion channels in parasite nerves and muscles of parasitic nematodes, resulting in an over-stimulation, spastic muscle contraction (***82***) and paralysis of the worms; the parasites are unable to move in the intestinal tract and are removed by the peristaltism of the host. Benzimidazoles are active against a range of species of nematodes (***83***); they ~~act by~~ block~~ing the~~ microtubular matrix formation by binding to tubulin (cytoskeletal protein), which is essential for various biological processes in the cell, including chromosome movement and cell division (***11, 81, 84***). Macrocyclic lactones act by opening glutamate-gated chloride channels, thus increasing the flow of chloride ions, and subsequently leading to defects in neurotransmission and flaccid paralysis (***84***). Recently, new classes of anthelmintics, ~~i.e. the~~in form of cyclooctadepsipeptides (e.g., emodepside), ~~the~~ amino acetonitrile derivatives (e.g., monepantel) and ~~the~~ 2-deoxy-paraherquamides (e.g., derquantel) have become available commercially (***85***). These compounds act by binding to presynaptic latrophilin-like receptors (emodepside), some acetylcholine receptors (monepantel) or B-subtype nicotinic acetylcholine receptors (derquantel), ~~leading to~~and cause spastic (monepantel) or flaccid (emodepside and derquantel) paralysis of some parasitic nematodes and subsequent death (***85***).

The relatively low cost, ease of administration and efficacy of anthelmintic drugs against various gastrointestinal parasitic nematodes of humans and animals has led to their extensive use and, consequently, to the emergence of resistance (***11***). Indeed, resistance in nematodes of

livestock to imidazothiazoles/tetrahydropyrimines, benzimidazoles and macrocyclic lactones has been reported, particularly in Africa, Australia, New Zealand, Asia and South America (***11, 12, 37, 84, 86***). Three mutations in the gene encoding the beta-tubulin isotype 1 in *H. contortus* were proposed to be involved in the mechanism of benzimidazole resistance (***87***). Although it was ~~propos~~suggested that a less frequent use of anthelmintics in humans (compared with their extensive use in livestock) should reduce the emergence of resistance in parasitic nematodes of humans (***88-91***), some studies (***92-97***) have reported a reduction in efficacy of mebendazole and pyrantel in *N. americanus* and *An. duodenale* in areas of Mali, Zanzibar and North Western Australia, pr~~oposed to be~~esumably attributed to resistance. Given the incomplete knowledge of the molecular mechanisms associated with resistance in parasitic nematodes (***11***), much attention is now directed to the identification of new drug targets, ~~and~~ development of new and effective anthelmintics (***98, 99***), as well as new effective strategies to prevent drug resistance (***100-102***).

Over the years, considerable research has focused on developing vaccines against selected parasitic nematodes (***8, 9, 57, 80, 103-108***). For instance, irradiated larvae were used as the basis for a vaccine against *H. contortus* and *An. caninum* infection in sheep and dogs, respectively (***109-112***). More recently, various proteins of the epithelial cell surface membrane of the digestive tract of some gastrointestinal nematodes have been evaluated as vaccine candidates in experimental murine models or in livestock (***9, 106, 107, 113***). For example, a 110 kDa integral membrane aminopeptidase of *H. contortus*, which is heavily glycosylated and localized in the brush border of the epithelial cells of the gut of the adult worm, was shown to be effective in reducing the intensity of *H. contortus* infection in different breeds and ages of sheep (***114-117***). However, protection is limited to native proteins, administered multiple times, usually in Freund's adjuvant (***118***). Another peptidase complex (P1), separated from the membrane aminopetidase H11 by ion-exchange chromatography, was identified (***119***) and shown to represent a ubiquitous component of the microvillar membrane of the intestinal cells of *H. contortus* (***119***). Although vaccination with this protein complex ~~was shown to~~ resulted in

a significant reduction (69%) in the number of *H. contortus* eggs in the faeces from vaccinated sheep following *H. contortus* challenge infection, P1 led only to a ~22-38% reduction of infection intensity (***115***). On the other hand, vaccination with the glucose-binding glycoprotein complex (H-gal-GP), separated by lectin affinity chromatography from other integral membrane proteins from the gut of adult *H. contortus* achieved ~53-72% protection and a > 90% reduction in the number of eggs in the faeces from vaccinated sheep (***120***). However, the vaccination of lambs (9 months of age) with prokaryotically expressed recombinant H-gal-GP failed to induce protective immunity against challenge infection with *H. contortus* L3s (***121***).

Other vaccine candidates have been derived from the excretory/secretory products (ES) from worms (***57, 80, 106, 122***). For example, proteases in ES from parasitic nematodes have been a major focus for vaccine development, given their inferred roles in the digestion of nutrients acquired from the host and/or during the penetration and migration through host tissues (***122***). Metalloproteases, ~~and~~ aspartic and cysteine proteases have received considerable attention for blood-feeding nematodes, such as *H. contortus*, *An. caninum* and *N. americanus* (***57, 106, 123-128***). For instance, vaccination with a cysteine protease-enriched fraction from membrane extracts from the microvillar surface of intestinal cells from adult *H. contortus* (***129***) was demonstrated to reduce infection intensity by 47%, and the number of eggs in faeces by 77% in sheep following a single challenge infection (***130***). Similarly, the vaccination of dogs with recombinant forms of a cyteine~~-~~ or aspart~~yl -~~ic protease from *An. caninum* (designated *Ac*-CP-2 and *Ac*-APR-1, respectively) resulted in partial protection against this hookworm, characterised by an absence of clinical signs and a reduced fecundity of the adult worms in dogs (***126, 131***). In addition, vaccination of hamsters with the *N. americanus* homologue of *Ac*-CP-2 (i.e., *Na*-CP-2) was shown to induce partial protection, achieving a ~30-46% reduction of infection intensity, following challenge infection with L3s (***132***).

Proteases from larval stages have also been the focus of vaccine research, because of their proposed role(s) in host invasion (***80, 133***). In ES of hookworm larvae~~s~~, for example, ~~a well characterised protease in larval ES is~~ an astacin-like zinc metalloprotease from *An. caninum*,

called *Ac*-MTP-1 (***134, 135***), ~~which~~ has been demonstrated to degrade fibronectin, laminin and collagen (***135***). Based on the results of a vaccine trial in hamsters, this protein was proposed as a potential candidate for the development of a multi-epitope vaccine (***132***). In addition, two cysteine-rich secretory proteins, known as '*Ancylostoma*-secreted proteins' (ASPs) (***136-139***), major components of ES of hookworm L3s, can represent vaccine candidates (***140***). However, the development of ASP-based vaccines ~~might be~~has been impaired ~~somewhat~~ by evidence that such molecules can cause allergic reactions in humans previously infected with *N. americanus* hookworms (***141, 142***).

