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Research review paper

Whole-organism phenotypic screening methods used in early-phase anthelmintic drug discovery

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ABSTRACT

Diseases caused by parasitic helminths (worms) represent a major global health burden in both humans and animals. As vaccines against helminths have yet to achieve a prominent role in worm control, anthelmintics are the primary tool to limit production losses and disease due to helminth infections in both human and veterinary medicine. However, the excessive and often uncontrolled use of these drugs has led to widespread anthelmintic resistance in these worms - particularly of animals - to almost all commercially available anthelmintics, severely compromising control. Thus, there is a major demand for the discovery and development of new classes of anthelmintics. A key component of the discovery process is screening libraries of compounds for anthelmintic activity. Given the need for, and major interest by the pharmaceutical industry in, novel anthelmintics, we considered it both timely and appropriate to re-examine screening methods used for anthelmintic discovery. Thus, we reviewed current literature (1977–2021) on whole-worm phenotypic screening assays developed and used in academic laboratories, with a particular focus on those employed to discover nematocides. This review reveals that at least 50 distinct phenotypic assays with low-, medium- or high-throughput capacity were developed over this period, with more recently developed methods being quantitative, semi-automated and higher throughput. The main features assessed or measured in these assays include worm motility, growth/development, morphological changes, viability/lethality, pharyngeal pumping, egg hatching, larval migration, CO_2- or ATP-production and/or enzyme activity. Recent progress in assay development has led to the routine application of practical, cost-effective, medium- to high-throughput whole-worm screening assays in academic or public-private partnership (PPP) contexts, and major potential for novel high-content, high-throughput platforms in the near future. Complementing this progress are major advances in the molecular data sciences, computational biology and informatics, which are likely to further enable and accelerate anthelmintic drug discovery and development.

1. Introduction

Parasitic roundworms (nematodes) and flatworms (platyhelminths: trematodes and cestodes) cause substantial morbidity and mortality in humans and other animals globally, and major losses to global food production annually (Charlier et al., 2015, 2020). Nematodes of multiple orders, including the Strongylida (strongylids), Ascaridida (ascarids) and Spirurida (spirurians), cause some of the most important diseases of livestock in Australia and worldwide, affecting hundreds of millions of food animals (including sheep, goats, cattle and pigs), with economic losses estimated at tens of billions of dollars per annum globally (Roeber et al., 2013; Charlier et al., 2020). Although infections with these parasites are often subclinical, many of them can cause gastrointestinal or respiratory diseases and death in severely affected animals. For instance, gastrointestinal trichostrongyloid nematodes of livestock animals, such as species of *Haemonchus*, *Teladorsagia*, *Ostertagia*, *Trichostrongylus* and *Cooperia*, are of particular economic importance, and are transmitted orally from contaminated pasture to the host through direct life cycles

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(cf. Beveridge and Emery, 2014). Eggs are excreted in host faeces; the first-stage larva (L1) develops inside the egg to then hatch (within 1 day) and develop to the second- and third-stage larval stages (L2s and L3s) in about a week; the infective L3s are then ingested by the host, exsheath (xL3) and develop through fourth-stage larvae (L4) to dioecious adults (within ~3 weeks) in the gut of the animal.

Despite decades of research, helminth vaccines have yet to achieve a prominent role in nematode intervention, such that control still relies heavily on the use of anthelmintics. However, drug resistance is emerging or is already widespread, particularly in nematodes of livestock animals (e.g., Kaplan and Vidyashankar, 2012; Kotze and Prichard, 2016; Kaplan, 2020), and no vaccines are available against the vast majority of them, such that the discovery and development of new anthelmintic compounds is crucial to ensure sustained control into the future. Although the drugs monepantel (Kaminsky et al., 2008; Prichard and Geary, 2008) and derquantel (Little et al., 2010) have provided hope for the discovery of new classes of anthelmintics, success in the identification of new lead anthelmintic compounds through conventional screening approaches has been limited since 2009.

Three main screening approaches have been used to discover active ('hit') compounds: (i) animal-based, (ii) target-based and (iii) phenotypic methods (Geary et al., 2015). Most early drug discovery programs used animal-based techniques, in which infected animals were treated with test compounds, and the reduction in parasite burden was measured (Geary and Thompson, 2003; Geary et al., 2015). These methods were historically successful, but are not used now, for animal ethics reasons, and because they are low-throughput, high cost, time-consuming, and require large amounts of compound (depending on size of host animal) (Geary et al., 2015). To circumvent these issues, in vitro (i.e., target-based and phenotypic) screening methods have been established and employed (Geary et al., 2015). Target-based platforms screen test and control compounds against one or more defined molecules (e.g., with essential functions in a key biological pathways or processes) (Zheng et al., 2013; Geary et al., 2015); this approach usually requires only small amounts of compounds, and is amenable to high throughput screening (cf. Geary et al., 2015). However, a lack of knowledge of targets in parasites, challenges in expressing functional recombinant parasite (target) proteins and the associated laboratory costs can be a 'bottleneck' in the use of target-based screening for drug discovery (Geary et al., 2015). Moreover, such methods are usually biochemical/molecular, and (at least initially) do not consider the bioavailability of compounds to the parasite. By contrast, phenotypic screening, which employs whole parasites in vitro, has become a practical and commonly used approach for anthelmintic discovery (Geary et al., 2015). Over the years, a range of such screening platforms have been developed for different helminth species and/or life cycle stages; they quantitatively assess or measure phenotypic features during and/or following treatment with compounds, ideally using the disease-causing stages for screening in vitro.

Here, we report a review of the literature on the development and/or use of whole-worm phenotypic screening platforms for the discovery of anthelmintic lead compounds, with a particular focus on nematocides. Given the adverse impact of parasitic worms on animals and people globally (Hay et al., 2017; Charlier et al., 2020), the lack of vaccines and the established or emerging problems with drug resistance in parasite populations, and the imperative to work toward achieving some of the key sustainable development goals (SDGs; Bangert et al., 2017) by developing improved treatments, it is both timely and appropriate to assemble this review, to complement or extend previous articles on the topic (e.g., Singh, 2012; Paveley and Bickle, 2013; Geary et al., 2015; Partridge et al., 2020; Sepúlveda-Crespo et al., 2020) and provide a perspective on some of the latest developments in this area.