Collectively, the results of studies focusing on the identification of suitable immunogens and the development of effective vaccines against gastrointestinal parasitic nematodes show that progress has been made over the years. However, there is still limited information on parasite-host interactions at the molecular level. Clearly, advanced molecular technologies provide unique opportunities to explore the molecular biology of parasitic nematodes, parasite-host interactions and diseases on a global scale, and should thus underpin the discovery of new intervention strategies. Indeed, high-throughput technologies are revolutionizing the way biology is done, allowing systems biological investigations of parasites and other pathogens, This statement applies to many areas, including genomics, proteomics and metabolomics, but also the detailed explorations of transcriptomes and associated molecular processes.

3. Some key techniques for transcriptomic investigations of parasitic nematodes

3.1. Conventional methods

The genome of any living organism includes coding regions that are transcribed into mRNAs, which are subsequently translated into proteins. Techniques, such as Northern blot (***143***), quantitative real-time, reverse transcription PCR (qRT-PCR; ***144***) and differential display (DD; ***145***) have been used to define patterns of transcription for single genes or small numbers of

molecules in parasitic nematodes, such as species of *Trichostrongylus*, *Haemonchus*, *Oesophagostomum* and *Ostertagia* (***146-153***). Another approach is the serial analysis of gene expression (SAGE) (***154***)~~. The SAGE technique~~, which is based on the generation of a short specific tag (14 bp) from each mRNA present in the sample; these tags are used for the construction of a SAGE library. The sequencing of these tags allows a relatively high-throughput determination of their frequencies in the library, which are correlated with relative amounts of the corresponding mRNAs. Despite its demonstrated utility in studies of yeast (***155***) and humans (***156, 157***), the application of SAGE for investigations of transcription in parasitic nematodes has remained limited (***158***). A single study (***159***) used SAGE to sequence and analyse ~3,000 transcripts from adult *H. contortus*, of which ~60% had homologues in public databases.

The analysis of conventional expressed sequence tag (EST) datasets has been a widely used approach for investigations of the transcriptomes of parasitic nematodes. *In vitro*, mRNAs are reverse-transcribed, resulting in stable complementary DNAs (cDNAs); ESTs usually represent single pass DNA sequence reads derived from cloned cDNAs (***160, 161***). Traditional sequencing (***162, 163***) involves the use of a DNA polymerase, an oligonucleotide primer and four deoxyribonucleotide triphosphates (dNTPs) ~~are used~~ to synthesize the complementary strand to the template sequence (***162-164***). The advent of EST sequencing marked a revolution in the field of parasitology and has been used in a range of studies aimed at investigating fundamental molecular processes in parasitic nematodes as well as drug and vaccine target discovery (e.g., ***17, 165-172***). For nematodes of animals, applications range from the analyses of stage- and gender-enriched molecules (e.g., ***149, 153, 167, 173, 174***) to global analyses of gene transcription (e.g., ***166, 170, 175-177***).

The~~Also~~ cDNA microarray technology (***178***) was a significant advance for large-scale studies of the transcriptomes of parasitic nematodes (***179***). In ~~cDNA~~these microarrays, thousands of oligonucleotides, usually cDNAs, EST clones or fragments of PCR products (which correspond to previously characterised genes/transcripts) are ‘spotted’ (= ‘arrayed’) on to glass slides or

chips in precise positions. The mRNAs from different stages or tissues are labelled with different fluorescent or radioactive markers and hybridized to the spots on the array. The relative abundance of hybridization for each mRNA population is then determined by comparing the relative signal intensity of each fluorescent marker (***178***). Supported by the increasing amount of sequence data available in public databases, microarray technology has allowed comparisons of levels of transcription of large numbers of mRNAs in, for instance, different tissues, developmental stages and sexes of these nematodes to be performed, ultimately providing researchers with the opportunity to identify molecules considered to play essential roles in fundamental biological pathways of survival, development and reproduction (***9, 179, 180***). The use of microarray technology has resulted in an expanded knowledge of the transcriptomes of socio-economically important strongylids, including *H. contortus*, *T. vitrinus*, *Oe. dentatum*, *Teladorsagia circumcincta* and *An. caninum* (***153, 174, 181-185***). In addition, the combined application of suppressive-subtractive hybridization (SSH) and microarray analysis has been useful in enabling rapid comparisons of transcriptional profiles between/among life cycle stages, genders and/or species of parasitic nematodes (***153, 183, 184, 186-188***).

Knowledge of the complement of molecules transcribed in the larval stages of strongylid nematodes should also aid the elucidation of pathways associated with infecti~~vity~~on and interactions with the vertebrate host. The molecular mechanisms that regulate the transition from the free-living to the parasitic stage of nematodes may allow the development of novel strategies to disrupt this transition. Previous studies have analysed differences in transcription between the ensheathed, free-living L3 and the exsheathed L3 of *H. contortus* (***152, 167, 189***) and the related strongylid, *An. caninum* (***182, 183***). The results of a cDNA microarray analysis, complemented by qRT-PCR of differentially transcribed molecules~~,~~ showed that, amongst others, most transcripts encoding ASPs were up-regulated in free-living L3s compared with parasitic, serum-stimulated larvae of *An. caninum* (***182***). However, a study using SSH-based microarray analysis (***183***) showed a substantial ‘up-regulation’ in the numbers and levels of transcripts encoding ASPs in serum-activated L3s (***183***).

To date, molecular studies of hookworms have mainly involved *An. caninum*, because of its use as a model for species infecting humans (***182, 183, 190-192***). Clearly, detailed knowledge and understanding of the molecules transcribed in all stages of different species of hookworms, including *N. americanus* and *An. duodenale* of humans, should facilitate the identification of conserved pathways linked to development, survival, reproduction, parasite interactions and disease, and could assist in the discovery of new intervention strategies.