2. Methodology

We undertook this review according to the recommendations provided in Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA; cf. Moher et al., 2015). Four data bases – PubMed, Scopus, Web of Science and SciELO – were systematically searched by two independent investigators (H.M.P.D.H. and A.C.T.) for peer-reviewed articles published until 30 December 2021 (Fig. 1). The literature search was performed using the following terms, employing Boolean operators "AND" and/or "OR": helminth, nematode, anti-parasitic, anthelmintic, phenotypic screening, high throughput, in vitro, automated, high content, microfluidic, drug discovery, motility, development inhibition, viability. Time and language restrictions were not applied during the search, and the abstracts and key parts of papers published in languages other than English were translated to English using 'Google translate'.

The following inclusion criteria were applied: studies that described (1) newly established phenotypic screening assays for particular helminth(s) and/or life cycle stage(s); (2) already published assays adapted to a different helminth/life cycle stage; and (3) assays developed by modifying or improving the technique or approach of an existing assay.

The following exclusion criteria were applied: (1) studies describing screening platforms developed for plant parasitic nematodes; (2) studies using already established phenotypic platforms; (3) studies using screening platforms that employ a non-phenotypic technique; (4) articles with no access to a full text; and (5) a case report or review article. The articles were transferred to the program EndNote (Clarivate), duplicates deleted, and titles and abstracts were examined against the eligibility criteria by two independent investigators (H.M.P.D.H. and A.C.T.). Then, the full-text articles were independently downloaded and searched against the eligibility criteria by these two researchers (H.M.P.D.H. and A.C.T.). A manual search of reference lists of extracted publications was performed to identify relevant studies. Discrepancies between the records of the two investigators were resolved in discussion with other investigators (R.B.G., T.G.G., A.J. and/or J.K.).

The data of the eligible full texts were extracted and entered into an Excel spreadsheet independently by two investigators (H.M.P.D.H. and A.C.T.). The discrepancies were resolved by consulting the other investigators (R.B.G., T.G.G., J.K., A.J. and/or A.R.). The following information on the assays was extracted from individual eligible articles: author, year of publication, helminth species and life cycle stage used, measurement approach, phenotypic character(s) assessed, the type of readout (i.e., subjective or objective), level of automation/throughput and type of vessels or plates used. Using this approach, 2239 articles were identified in databases, and a small number ($n = 29$) was obtained in a manual search. After duplicates were removed, the titles and abstracts of the remaining 1936 articles were screened, and 1792 ineligible articles removed. A critical, detailed appraisal of 144 pertinent articles identified 84 eligible articles for inclusion (Fig. 1; Table 1).

3. Screening approaches

Most assays developed use whole worms (Table 1), while some are cell-based, employing embryonic cells of free-living nematode *Caenorhabditis elegans* or *Wolbachia*-infected cells as targets (Serbus et al., 2012; Lai et al., 2014; Clare et al., 2015, 2019a, 2019b). Worm tissue-based assays are not commonly employed, although Comley and Rees (1989) demonstrated the use of tissues of *Onchocerca* sp. to measure alterations in viability following compound treatment.

Most screening assays have been developed for nematodes of veterinary importance, including species of *Haemonchus*, *Ancylostoma*, *Ascaris*, *Trichuris*, *Dirofilaria* and *Brugia* (see Table 1). Although there has been some debate about the suitability of using *Caenorhabditiselegans* as a surrogate for parasitic nematodes (Geary and Thompson, 2001; Gilleard, 2004; Keiser, 2015), a significant number of assays have been developed for this species (Table 1). This free-living nematode is related to strongylid nematodes (most of which are socioeconomically important; clade V), with a relatively high synteny and orthology of genes, except those that relate to parasitism (Dorris et al., 1999; Gilleard,

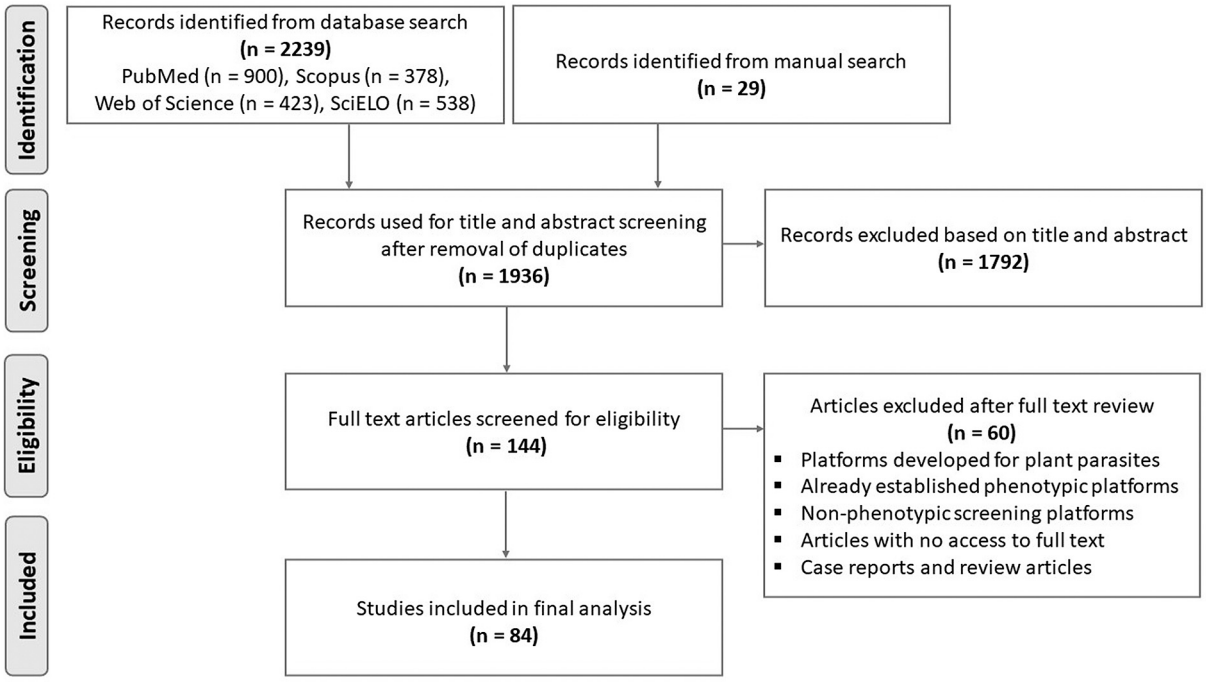

Fig. 1. PRISMA flow chart, showing the literature search strategy and selection criteria.

2004). Furthermore, a powerful genetic system available for *Caenorhabditiselegans* makes it suitable for functional analyses and mechanism of action studies (Gilleard, 2004; Sepúlveda-Crespo et al., 2020). These features make *Caenorhabditiselegans* a useful tool to aid anthelmintic discovery efforts. On the other hand, assays have been established to target the bacterium *Wolbachia* within some filarioid nematodes (Table 1; cf. Section 5). This bacterium maintains a symbiotic relationship with these worms and is essential for their survival, and, thus, represents a useful target for new therapeutics against the filarioids themselves. Other assays have been developed for trematodes (flukes), including species of *Schistosoma*, *Echinostoma* and *Fasciola* as well as cestodes (tapeworms) of the genus *Echinococcus* (Table 1).

In the 84 articles selected and evaluated here, 25 helminth genera have been used to develop whole-organism screening platforms (Table 1), with *Caenorhabditiselegans* being employed in both whole-organism and cell-based assays. Most assays used larvae rather than adult worms (Table 1). Larvae of some species, such as *Haemonchus contortus*, are amenable to screening in medium- or high-throughput assays, as they can be artificially exsheathed and maintained in culture in vitro in relatively large numbers for relatively long periods (1 week) (Geary, 2016). Assays using adult worms have been developed for a range of species including the nematodes *Caenorhabditiselegans*, *Acanthocheilonema viteae*, *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Brugia malayi*, *Brugia pahangi*, *Onchocerca gutturosa*, *Onchocerca volvulus* and *Trichuris muris* (Table 1). In many cases, the use of adult worms is preferred, as they almost always represent the ultimate target stage for anthelmintics. However, adult stages of many helminths can be challenging to produce due to the difficulty/impossibility to culture and maintain them in vitro, relatively long developmental times and prepatency periods, the difficulty in obtaining large numbers and/or the reliance on experimental animals for the production of parasites.

While early screening assays were low-throughput and assessed phenotypic alterations in a relatively subjective/qualitative manner (see Subsection 4.1; Fig. 2), there has been significant progress in the development of technologies amenable to medium- or high-throughput assays for different helminth species and life cycle stages that measure phenotypes in an objective/quantitative way (see Section 5; Fig. 2; cf. Table 1).

4. Low-throughput methods

4.1. Microscopy-based assays

Bright field microscopy initially served as the "gold standard" for phenotypic screening. Worms were cultured, exposed to test compounds and observed under microscopy for changes in phenotypic characters. Some of these assays were used to measure the motility or viability of larvae of nematodes such as *Ancylostoma ceylanicum, Ancylostoma caninum*, *Haemonchus contortus*, *Necator americanus, Onchocerca ochengi, Strongyloides stercoralis*, *Strongyloides ratti* and *Trichuris muris* (see Gill et al., 1991; Kotze et al., 2004; Panic et al., 2013; Wimmersberger et al., 2013; Abongwa et al., 2021; Jacob et al., 2021) and select flatworms (cf. Table 1). In addition, O'Grady and Kotze (2004) developed a microscopy-based assay for adults of *Haemonchus contortus*. The results were expressed as the percentage of motile worms in wells containing treated worms in relation to untreated controls (Gill et al., 1991; Kotze et al., 2004), or a motility reduction score (O'Grady and Kotze, 2004; Panic et al., 2013; Wimmersberger et al., 2013).

Another approach was the microscopic assessment of larval migration inhibition. Here, the number of larvae that migrated through a mesh following treatment with test compounds (and controls) were counted using a microscope (Douch et al., 1983; Wagland et al., 1992; Evans et al., 2013; Zhao et al., 2017). Larval migration assays were developed for species including *Ascaris suum*, *Dirofilaria immitis* and *Trichostrongylus colubriformis* (see Douch et al., 1983; Wagland et al., 1992; Evans et al., 2013; Zhao et al., 2017). Furthermore, Taylor (1990) developed a microscopy-based assay to measure inhibition of larval development in *Haemonchus contortus* and *Teladorsagia circumcincta*. Here, eggs were cultured in Petri dishes and allowed to develop to first-stage larvae (L1s); the test compounds were added to the plates, incubated for 6 days, and the number of third-stage larvae (L3s) was counted under microscope (Taylor, 1990). Later, Athanasiadou et al. (2001) modified this assay to test the effect of condensed tannins on the development and viability of larvae of *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus vitrinus* in 24-well plates (Athanasiadou et al., 2001). Other phenotypic screening assays were based on the ability of worms, such as *Haemonchus contortus*, and the blood fluke *Schistosoma mansoni*,

Table 1
Information extracted from key articles ($n = 84$) selected for this review of whole-worm phenotypic screening assays/platforms for the discovery of anthelmintic compounds, with a particular focus on nematocides.