3.2. High-throughput sequencing techniques

Recent advances in sequencing technologies (***193-196***; Table 1) now provide the unique opportunity to perform *de novo* analyses of the whole transcriptomes of different species, sexes and/or developmental stages of nematodes of socio-economic importance. Currently available massively parallel sequencing platforms include the 454/Roche (***193***; www.454.com), the Illumina/Solexa (***194***; www.illumina.com) and the SOLiD (= Supported Oligonucleotide Ligation and Detection) (***195***; http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Sequencing/Next-Generation-Sequencing.html) (Table 1). Due~~Thanks~~ to their capacity of generating millions or hundreds of millions of sequences simultaneosly, these platforms have been at the forefront of the genomic and transcriptomic research (***197-199***) and are powerful tools for investigating the transcriptomes of parasitic nematodes on an unprecedented scale~~ like never before~~.

The 454/Roche platform (***193***; www.454.com) uses a sequencing-by-synthesis approach. For transcriptomic studies, cDNA is randomly fragmented (by 'nebulization') into ~~fragment~~sections of variable size; adaptors are ligated to each end of these fragments, which are then mixed with a population of agarose beads whose surfaces anchor oligonucleotides complementary to the 454-specific adapter sequence, such that each bead is associated with a single fragment. Each of these complexes is transferred into individual oil-water micelles containing amplification reagents and is then subjected to an emulsion PCR (emPCR) step, during which ~10 million copies of each cDNA are produced and bound to individual beads. Subsequently, in the

sequencing phase, the beads anchoring the cDNAs are deposited on a pico-titre plate, together with other enzymes required for the pyrophosphate sequencing reaction (i.e., ATP sulfurylase and luciferase) and ~~the~~ sequencing is carried out by flowing the~~sequencing~~ reagents (nucleotides and buffers) over a plate (***200***).

Following the introduction of the 454 technology, the first Illumina (formerly Solexa) sequencer became available (***194***; www.illumina.com). This technology involves fragmentation of cDNA sample into a shotgun library, followed by the *in vitro* ligation of Illumina-specific adaptors to each cDNA template; the 3' or 5'? termini of the template are covalently attached to the surface of a glass slide (or flow cell). Attached to the flow cell are primers complementary to the other end 5' or 3'? of the template, which bend the cDNAs to form bridge-like structures. During the amplification step (bridge-PCR), clonal clusters, each consisting of ~1000 amplicons, are generated; subsequently, the cDNAs are linearised, and the sequencing reagents are directly added to the flow cell, with four types of fluorescently labelled nucleotides. After the incorporation of a fluorescent base, the flow cell is interrogated with a laser in several locations, which results in several image acquisitions at the end of a single synthesis cycle (***200***). This technology is considered ideal for both *de novo* and re-sequencing projects, targeted sequencing, single nucleotide polymorphism (SNP) analyses and gene transcription studies.

The sequencing process of the SOLiD platform (***195***; www.appliedbiosystems.com) different web site than above? employs the enzyme DNA ligase, instead of a polymerase (***200***). Briefly, after an emPCR step, the adaptor sequences of the cDNA templates bind to complementary primers that are covalently anchored to a glass slide. Subsequently, a set of four fluorescently labelled di-probes (octamers of random sequence, except known dinucleotides at the 3'-terminus) is added to the sequencing reaction. In case an octamer is complementary to the template, it will be ligated, and the two specific nucleotides can be called; subsequently, an image is acquired and the fluorescent dye is removed, so that other octamers can be ligated. After multiple ligations (e.g., 7 ligations for a 35 bp read), the newly synthesized cDNA is removed and the primer is inactivated. This process is repeated multiple times from different

starting points of the cDNA templates, so that each position is sequenced at least twice. This technique, known as 'two-base-calling', allows the correction of sequencing errors, thus providing accurate base calling (***200***). Because of the short read-length, the range of applications of the SOLiD system is considered similar to that of the Illumina technology and includes (targeted) re-sequencing projects, SNP detection and gene transcription studies.

In the last few years, numerous studies have demonstrated the utility of high-throughput sequencing for investigating, for example, aspects of the systematics, population genetics and molecular biology of helminths (***192, 201-211***). For instance, Illumina technology alone has been used to sequence the entire genomes of *A. suum* (***202***) and the human blood fluke, *Schistosoma haematobium* (***211***), whereas the 454 technology has been instrumental for *de novo* sequencing of the transcriptomes of important parasitic worms, such as *N. americanus* *Clonorchis sinensis*, *Opisthorchis viverrini*, *Fasciola hepatica* and *F. gigantica* (***205, 208-210***) of humans and other animals. Several thousands of unique and novel sequences were characterised for each of these parasites, demonstrating the capacity of this technology to generate large and informative datasets. The development of appropriate bioinformatic tools has become crucial for the detailed analyses of such datasets.

3.3. Bioinformatics

The increasing number of high-throughput sequence datasets in public databases has been accompanied by an expansion of bioinformatic tools for the analysis of such datasets, both at the cDNA, genomic DNA and protein levels. This ~~expansion~~ has resulted in the development of a number of web-based programs and/or integrated pipelines (***16, 206, 212-218***). In brief, following the acquisition of sequence data, these are firstly screened for sequence repeats, contaminants and/or adaptor sequences (***215, 219***). Following the pre-processing, sequences are 'clustered' (= assembled) into contiguous sequences (of maximum length) based on sequence similarity.

3.3.1 Assembly

The main goal of sequence assembly is to determine, with confidence, the sequence of a target transcript/gene. This process involves the alignment and merging of fragments of nucleic acids to form long contiguous sequences (i.e., contigs) (***18, 215***). Long- (e.g., generated by Sanger sequencing or 454 technology), and short-reads (e.g., Illumina or SOLiD platform) are assembled using ~~the~~ algorithms for 'overlap-layout-consensus' (***220***) and 'de Bruijn graph' (***221, 222***), respectively.

For the former algorithm (***220***), all pairwise overlaps among reads are computed and stored in a graph; all graphs are used to compute a layout of reads and then a consensus sequence of contigs (***223, 224***). Some of the assemblers designed to support long-read assembly include PHRAP (***225***), the *c*ontig *a*ssembly *p*rogram v.3 (CAP3; ***212***), the TIGR assembler (***226***), the *p*arallel *c*ontig *a*ssembly *p*rogram (PCAP; ***227***) and the *m*imicking *i*ntelligent *r*ead *a*ssembly program (MIRA; ***228***).