Authors (alphabetical)	Species used in the assay	Technology used in the assay	Life-cycle stages used[a]	Phenotypic characters assessed	Read-out	Characteristics	Through-put potential
Abongwa et al. (2021)	*Brugia pahangi*	Image-based	Adults	Motility	Objective	Automated	High
	Onchocerca ochengi	Bright-field microscopy	Microfilariae	Motility	Subjective	Manual	Low
	Onchocerca ochengi	Bright-field microscopy (MTT)	Adults	Viability	Subjective	Manual	Low
Abriola et al. (2019)	*Ancylostoma ceylanicum*	Fluorescence-based	Eggs	Egg hatch	Objective	Automated	High
Aguiar et al. (2017)	*Schistosoma mansoni**	Absorbance-based (XTT)	Schistosomula	Viability	Objective	Automated	High
Atakan et al. (2019)	*Caenorhabditis elegans*	Microfluidic (image-based)	Embryos to adults	Motility	Objective	Automated	Low
Athanasiadou et al. (2001)	*Haemonchus contortus*	Bright-field microscopy	Eggs, L3s	Larval development, viability	Subjective	Manual	Low
	Teladorsagia circumcincta	Bright-field microscopy	Eggs, L3s	Larval development, viability	Subjective	Manual	Low
	Trichostrongylus vitrinus	Bright-field microscopy	Eggs, L3s	Larval development, viability	Subjective	Manual	Low
Aubry and Lu (2017)	*Caenorhabditis elegans*	Microfluidic (image-based)	Young adults	Trashing activity, morphology	Objective	Automated	Low
Bakowski et al. (2019)	*Wolbachia* - endosymbiont of filarial worms	Fluorescence-based	Cells	Cell viability - bacterial load	Objective	Automated	High
Bennett and Pax (1986)	*Nippostrongylus brasiliensis*	Micro-motility meter	L4s	Motility	Objective	Automated	Low
	Ascaris suum	Micro-motility meter	L4s	Motility	Objective	Automated	Low
	Caenorhabditis elegans	Micro-motility meter	L4s	Motility	Objective	Automated	Low
	Brugia pahangi	Micro-motility meter	Adults	Motility	Objective	Automated	Low
Buckingham and Sattelle (2009)	*Caenorhabditis elegans*	Image-based	Adults	Thrashing activity	Objective	Automated	Medium
	Haemonchus contortus	Image-based	L3s	Thrashing activity	Objective	Automated	Medium
Carr et al. (2011)	*Caenorhabditis elegans*	Microfluidic (image-based)	L4s	Motility	Objective	Automated	Low
	Oesophagotomum dentatum	Microfluidic (image-based)	L3s	Motility	Objective	Automated	Low
Chawla et al. (2018)	*Schistosoma mansoni**	Microfluidic (impedance-based)	Schistosomula	Motility	Objective	Automated	Low
Chen et al. (2020)	*Schistosoma mansoni**	Image-based	Schistosomula	Body morphology	Objective	Automated	High
Cintra et al. (2019)	*Caenorhabditis elegans*	Fluorescence-based	L1s	Viability	Objective	Automated	Medium-high
Clare et al. (2019a)	*Wolbachia* - endosymbiont of filarial worms	Fluorescence-based	Cells	Cell viability - bacterial load	Objective	Automated	High
Clare et al. (2019b)	*Wolbachia* - endosymbiont of filarial worms	Fluorescence-based	Cells	Cell viability - bacterial load	Objective	Automated	High
Clare et al. (2015)	*Wolbachia* - endosymbiont of filarial worms	Fluorescence-based	Cells	Cell viability - bacterial load	Objective	Automated	High
Comley and Rees (1989)	*Acanthocheilonema viteae*	Radiorespirometry-based	Macrofilariae	CO_2 production	Objective	Automated	Low
	Onchocera gibsoni	Radiorespirometry-based	Macrofilariae	CO_2 production	Objective	Automated	Low
Comley et al. (1989a, 1989b, 1989c)	*Onchocerca volvulus*	Micro-motility meter	Adults	Motility	Objective	Automated	Low
Comley et al. (1989a, 1989b, 1989c)	*Acanthocheilonema viteae*	Absorbance-based (MTT)	Tissues, adults	Viability	Objective	Automated	Low
	Brugia pahangi	Absorbance-based (MTT)	Adults	Viability	Objective	Automated	Low
Comley et al. (1989a, 1989b, 1989c)	*Onchocerca gutturosa*	Absorbance-based (MTT)	Adults	Viability	Objective	Automated	Low
	Acanthocheilonema viteae		Adults	Viability	Objective	Automated	Low
Das et al. (1988)	*Ancylostoma ceylanicum*	Micro-motility meter	Adults	Motility	Objective	Automated	Low
	Nematospiroides dubius[b]	Micro-motility meter	Adults	Motility	Objective	Automated	Low
Dey and Roy (2018)	*Raillietina echinobothrida**	Bright-field microscopy	Adults	Motility	Subjective	Manual	Low
Ding et al. (2017)	*Caenorhabditis elegans*	Microfluidic (image-based)	Adults	Motility	Objective	Automated	Low
Douch et al. (1983)	*Trichostrongylus colubriformis*	Bright-field microscopy	xL3s	Larval migration	Subjective	Manual	Low
Evans et al. (2013)	*Dirofilaria immitis*	Bright-field microscopy	L3s	Larval migration	Subjective	Manual	Low
	Brugia pahangi	Bright-field microscopy	L3s	Larval migration	Subjective	Manual	Low
Fateh et al. (2021)	*Echinococcus granulosus**	Bright-field microscopy (eosin exclusion)	Protoscoleces	Viability	Subjective	Manual	Low
Fetterer et al. (1977)	*Schistosomamansoni**	Electrophysiology-based	Adults	Motility	Objective	Automated	Low
Folz et al. (1987)	*Trichostrongylus colubriformis*	Micro-motility meter	xL3s	Motility	Objective	Automated	Low
Ge et al. (2019)	*Caenorhabditis elegans*	Microfluidic (fluorescence-based)	Adults	Motility	Objective	Automated	Low
Gill et al. (1991)	*Haemonchus contortus*	Bright-field microscopy	L3s	Motility	Subjective	Manual	Low
Hordegen et al. (2006)	*Haemonchus contortus*		xL3s	Viability	Subjective	Manual	Low

(*continued on next page*)

Table 1 (*continued*)

Authors (alphabetical)	Species used in the assay	Technology used in the assay	Life-cycle stages used[a]	Phenotypic characters assessed	Read-out	Characteristics	Through-put potential
		Bright-field microscopy (dye uptake)					
Howe et al. (2015)	*Schistosoma mansoni**	Fluorescence-based	Schistosomula	Viability	Objective	Automated	Medium
Jacob et al. (2021)	*Angiostro*	Bright-field microscopy	L3s	Motility	Subjective	Manual	Low High
		Image-based	L3s	Viability	Objective	Automated	
James and Davey (2007)	*Caenorhabditis elegans*	Absorbance-based (MTT)	L1s	Viability	Objective	Automated	Medium
	Haemonchus contortus	Absorbance-based (MTT)	L1s	Viability	Objective	Automated	Medium
Jasmer et al. (2020)	*Ascaris suum*	Fluorescence microscopy	L3s	Viability	Subjective	Manual	Low
Jeyathilakan et al. (2012)	*Fasciola gigantica**	Bright-field microscopy	Adults	Motility	Subjective	Manual	Low
Jiraungkoorskul et al. (2005)	*Eurytrema pancreaticum**	Bright-field microscopy	Adults	Motility	Subjective	Manual	Low
Keiser et al. (2013)	*Fasciola hepatica**	Heat flow-based	Juveniles	Motility	Objective	Automated	Medium
Kirchhofer et al. (2011)	*Fasciola hepatica**	Heat flow-based	Juveniles, adults	Motility	Objective	Automated	Medium
Kotze et al. (2004)	*Haemonchus contortus*	Bright-field microscopy	L3s	Motility	Subjective	Manual	Low
	Ancylostoma ceylanicum	Bright-field microscopy	L3s	Motility	Subjective	Manual	Low
	Ancylostoma caninum	Bright-field microscopy	L3s	Motility	Subjective	Manual	Low
	Necator americanus	Bright-field microscopy	L3s	Motility	Subjective	Manual	Low
	Strongyloides stercoralis	Bright-field microscopy	L3s	Motility	Subjective	Manual	Low
	Strongyloides ratti	Bright-field microscopy	L3s	Motility	Subjective	Manual	Low
Kundu et al. (2017)	*Hymenolepis diminuta**	Bright-field microscopy	Adults	Motility	Subjective	Manual	Low
Lai et al. (2014)	*Caenorhabditis elegans*	Absorbance-based (MTS)	Embryonic cells, L2s	Viability	Objective	Automated	High
Lalli et al. (2015)	*Schistosoma mansoni**	Luminescence-based	Schistosomula	Viability	Objective	Automated	Medium
Liu et al. (2019)	*Cooperia oncophora*	Image-based	xL3s	Motility	Objective	Automated	Medium
	Ostertagia ostertagi	Image-based	xL3s	Motility	Objective	Automated	Medium
	Haemonchus contortus	Image-based	xL3s	Motility	Objective	Automated	Medium
	Teladorsagia circumcincta	Image-based	xL3s	Motility	Objective	Automated	Medium
Lockery et al. (2012)	*Caenorhabditis elegans*	Microfluidic (electrophysiology-based)	Adults	Pharyngeal pumping	Objective	Automated	Low
Mazhangara et al. (2020)	*Paramphistomum cervi**	Bright-field microscopy	Adults	Motility	Subjective	Manual	Low
Manneck et al. (2011)	*Schistosoma mansoni**	Isothermal microcalorimetry	Schistosomula	Metabolic activity-heat production	Objective	Automated	Low
Mansour and Bickle (2010)	*Schistosoma mansoni**	Fluorescence-based	Schistosomula	Viability	Objective	Automated	Medium
Marcellino et al. (2012)	*Brugia malayi*	Image-based	Adults	Motility	Objective	Automated	Medium
Mathew et al. (2012)	*Caenorhabditis elegans*	Image-based	L1s	Mortality, motility, fecundity, size	Objective	Automated	Medium-high
Mathew et al. (2016)	*Caenorhabditis elegans*	Image-based	L4s	Mortality, motility, fecundity, length	Objective	Automated	Medium-high
Mitsui and Kato (2018)	*Schistosomamansoni**	Absorbance-based (methylene blue, neutral red, trypan blue)	Adults	Motility, body morphology	Subjective	Manual	Low
de Moura and Rozental (1983)	*Schistosomamansoni**	Electrophysiology-based	Adults	Motility	Objective	Manual	Low
Mukherjee et al. (1998)	*Acanthocheilonema viteae*	Bright-field microscopy Absorbance-based (MTT)	Microfilariae	Motility	SubjectiveObjective	Manual	Low
	Acanthocheilonema viteae		Adults	Viability		Automated	
O'Grady and Kotze (2004)	*Haemonchus contortus*	Bright-field microscopy	Adults	Motility	Subjective	Manual	Low
Panic et al. (2013)	*Echinostoma caproni**	Bright-field microscopy	Newly excysted larvae	Motility	Subjective	Manual	Low
Partridge et al. (2018)	*Caenorhabditis elegans*	Image-based	L1s, young adults	Growth, motility	Objective	Automated	High
	Haemonchus contortus	Image-based	xL3s	Motility	Objective	Automated	High
	Teladorsagia circumcincta	Image-based	xL3s	Motility	Objective	Automated	High
	Trichuris muris	Image-based	Adults	Motility	Objective	Automated	Medium
Paveley et al. (2012)	*S. mansoni**	Image-based	Schistosomula	Morphology, motility	Objective	Automated	High
Peak et al. (2010)	*S. mansoni**	Fluorescence-based	Schistosomula	Viability	Objective	Automated	High
Phiri et al. (2017)	*Caenorhabditis elegans*	Absorbance-based (neutral red)	Adults	Cuticular damage	Objective	Automated	Low
Preston et al. (2015)	*Haemonchus contortus*	Image-based	xL3s	Motility	Objective	Automated	Medium
Puckering et al. (2019)	*Caenorhabditis elegans*	Image-based	L4s	Motility	Objective	Automated	Medium
Rapson et al. (1986)	*Nipostrongylus brasiliensis*	Absorbance-based	L4s, young adults	Acetylcholinesterase secretion	Objective	Automated	Low

(*continued on next page*)

Table 1 (*continued*)