For the 'de Bruijn graph' (***221, 222***), reads are fragmented into short segments, called 'k-mers', where 'k' represents the number of nucleotides in each segment.~~;~~ ~~o~~Overlaps between or among k-mers are captured and stored in graphs, which are subsequently used to generate the consensus sequences (***223, 224***). Examples of programs specifically designed for the assembly of short-reads include the *s*hort *s*equence *a*ssembly by *k*-mer search and 3'-read *e*xtension (SSAKE; ***229***), Velvet (***222***), Oases (***230***), the *e*xact *de novo a*ssembler (EDENA; ***231***), Euler-SR (***232***), the *a*ssembly *by s*hort *s*equencing (ABYSS; ***233***), the *s*hort *o*ligonucleotide *a*nalysis *p*ackage (SOAP; ***234***) and Trinity (***235***).

3.3.2. Annotation and analyses

Following assembly, the contigs and single reads (or singletons) are compared with known sequence data available in public databases, in order to assign a predicted identity to each query sequence if significant matches are found (***206, 215***). In addition, assembled nucleotide sequences are usually conceptually translated into predicted proteins using algorithms that

identify protein-coding regions (open reading frames, ORFs) from individual contigs. Examples of such algorithms are OrfPredictor (***236***), ESTScan (***213***), DECODER (***237***) and ORFcor (***238***). Once peptide sequences are predicted, these are anslysed for protein ~~analyses~~identity, which includ~~ing~~es amino acid sequence comparisons with data available in public databases, in order to infer ~~and~~ known protein domains ~~are then inferred~~ (***206, 215***). For instance, the software InterProScan (***216***) provides an integrated tool for the characterization of a protein family, or an individual protein sequence, domain and/or functional site by comparing sequences with information available in the databases PROSITE (***239***), PRINTS (***240***), Pfam (***241***), ProDom (***242***), SMART (***243***) and/or Gene Ontology (GO; ***244***). In addition, other programs are available for the prediction of transmembrane domains (e.g., TMHMM; ***245***) and/or signal peptide motifs (e.g., SignalP; ***246***).

Different types of the Basic Local Alignment Software Tool (BLAST; ***247***) are used for comparing the nucleotide sequence data with DNA or cDNA (BLASTn), or amino acid (BLASTx) sequences or conceptually translated peptides with protein sequences (BLASTp), available in databases (***206, 215***). Public databases represent comprehensive collections of nucleotide and amino acid sequences. Due to the rapid progress in the discovery and characterization of novel genes and proteins, online public databases have become one of the primary resources for sequence data storage, analysis and annotation. For example, the International Nucleotide Sequence Database Collaboration includes three ‘sister’ databases, namely GenBank (***248***), the Enterprise Management Technology Transfer nucleotide database curated by the European Molecular Biology Laboratories (EMBL; ***249***) and the DNA Databank of Japan (DDBJ; ***250***). In these databases, all publicly available nucleotide sequences are stored and curated; in addition, each sequence is stored as a separate record and linked to information, such as primary source references and predicted and/or experimentally verified biological features. For high-throughput sequencing projects, raw sequence data are often stored in subdivisions of these nucleotide databases, such as UniGene (***251***) and the Sequence Read Archive (***252***). Various databases, which exclusively store known amino acid sequence data, are

also available. For instance, the Protein Data Bank (PDB; ***253***), maintained by the Research Collaboratory for Structural Bioinformatics, represents the primary source for protein structures, whereas the SWISS-PROT database (***254***) is a protein sequence database for a number of prokaryotes and eukaryotes. The TrEMBL (***255***) division of SWISS-PROT contains a non-redundant set of translations for all coding sequences in the EMBL nucleotide sequence database that do not correspond to existing SWISS-PROT entries. In addition to these comprehensive general databases, there is a number of specialized collections of gene and protein information on particular organisms. Examples include the databases for *Saccharomyces cerevisiae* (yeast) (http://www.yeastgenome.org/; ***256***), *Drosophila melanogaster* (vinegar fly) (http://flybase.org/; ***257***), *Mus musculus* (mouse) (http://www.informatics.jax.org/; ***258***) and *C. elegans* (free-living nematode) (WormBase at http://www.wormbase.org; ***259***). WormBase is a comprehensive repository of information on *C. elegans* and related nematodes, such as *C. briggsae* (***259***). Here, essentially all information and data on classical genetics, cellular biology, structural and functional genomics of these free-living nematodes are stored and continually curated (***259-262***).

The functional annotation of sequence data for parasitic nematodes has often relied on pairwise homology-based comparative analyses with already annotated and curated sequence data sets for a range of organisms (***203, 204***). However, many genes, transcripts and gene products of these worms (often ≥ 50%) cannot be functionally annotated using this approach, because closely related, homologous molecules do not exist in transcriptomic and/or genomic data sets available in public databases and/or because sequence data sets are incomplete. In addition, as functional genomic tools are not yet practical or established for most parasitic helminths, improved bioinformatic approaches need to be established to achieve enhanced functional annotation of genes and gene products. Recently Mangiola et al. (***263***) tackled this issue and compiled transcriptomic data sets of key, socioeconomically important parasitic helminths, constructed and validated a curated database (HelmDB; www.helmdb.org) and showed how data integration and clustering can achieve improved functional annotations. HelmDB provides

a practical and user-friendly toolkit for sequence browsing and comparative analyses among divergent helminth groups (including nematodes and trematodes), and should be readily adaptable and applicable to a wide range of parasites.

4. *Caenorhabditis elegans* as major resource for comparative studies

The annotation and analysis of sequence data derived from many parasitic nematodes, particularly Strongylida, relies on information available for *C. elegans* (in WormBase). The latter nematode is simple in its anatomy (959 somatic cells in the hermaphrodite and 1031 in the male), has a short life cycle (~3 days) and is easy to culture *in vitro* (***264***). The genome of *C. elegans* is ~100 Mb in size (***265***). Currently, WormBase (www.wormbase.org) contains detailed and curated information on ~20,000 *C. elegans* genes and associated data on, for instance, transcription/expression profiles in different developmental stages, tissues and cells, mutants and their phenotypes, genetic and physical maps, SNPs, information on gene-gene and protein-protein interactions as well as all peer-reviewed literature pertaining to *C. elegans*.

The advent of double-stranded RNA interference (RNAi; ***266***) has revolutionised the study of gene function in metazoan organisms and led to detailed information on the functions of ~96% genes in *C. elegans* (***267-271***). The principle of RNAi relies on the introduction of double-stranded RNA (dsRNA) into the cells of a living organism, which induces the degradation of the homologous (target) mRNA (***266***). The dsRNA can be introduced directly into *C. elegans* by injection (***266***), by soaking worms in solution (***272***) or by feeding worms *Escherichia coli* expressing a dsRNA fragment of a target gene (***273***); it can also be introduced using a transgene expressing dsRNA (***274, 275***). This gene silencing approach opened up avenues for large-scale studies of molecular function in *C. elegans* (***267-270, 274, 276, 277***) as well as for comparative studies (e.g., comparison with parasitic nematodes or humans) (***278-282***).

Transgenesis of *C. elegans* has also been widely used for assessing gene function (***283, 284***). This technique can involve the microinjection of expression constructs, which usually comprise plasmid or cosmid DNA, often incorporating green fluorescent protein (GFP; ***285***) into the

syncytium (mitotically active) region of the adult hermaphrodite gonad (= 'gonadal microinjection'); alternatively, the DNA constructs can be transferred directly into target cells *via* high density microparticles of gold or tungsten (= 'biolistics' or 'particle bombardment') (***286***). Introduced DNA does not usually integrate into the chromosome, rather it forms a multi-copy extrachromosomal array, which can be inherited. Labelling with green fluorescent protein (GFP) allows the study of a number of (temporal and spatial) biological processes, including gene expression, protein localization and dynamics, protein-protein interactions, cell division, chromosome replication and organization, intracellular transport pathways, organelle inheritance and biogenesis (***287***).

In addition to investigations of gene expression and localization, patterns of gene transcription during key developmental and reproductive processes have also been studied in *C. elegans*, employing microarray technology (***288-290***). In an early study (***288***), various groups of molecules were demonstrated to have high expression levels in the germline tissues of *C. elegans*, i.e. the 'germline intrinsic' molecules (expressed in the germline of hermaphrodites producing either sperm or oocytes, and proposed to play key roles in biological processes linked to meiosis, stem cell recombination and germline development), and molecules highly expressed either in oocytes-producing or sperm-producing hermaphrodites (***288***). The latter group included a large number of molecules, such as protein kinases and phosphatases, associated with spermatogenesis, in accordance with other studies investigating gender-enriched transcriptional patterns in parasitic nematodes (e.g., ***153, 174, 181***). Previously, genetic studies had indicated that ~50-70% of genes in parasitic nematodes have orthologues in *C. elegans* (***27, 171***), which supported the grouping of this free-living nematodes into 'clade V' of the phylum Nematoda, together with parasitic nematodes of the order Strongylida (***27, 291***). These results, together with similarities in various characteristics (such as body plan and moulting) between *C. elegans* and some parasitic nematodes (e.g., ***5, 292***) indicate that this free-living nematode provides a useful system for comparative investigations of many conserved biochemical and molecular pathways linked to development in related nematodes.

5. Understanding nematodes of socioeconomic importance through genomics and transcriptomics – examples

High-throughput sequencing technologies (Table 1) and improved bioinformatic tools are providing unparalleled opportunities for global analyses of the genomes and transcriptomes of key nematodes, such as *As. suum* (***202***) and *Trichinella spiralis* (trichina; ***293***). Recent studies have utilised such technologies to explore the transcriptomes of different developmental stages and both sexes of key strongylid nematodes, including *N. americanus*, *H. contortus*, *T. colubriformis* and *Oe. dentatum* (***203-206***).

Although human hookworms are of major socioeconomic importance (***1, 3, 6, 7***), genomic and molecular studies have mostly involved *An. caninum* (e.g., ***182, 183, 190-192***). Recently, 454 sequencing and bioinformatic analyses were conducted to investigate, for the first time on a large scale, the transcriptome of the adult stage of *N. americanus* (***205***). The results showed that transcripts encoding proteases and Kunitz-type protease inhibitors were most abundantly represented in the transcriptome of this nematode, supporting the fundamental roles that these molecules play in multi-enzyme cascades to digest haemoglobin and other serum proteins (***294, 295***), as well as ~~and~~ in preventing homeostasis and inhibiting host proteases (***296, 297***)~~,~~ ~~respectively~~. Using a combination of orthology-mapping and functional data available for *C. elegans*, Cantacessi et al. (***205***) predicted 18 potential drug targets in the transcriptome of the adult stage of *N. americanus*, which included, for instance, mitochondrial-associated proteins known to be essential in *C. elegans* (***298***).

In *H. contortus*, high-throughput sequencing and bioinformatic analyses were used to explore differences in gene transcription between the free-living (L3) and the parasitic (xL3) third larval stage and to predict the roles that key transcripts play in the metabolic pathways linked to larval development (***204***). These analyses revealed that transthyretin-like proteins (TTLs) and calcium-binding proteins were highly represented in the transcriptome of both *H. contortus* L3 and xL3, whereas selected transcripts encoding collagens and neuropeptides were present

exclusively in L3 and proteases in xL3 (***204***). In nematodes, the synthesis of collagens has been observed to increase significantly prior to a moult (***299***), whereas proteins involved in the development of the nervous system are essential in the cascade of events that lead to the growth and development of the larval stages (***300***). Therefore, increased transcription of neuropeptides in L3s of *H. contortus* might relate to axon guidance and synapse formation during the L3's transition to parasitism (***204***). This statement is supported by the fact that, in *H. contortus*, the transition from the free-living to the parasitic L3 is triggered by gaseous CO_2, detected by chemosensory neurons of amphids, which are located in the anterior end of the L3 stage, ultimately leading to the secretion of the neurotransmitter noradrenaline (***5***). Conversely, the largest number of *C. elegans* orthologues of *H. contortus* xL3-specific transcripts encoded peptidases and other enzymes involved in amino acid catabolism, supporting previous evidence that cysteine proteases play a crucial role in the catabolism of globin, as is the case for *An. caninum* and *N. americanus* (***146, 294, 295, 301***). A similar spectrum of proteases and other molecules linked to catalytic activity had been shown also to be highly represented in the transcriptomes of activated xL3 stages of both *H. contortus* and *An. caninum* by comparison with their L3s (***183, 204***). This finding, for two haematophagous bursate nematodes with differing life histories, is likely to reflect the key roles that these molecules play in host tissue invasion, degradation and/or digestion.