Authors (alphabetical)	Species used in the assay	Technology used in the assay	Life-cycle stages used[a]	Phenotypic characters assessed	Read-out	Characteristics	Through-put potential
Ravaynia et al. (2020)	*Schistosoma mansoni**	Impedance-based	Schistosomula	Motility	Objective	Automated	Medium-high
Rinaldi et al. (2015)	*Schistosoma mansoni**	Impedance-based	Eggs, cercariae, adults	Motility, egg hatch	Objective	Automated	Medium
Ritler et al. (2017)	*Echinococcus multilocularis**	Image-based	Protoscoleces	Motility	Objective	Automated	Medium
Satou et al. (2001)	*Strongyloides ratti*	Image-based	L3s	Motility	Objective	Automated	Medium
	Strongyloides venezuelensis	Image-based	L3s	Motility	Objective	Automated	Medium
Serbus et al. (2012)	*Wolbachia* - endosymbiont of filarial worms	Fluorescence-based	Cells	Cell viability-bacterial load	Objective	Automated	High
Silbereisen et al. (2011)	*Trichuris muris*	Heat flow-based	L4s	Motility	Objective	Automated	Medium
	Trichuris muris	Impedance-based	L4s, adults	Motility	Objective	Automated	Medium
da Silva et al. (2018)	*Schistosoma manson**	Bright-field microscopy	Adults	Motility	Subjective	Manual	Low
Simonetta and Golombek (2007)	*Caenorhabditis elegans*	Image-based	L4s	Motility	Objective	Automated	Medium
Smout et al. (2010)	*Haemonchus contortus*	Impedance-based	Eggs, L3s	Motility, egg hatch	Objective	Automated	High
	Strongyloides ratti	Impedance-based	L3s	Motility	Objective	Automated	High
	Ancylostoma caninum	Impedance-based	Adults	Motility	Objective	Automated	High
	*Schistosoma mansoni**	Impedance-based	Adults	Motility	Objective	Automated	High
Stadelmann et al. (2010)	*Echinococcus multilocularis**	Absorbance-based	Metacestode (larval)	Phosphoglucose isomerase release	Objective	Automated	Low
Storey et al. (2014)	*Cooperia punctata*	Image-based	L3s	Motility	Objective	Automated	High
	Brugia malayi	Image-based	Microfilariae, adults	Motility	Objective	Automated	High
	Dirofilaria immitis	Image-based	Microfilariae	Motility	Objective	Automated	High
Stroustrup et al. (2013)	*Caenorhabditis elegans*	Image-based	Young adults	Worm survival	Objective	Automated	Medium
Taki et al. (2021a, 2021b)	*Caenorhabditis elegans*	Image-based	Young adults	Motility	Objective	Automated	High
Taki et al. (2021a, 2021b)	*Haemonchus contortus*	Image-based	xL3s	Motility	Objective	Automated	High
Taylor (1990)	*Haemonchus contortus*	Bright-field microscopy	Eggs	Larval migration	Subjective	Manual	Low
	Teladorsagia circumcincta	Bright-field microscopy	Eggs	Larval migration	Subjective	Manual	Low
Townson et al. (1989)	*Onchocerca volvulus*	Absorbance-based (MTT)	Adults	Viability	Objective	Automated	Low-medium
	Onchocerca gutturosa		Adults	Viability	Objective	Automated	Low-medium
Tritten et al. (2012)	*Ancylostoma ceylanicum*	Impedance-based	Adults	Motility	Objective	Automated	Low-medium
Wagland et al. (1992)	*Trichostrongylus colubriformis*	Bright-field microscopy	L3s	Larval migration	Subjective	Manual	Low
Weeks et al. (2016)	*Ancylostoma ceylanicum*	Microfluidic (electrophysiology-based)	L3s, L4s	Pharyngeal pumping	Objective	Automated	Medium
	Ancylostoma caninum	Microfluidic (electrophysiology-based)	L3s	Pharyngeal pumping	Objective	Automated	Medium
Wimmersberger et al. (2013)	*Trichuris muris*	Bright-field microscopy	L1s	Motility	Subjective	Manual	Low
Zhao et al. (2017)	*Ascaris suum*	Bright-field microscopy	L3s	Larval migration	Subjective	Manual	Low

[a] Abbreviations: L1s = first-stage larvae; L2s = second-stage larvae; L3s = (infective) third-stage larvae; xL3s = exsheathed third-stage larvae; L4s = fourth-stage larvae; MTS = 3-(4,5,dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2–4-sulfophenyl)-2H-tetrazolium; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; XTT = 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

[b] Now called *Heligmosomoides polygyrus* or *Heligmosomoides bakeri*. Species marked with an asterisk are platyhelminths (flatworms: cestodes or trematodes).