In the transcriptome of *T. colubriformis*, molecules encoding peptides that are predicted to be associated with the nervous system (i.e., 'transthyretin-like' and 'neuropeptide-like'proteins; TTLs and NLPs, respectively), digestion of host proteins or inhibition of host proteases (i.e., proteases and protease inhibitors, respectively) were highly represented (***203***), with serine- and metallo-proteases and 'Kunitz-type' protease inhibitors being the vast majority of molecules characterised (***203***). In strongylid nematodes, these molecules play fundamental roles in the invasion of the vertebrate host by mediating, for example, tissue penetration, feeding and/or immuno-evasion by (i) digesting antibodies; (ii) cleaving cell-surface receptors for cytokines and/or (iii) causing the direct lysis of immune cells (***302-306***).

In an effort to predict and prioritize molecules that could represent novel drug targets and are expressed across different stages of development, Cantacessi et al. (***206***) employed high-throughput sequencing and predictive algorithms to explore similarities and differences in the transcriptomes of the L3, L4 and adult male and female of *Oe. dentatum* (***206***). Most of the molecules unique to adult male and female of *Oe. dentatum* could be linked to pathways associated with reproductive processes. For instance, a large number of *Oe. dentatum* male-specific molecules encoded major-sperm proteins (MSPs), in accordance with previous studies of male-enriched datasets of other species of trichostrongylid nematodes, including *T. vitrinus* and *H. contortus* (***174, 181***). Based on the observation that MSPs from various nematodes, including *C. elegans*, are characterised by a significant amino acid sequence conservation (~67%; ***307***), a similar role has been proposed for these proteins in processes linked to the maturation of oocytes in the uterus of female nematodes (***308, 309***). In addition, a large proportion (17%) of molecules unique to the larval stages of *Oe. dentatum* represented proteases that, in this species, have been reported to evoke immunological and/or inflammatory reactions (including infiltrations of neutrophils and eosinophils) surrounding the encapsulated larvae (***77, 180***). In addition, somatic extracts of and supernatants from *in vitro* maintenance cultures of *Oe. dentatum* L4s have been shown to induce the proliferation of porcine mononuclear cells *in vitro* (***310***), which supports the hypothesis that L4-specific proteases play an active role in the modulation of the host's immune response (***302-304***). The results from a recent study (***206***) showed also that a high proportion (27-32%) of transcripts encoding protein kinases and phosphatases were common among all developmental stages of *Oe. dentatum* investigated (***206***). Supported by investigations of the free-living nematode, *C. elegans*, other studies have predicted, for instance, that some kinases and phosphatases could represent targets for novel nematocidal drugs (***311, 312***). Some cantharidin/norcantharidin analogues (***313-315***) are known to display exquisite and specific inhibitory activity against PP1 and PP2A phosphatases, which indicated that some of them could be designed to selectively inhibit essential serine/threonine phosphatase (STPs) of nematodes (***312***) (see section 6). In addition to phosphatases, other

molecules, such as chitin-binding proteins or proteases, might be interesting drug target candidates, given that they are proposed to have crucial roles in pathways linked to developmental and reproductive processes in some nematodes (***180, 206, 316***).

Highly represented in the transcriptomes of a number of strongylid nematodes (***203-206***) are proteins containing a 'sperm-coating protein (SCP)-like extracellular domain' (InterPro: IPR014044), also called SCP/Tpx-1/Ag5/PR-1/Sc7 (SCP/TAPS; Pfam accession number no. PF00188), or ASPs (***139***). Due to their abundance in the excretory/secretory (ES) products from serum-activated L3s (= aL3s) of *An. caninum* and high transcriptional levels of mRNAs encoding ASPs in activated L3s compared with non-activated, ensheathed L3s, these molecules have been hypothesized to play a major role in the transition from the free-living to the parasitic stages of this hookworm (***137, 183***). Other ASP homologues have been characterised for the adult stage of hookworms, and are proposed to play a role in the initiation, establishment and/or maintenance of the host-parasite relationship (***183, 317, 318***). Due to the~~ir~~ immunogenic properties of ASPs, one ~~ASP~~member of this family (i.e., *Na*-ASP-2) has been under investigation as a vaccine candidate against necatoriasis in humans (***57, 132, 319-321***). Whether SCP/TAPS proteins or their genes represent drug target candidates still remains to be determined. For ASPs, a focus of future research could be on studying their structure and function in parasitic helminths, to pave the way for applied outcomes, such the development of vaccines and/or drugs (***321a***).

6. Opportunities for drug discovery using global datasets

For parasitic nematodes, the prediction of drug target candidates from global genomic and transcriptomic datasets can be assisted by using extensive information on the functionality and essentiality of homologues in *C. elegans*, *D. melanogaster*, *M. musculus* and/or *S. cerevisiae* (accessible *via* public databases http://www.wormbase.org, http://flybase.org/, http://www.informatics.jax.org/ and http://www.yeastgenome.org/) (***202-206, 211***). Since most effective drugs achieve their activity by competing with endogenous small molecules for a

binding site on a target protein (***322***), the amino acid sequences produced from essential genes can be screened for the presence of conserved ligand-binding domains (***322, 323***) and lists of prioritized inhibitors compiled (***323***). The comparison of various studies shows consistently that some proteases, GPCRs, and guanosine triphosphatases (GTPases), kinases, phosphatases are salient among essential molecules and, thus, represent potential targets for nematocides (***202-206***).

Protein kinases (PTKs) have shown considerable promise as drug targets in protists, such as *Plasmodium* and *Giardia* (***324-326***) and in helminths, including *Schistosoma mansoni* and *Echinococcus multilocularis* (***327***). In the latter two species, for example, PTK inhibitors (i.e., tyrphostins AG1024 and AG538) have been shown to affect the survival and development of the parasite through the inhibition of glucose uptake (***327***). In another study, the inactivation of *S. mansoni* PTKs with herbimicin A (an Src kinase inhibitor) was shown to disrupt mitosis, thus reducing the expression of proteins essential for egg production, including the formation of the eggshell, in adult females (***328***). Although ~~the~~ crystal structures of PTKs from parasitic nematodes have not yet been de~~fin~~termined, some advances have been made in the identification and design of effective inhibitors based on homology models for protein kinases from humans (***327***). There is evidence that the active sites of parasite PTKs display a variable degree of structural divergence compared with their human counterparts (***326, 327***), which seems promising for designing selective kinase inhibitors for helminths.