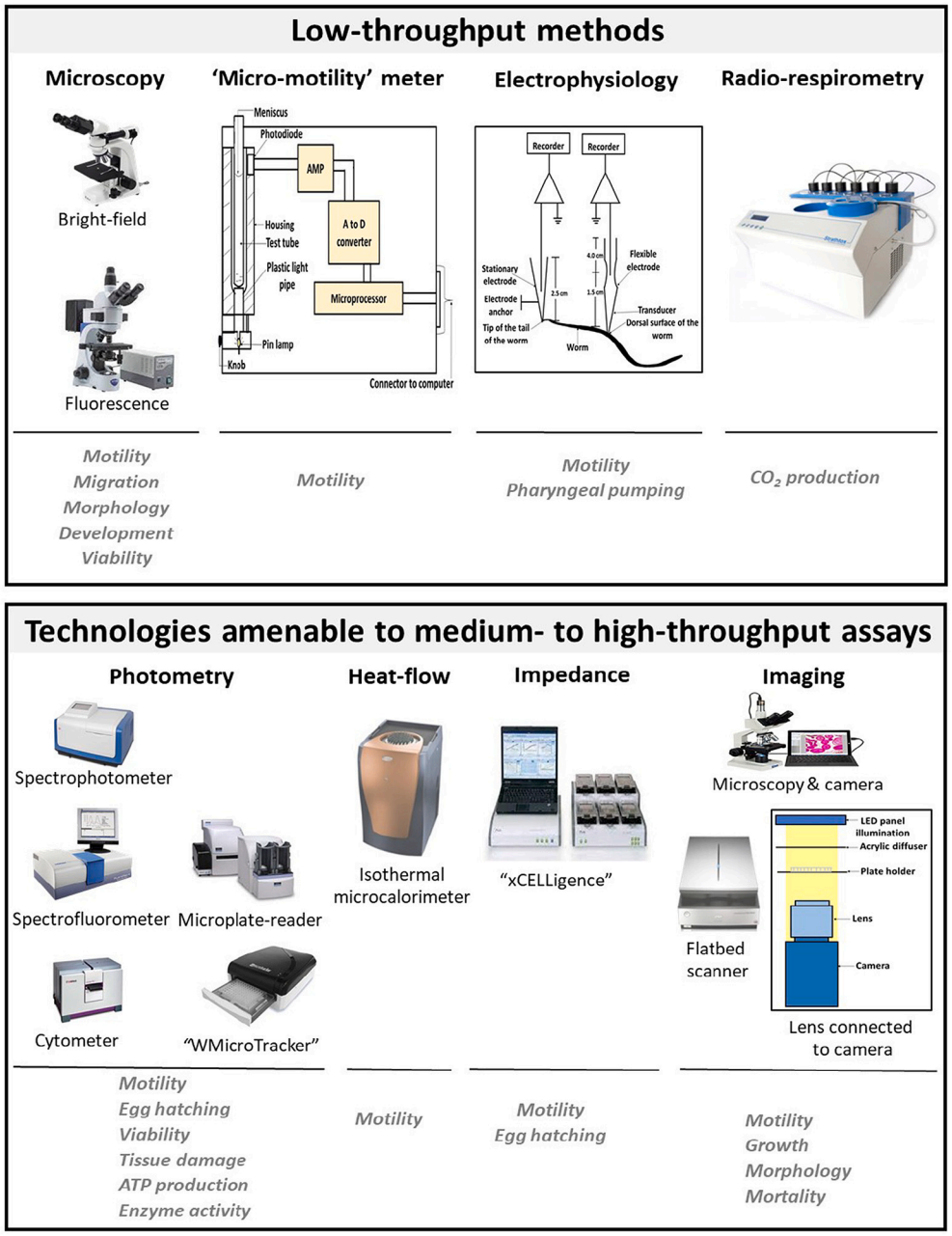

Fig. 2. A summary of techniques used in phenotypic screening assays for helminths employed for anthelmintic discovery. Low-throughput assays have relied on bright-field or fluorescence microscopy, micro-motility meters, electrophysiology or radio-respirometers. Technologies amenable to medium- or high-throughput assays include photometry-, heat-flow- or impedance-based instruments or imaging systems. Phenotypic parameters assessed or measured are indicated in grey font (cf. Table 1).

to accumulate dyes (Table 1). Worms were treated with test compound, and the colour change of the worms stained with a vital dye was recorded under microscopy to classify dead or live worms. Hordegen et al. (2006) used a method, modified from that used by Richardson et al. (1986), to assess the viability of xL3s of *Haemonchus contortus* employing the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (abbreviated as MTT; Hordegen et al., 2006), originally used by Townson and colleagues for anti-filarial screening (Townson et al., 1989; Comley et al., 1989a, 1989c). The colour-change from yellow (tetrazole/MTT) to blue (formazan) in live larvae differentiated them from dead ones (with no colour change) (Hordegen et al., 2006). A similar assay applied other dyes, such as methylene blue, neutral red and trypan blue, to the adult worms of *Schistosoma mansoni*, and allowed the viability status to be assessed using a stereoscopic microscope (Mitsui and Kato, 2018). These microscopy-based colorimetric assays reduce the subjectivity of observations/measurements to some extent; however, they are not completely objective due to the need for manual observation. Recently, Jasmer et al. (2020) used two fluorescent nuclear probes, bisbenzimide and propidium iodide, to assess pathological changes in L3s, L4s and adults of *Ascaris suum* caused by exposure to nematode intestinal toxins (NITs) by fluorescence microscopy. Although applied to *Ascaris suum*, the authors suggested that this approach would be applicable to other worm species. All of these microscopy-based techniques involve manual observation and evaluation of worm phenotypes by a trained operator, such that they can be quite time-consuming and labour-intensive to carry out, and are not readily adaptable to a high-throughput format.

4.2. Micro-motility meter/recorder

Bennett and Pax (1986) developed an instrument called the "micro-motility meter" to quantitatively measure the motility of larvae and adult helminths. The equipment measures the voltage signal generated from the light perturbed by the presence of worms in comparison to the

average voltage signal generated in the absence of worms (Bennett and Pax, 1986). This instrument was initially tested using L4s of *Ascaris suum* and *Nippostrongylus brasiliensis*, and adults of *Caenorhabditiselegans* and *Brugia pahangi* (see Bennett and Pax, 1986), and was later adapted to xL3s of *Trichostrongylus colubriformis* and adults of *Onchocerca volvulus* (see Folz et al., 1987; Comley et al., 1989b). Das et al. (1988) developed a micro-motility recorder, and tested it on *Ancylostoma ceylanicum* and *Nematospiroides dubius* (now called *Heligmosomoides polygyrus* or *Heligmosomoides bakeri*; cf. Behnke et al., 2009); this assay recorded changes in voltage signal as a result of light-scattering resulting from the movement of worms. In this instrument, the read-out was "paralysis time" rather than "motility index" recorded in the micro-motility meter (Bennett and Pax, 1986). These micro-motility-based assays provided relatively objective, quantitative motility measurements for larval or adult helminths, but have not been amenable to a high-throughput format.

4.3. Radio-respirometry-based assays

Comley and Rees (1989) developed an assay using *Acanthocheilonema viteae* and *Onchocerca gibsoni* to measure inhibition of CO_2 evolution following exposure to test compounds. This radio-respirometric assay quantified $^{14}CO_2$ released from either individual worms or fragments of filarial tissues. Of the four substrates (i.e., L-[U-^{14}C] glutamine, [l-^{14}C] acetate, [l-^{14}C] octanoate and D-[U-^{14}C] glucose) assessed, glutamine showed a linear rate of $^{14}CO_2$ production. This method provided a sensitive measure of the viability of filarioid worms, but the requirement to use radioisotope-labelling rendered this approach untenable for high-throughput screening (Comley and Rees, 1989).

4.4. Electrophysiology-based assays

Fetterer et al. (1977) developed an apparatus to assess the motility of the blood fluke *Schistosoma mansoni* by measuring contractions occurring as a result of electrical stimulation. This system provided a simultaneous recording of electrical (on the surface) and motor (i.e., worm shortening) activities for individual worms. Subsequently, a similar assay was developed for the same species (de Moura and Rozental, 1983). Then, Lockery et al. (2012) developed a microfluidic-based platform to measure pharyngeal pumping activity in *Caenorhabditiselegans*; this platform was later adapted to screen L4s of *Ancylostoma ceylanicum* and L3s of *Ascaris suum* for anthelmintic candidates (Weeks et al., 2016). Although microfluidic-based platforms are not suited to a high-throughput format, they allow detailed, real-time measurements of the effects of compounds/drugs on individual worms, which has major benefits for studying lead compounds or anthelmintics with unknown mechanisms/modes of action.

5. Technologies amenable to medium- or high-throughput assays

The integration of technologies to develop sophisticated platforms for phenotype recording and data analysis, and the establishment of automated systems, enabled implementation of anthelmintic assays with increased throughput (Paveley and Bickle, 2013; Buckingham et al., 2014; Hahnel et al., 2020; Sepúlveda-Crespo et al., 2020). For instance, photometry-based assays can measure absorbance, fluorescence, luminescence or light scattering to identify phenotypic alterations of/in parasites (see Subsection 5.1; Fig. 2). Furthermore, integration of physics, engineering, chemistry and biology facilitated the assessment of compound-induced phenotypic alterations by obtaining quantitative measurements of changes in heat production or impedance (see Subsection 5.2; Fig. 2). The use of high-resolution cameras, coupled with microscopy or other image acquisition instruments, enabled the development of improved high-throughput and/or high-content image-based screening platforms (see Subsection 5.3; Fig. 2). In addition, coupling microfluidic chips to phenotype analysis platforms allows detailed, real-time studies of compound effects on individual worms (see Subsections 5.2 and 5.3). These advances have resulted in a range of medium- to high-throughput phenotypic screening assays for multiple helminth species (see Subsections 5.1–5.3).