Recent work has also shown potential for atypical protein kinases (aPKs; ***324***) as targets for the development of novel intervention strategies. Amongst these aPKs, the RIO kinases (= RIOKs: RIOK-1, RIOK-2 and RIOK-3) are considered essential for life (***329***). RIOK of parasitic strongylid nematodes have close homologues in *C. elegans* (***329, 330***); however, almost nothing is known about the function or biology of RIOKs in parasitic nematodes and most other metazoans. Although there are some conserved elements in each of the three RIOKs of different organisms, these aPKs from nematodes cluster (with high statistical support) to the exclusion of those of other eukaryotic organisms, including mammals (***329***), indicating prospects for the

design of a new class of nematode-specific inhibitors of these aPKs. Using *in silico* screening of the SPECS database (www.specs.net), Campbell et al. (***329***) recently identified compounds that bind *in silico* to RIOK-1 of *H. contortus* (*Hc*-RIOK-1). For some of these compounds, ~~primary and secondary~~multiple highly scored binding modes were observed, indicating an increased likelihood that these aPKs would display productive interactions in an *in vitro* assay (***329***). In addition, the hydrogen-bond interactions between the compounds identified and the *Hc*-RIOK-1 model involved multiple conserved side chains in the active site (including the P-, catalytic and metal binding loops); however, all compounds identified were also involved in interactions with residues that are not conserved and specific to *Hc*-RIOK-1 (***329***) and are thus considered important for the design of selective inhibitors of *Hc*-RIOK-1. A screen of the BRENDA database (www.brenda-enzymes.info; ***323***) for compounds with similar chemical structures to known kinase effectors identified two molecules with significant similarity to the protein kinase inhibitor emodol (an anthraquinone found in several plants), providing a useful starting point for drug development (***329***). Also identified were molecules with some structural similarity to known kinase effectors, such as the flavonoids apigenin and kaempferol (known to possess cancer-protective effects; ***331-333***) and prunitrin, a naturally occurring isoflavonoid in species of *Trifolium* (clover) and *Prunus*, characterized by a naphthaquinone scaffold and a carbohydrate moiety (***329***). In the future, an integrated approach, using advanced functional genomic, bioinformatic, cheminformatic and structural biological tools, could be used to elucidate the functions and structures of RIOKs, whose roles are proposed to be essential and involved intimately in developmental processes.

From a functional perspective, current information on *C. elegans* shows that *riok-1* encodes two isoforms (*via* alternative splicing) required for viability, fertility, endocytosis and fat storage. *C. elegans riok-2* also encodes a RIOK required for viability and fertility, and *riok-3* encodes a RIOK expressed in the larval and adult intestine of *C. elegans* (***329***). In addition, preliminary experiments have predicted null mutations in *riok-1* and *riok-2*, both of which are lethal, and an uncharacterised predicted null allele of *riok-3* (unpublished). From a structural biology

perspective, preliminary comparisons show that the RIOK domain harbouring the catalytic site is a conserved fold for nematode RIOKs. However, despite this fold, there are several amino acid substitutions in functionally important, conserved secondary structure elements, whose impact can only be assessed from three-dimensional structures determined experimentally (***329***). Thus, structural studies need to assess the particular binding modes of ligands, particularly the phosphate-donating nucleotides to provide a solid basis for structure-based drug design. Furthermore, the mechanistic aspects of RIOKs are poorly understood, thus requiring detailed structural information. The working model described by Campbell et al. (***329***) assumes that the two flexible elements in the RIOK domain, the hinge and the flexible loop, serve as docking points for the substrate and might undergo conformational change in the substrate-bound state. Such a process may be further aided by phosphorylation of Ser165 (in relation to RIOK-1), which is located in the flexible loop and seems to be a conserved residue for RIOKs. Crystal structures of substrate-bound and phosphorylated nematode RIOKs should assist in elucidating the biology of these proteins, providing clues as to how to best design selective and specific inhibitors.

Serine/threonine phosphatases (STPs) are also proposed to be involved in essential biological pathways and, thus, might represent viable anthelmintic targets (***99, 311***). *In silico* structural comparisons between *Hc*-STP-1 and homologues from other parasitic nematodes, including *Oe. dentatum* and *T. vitrinus*, have revealed conservation of residues and features putatively involved in catalytic activity, whereas phylogenetic analyses of STP sequence data from a range of eukaryotes confirmed the close relationship of nematode STPs, which clustered to the exclusion of homologues from other organisms (***99***). In a recent study, Campbell et al. (***312***) tested the activity of a series of norcantharidin-derived analogues against *H. contortus*; cf. section 5*)*. Three of these analogues reproducibly displayed 99-100% lethality against *H. contortus* in a larval development assay (***312***) and no toxic effects on multiple, independent mammalian (human cancer) cell lines. However, given the difference in structure between these analogues and the original norcantharidin ~~‘backbone’~~chemotype, it was proposed that these

molecules might have targets other than STPs (***312***). Further studies are needed to establish the precise mode of action of these effective norcantharidin-derived compounds in nematodes, which show considerable promise as anthelmintics.

7. Challenges and prospects

Due to the lack of complete genomic sequences for most parasitic nematodes, newly generated transcriptomic and genomic sequence datasets need to be assembled *de novo*, which means that pooled reads are assembled without a bias towards known sequences (***222***). Due to the amount of RNA required for high-throughput sequencing (~5-10 µg; ***334, 335***), transcriptomes from small nematodes usually originate from multiple individuals, potentially leading to an increased complexity of the sequence data acquired (linked, for instance, to single nucleotide polymorphisms [SNPs] and other types of sequence variation) and posing some challenges for the assembly. In terms of complexity, computational and time requirements, *de novo* assemblies are orders of magnitude slower and much more computationally intensive than knowledge-based (mapping) assemblies, in which reads are aligned and assembled against an existing "backbone" sequence (***336***). In addition, reliable *de novo* assemblies are heavily dependent on the availability of long reads (>100 bases) and of high-coverage, paired-end sequence data (***336, 337***). In previous studies, the complementary nature of the 454 and Illumina sequencing platforms has allowed the assembly of raw reads into large scaffolds without need for a reference sequence (***338-340***). Thus, clearly, the 454 sequence data assembled in previous studies (***203-206***) should assist future *de novo* assemblies of Illumina data (both transcriptomic and genomic) for the species investigated to date.

Some transcriptomic studies have employed 454 sequencing of normalized cDNA libraries (***203-206***). Normalisation allows transcripts to be studied qualitatively, but this approach does not allow differential gene expression to be investigated quantitatively (***203-206***). Exploring differential transcription among stages, sexes and tissues of parasitic nematodes and other helminths provides unique insights into molecular changes occurring, for example, during

development and reproduction. Future studies involving the sequencing of non-normalised cDNA libraries by, for instance, Illumina technology (www.Illumina.com; ***194***) will provide an avenue to explore essential biological pathways in parasitic nematodes, such as those linked to the development of neuronal tissue, the formation of cuticle and the digestion of host haemoglobin in *H. contortus* (***204***) and in mitochondrial and amino acid metabolism in *N. americanus* (***205***). However, the incorporation of gene expression data will inevitably pose new computational challenges for the correct assembly and analysis of sequence datasets and, for instance, for the accurate prediction of alternatively spliced transcripts.

The accurate assembly of ESTs is a crucial step for examining coding genes and, ultimately, addressing biological questions regarding gene and protein function (***263***). Knowledge of the function of genes and gene products from organisms is predicted using a process known as 'sequence annotation', which has been defined as "the process of gathering available information and relating it to the sequence assembly both by experimental and computational means" (***341***). Currently, the annotation of sequence data from parasitic nematodes is primarily based on comparisons with data available in public databases available *via* multiple portals (***203-206***) and updated at different rates. The Swiss-Prot database (http://au.expasy.org/sprot/), for instance, accepts corrections from its user community, whereas GenBank (http://www.ncbi.nlm.nih.gov/genbank/) only accepts corrections from the author of an entry (***342***), thus significantly affecting the accuracy and speed with which new sequences are annotated. In addition, some information-management systems evolve to efficiently incorporate data from large-scale projects, but often, the annotation of single records from the literature is slow and cumbersome (***343***). Given that, presently, the annotation of sequence data for parasitic nematodes relies heavily on the use of bioinformatic approaches and already annotated/curated sequence data for a wide range of organisms (***203-206***), these observations are particularly crucial and deserve further consideration. For instance, the analyses and annotation of large-scale transcriptomic sequence datasets for parasitic nematodes could be considerably facilitated through the establishment of a 'reference' website, which

could provide regular releases of newly developed and validated bioinformatic pipelines for the analyses of sequence datasets as well as links to regularly updated databases. In the future, the establishment of a 'centralised' consortium to facilitate the sharing and optimization of bioinformatic pipelines for sequence processing and annotation and, more broadly, to allow access to new sequence data, as well as experimental protocols and relevant literature would be very useful to the scientific community.

Typically, the annotation of peptides inferred from the transcriptomes of parasitic nematodes is performed by assigning predicted biological function/s based on comparison with existing information available for *C. elegans* and for other organisms in public databases (e.g., WormBase, www.wormbase.org; InterPro, http://www.ebi.ac.uk/interpro/; Gene Ontology, http://www.geneontology.org/; OrthoMCL, http://www.orthomcl.org/; BRENDA, http://www.brenda-enzymes.org/) (***203-206***). Using this approach, predictions for key groups of molecules were made in relation to their function and essential roles in biological processes (***203-206***). Such groups included the SCP/TAPS proteins and molecules linked to the physiology of the nervous system, to the formation of the cuticle, proteases and protease inhibitors, and protein kinases and phosphatases (***203-206***). However, in order to support data inferred from bioinformatic analyses of sequence data, experimental validation is now required. In particular, extensive laboratory experiments need to be conducted to evaluate the functions of molecules in the parasites studied and/or in a suitable surrogate organism. RNAi has been applied to a number of strongylid nematodes of animals, but success has been relatively limited (e.g., ***279, 344-351***). Current evidence (***279, 349***) suggests that a number of nematodes of animals, including *H. contortus*, lack critical components of the RNAi machinery (***279, 350, 352***). Transgenesis and gene complementation studies have shown considerable promise for evaluating the function of genes from some parasitic nematodes (e.g., ***353-355***). Indeed, a study demonstrating successful transgenesis in the parasitic nematode *Parastrongyloides trichosuri* (Rhabditida) (***356***) as well as the use of *C. elegans* as a surrogate system for the analysis of the function of some genes from selected members of the Strongylida and Rhabditida (***353-355***)

provide substantial promise and scope for the application of this methodology to functional genetic studies of selected groups of parasitic nematodes.

In the future, improved bioinformatic prediction and prioritization of potential drug targets in parasitic nematodes will depend on the availability of complete genome sequences. Global repertoires of drug targets could be inferred. For instance, the parasite kinome (= the complete set of kinase genes in the genome) could represent a unique opportunity for the design of parasite-selective inhibitors (***327***). In addition, the integration of genomic, transcriptomic and proteomic data will be crucial to identify groups of molecules essential to parasite survival and development, which could represent drug target candidates. Clearly, high-throughput sequencing, such as Illumina, provides the efficiency and depth-of-coverage required to rapidly define genomes and transcriptomes of eukaryotic pathogens of socio-economic importance (***202, 211, 293***). The combined use of innovative bioinformatic tools will open the door to understanding the molecular biology of parasites and other pathogens on an unprecedented scale. A deep understanding of these pathogens at the molecular level will provide exciting opportunities for the development of novel interventions and diagnostic methods.

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Table 1. Technical features of next-generation sequencing platforms (i.e., 454/Roche, Illumina/Solexa and SOLiD)[a].

Description	454/Roche	Illumina/Solexa	SOLiD
Platform	Genome Sequencer FLX	Genome Analyzer IIx	SOLiD 3 Plus System
Sequencing method	Emulsion PCR of bead-bound oligos	Isothermal bridge amplification on flowcell	Emulsion PCR of bead-bound oligos
Sequencing chemistry	Pyrosequencing using polymerase	Ligation (‘dual-base encoding’ octamers)	Reversible terminator using polymerase
Reads per run	~1 million	Up to 3 billion	1.2 to 1.4 billion
Read length	1000 bp	50 to 250 bp	100 bp
Run time	~12 hours	~2-9 days	~3 days
Peer-reviewed manuscripts	++++	+++	++
Examples of applications	*De novo* sequencing, metagenomics, targeted sequencing	Resequencing, RNA-Seq, DNA methylation studies	Resequencing, RNA-Seq

[a] Based on information available on July 2012.