5.1. Photometry-based techniques

5.1.1. Absorbance

Absorbance-based techniques typically measure worm or cell viability. Initial absorbance-based assays used a standard spectrophotometer to obtain readings, but, more recently, robust and efficient microplate readers have been introduced. These instruments can usually read 96-, 384- or 1536-well microplates in a few minutes, and measure the amount of light absorbed by the product of a chromogenic reaction at a specific wavelength (usually 410, 490 or 575 nm). Microplate readers provide objective, quantitative measurements in a medium- (using 96-well plates) or high- (using 384 or 1536-well plates) throughput format.

Colorimetric MTT-based assays have been useful for phenotypic screening (Comley et al., 1989a, 1989c; Mukherjee et al., 1998; James and Davey, 2007). Live larvae metabolise MTT, which reduces to formazan (dark blue) and can be assessed microscopically (cf. Subsection 4.1) or quantified by measuring the absorbance in a standard spectrophotometer or plate reader (Fig. 2). This approach has been used to develop screening platforms to measure the viability of species including *Caenorhabditiselegans*, *Haemonchus contortus*, *Acanthocheilonema viteae*, *Brugia pahangi* and *Onchocerca* spp. (see Comley et al., 1989a, 1989c; Mukherjee et al., 1998; James and Davey, 2007; cf. Table 1). Similar colorimetric assays were developed based on the reduction of the tetrazolium salt 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (abbreviated as XTT) and 3-(4,5,dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2–4-sulfophenyl)-2H-tetrazolium (abbreviated as MTS) to formazan, to assess the viability of *Schistosoma mansoni* and *Caenorhabditiselegans*, respectively (Lai et al., 2014; Aguiar et al., 2017). Phiri et al. (2017) established a platform for *Caenorhabditiselegans* based on the ability of neutral red dye to incorporate into lysosomes and released following cellular damage. Here, live worms were stained with the dye, treated with test compound, and dye-leakage due to cuticle damage was measured spectrophotometrically (Phiri et al., 2017). Absorbance-based assays using the nematode *Nippostrongylus brasiliensis* and the cestode *Echinococcus multilocularis* were also developed to measure acetylcholinesterase secretion and phosphoglucose isomerase activity, respectively (Rapson et al., 1986; Stadelmann et al., 2010). These absorbance-based techniques overcame the time-consuming assessment of dye uptake using a microscope, and provided relatively objective and quantitative measurements in colorimetric assays.

5.1.2. Fluorescence

Advances in instruments, including spectrofluorometers, fluorescence microplate readers and cytometers, enabled the establishment of fluorescence-based screening platforms using multiple helminth species. These assays utilise fluorescent dyes or probes, and obtain phenotypic measures for worm or cell viability (Fig. 2). For instance, Mansour and Bickle (2010) applied Alamar blue to schistosomula of *Schistosoma mansoni* to measure compound-induced fluorescence intensity changes in a plate reader, while Silbereisen et al. (2011) applied this dye to L4s of *Trichuris muris* to measure fluorescence changes employing a spectrofluorometer. Peak et al. (2010) developed a platform employing fluorescein diacetate (FDA) to detect live worms and propidium iodide (PI) to detect dead *Schistosoma mansoni*. Furthermore, Cintra et al. (2019) measured the viability of *Caenorhabditiselegans* employing a 2,1,3-benzothiadiazole (BTD) fluorescent derivative (DB-1, to identify worms in the population) and PI (to identify dead worms). In the same year, Abriola et al. (2019) developed an assay to measure egg hatch-inhibition

in *Ancylostoma ceylanicum*. This assay was developed based on the principle that chitinase released from hatched eggs emits a pronounced fluorescent signal compared with unhatched eggs (Abriola et al., 2019). The amount of lactate was also successfully assessed as a surrogate marker for viability in *Schistosoma* sp. using a fluorometric L-lactate assay kit (Howe et al., 2015).

Fluorescence-based assays were also applied to the bacterium *Wolbachia* as a target in filarioids. For instance, Serbus et al. (2012) used a *Wolbachia*-infected, fluorescently labelled (Jupiter-GFP) *Drosophila* JW18 cell line and assessed the bacterial content using DAPI (4′,6-diamidino-2-phenylindole). Clare et al. (2015) used the green, fluorescent nucleic acid stain SYTO 11 to develop an assay to measure bacterial load and host cell numbers based on a texture analysis. Another assay was developed using *Wolbachia*-infected LDW1 cells to assess *Wolbachia* content using fluorescent in situ hybridization (FISH) and LDW1-content employing DAPI (Bakowski et al., 2019). Furthermore, Clare et al. (2019a, 2019b) developed an assay to measure *Wolbachia*-linked fluorescence in compound-treated and relevant controls using a cytometer, and the assay was validated by screening AstraZeneca's 1.3 million in-house compound library. This semi-automated, high throughput assay was the result of a successful public-private partnership between the Anti-*Wolbachia* (A·WOL) Consortium at the Liverpool School of Tropical Medicine, UK, and the Global High-Throughput Screening Centre at AstraZeneca (Clare et al., 2019a, 2019b). These fluorescence-based assays provide sensitive and quantitative measurements of worm and/or cell viability.

5.1.3. Luminescence

The development of instruments that quantify emitted light photons, as a result of a chemical, biochemical or enzymatic reaction, facilitated the implementation of luminescence-based screening platforms (Fig. 2). Although these types of screening platforms are not commonly used for anthelmintic discovery, Lalli et al. (2015) established a luminescence-based assay to assess the viability of schistosomula of *Schistosoma mansoni* by quantifying the amount of ATP that accumulated. The availability of luminescence-plate readers allows the establishment of sensitive and high- throughput luminescence-based platforms. Additionally, microplate readers with multiple modes (e.g., absorbance, fluorescence and luminescence) are beneficial for developing high-throughput assays with multiple readouts.

5.1.4. Infrared scattering

Assays that rely on infrared microbeam measurements have also been used to screen compounds for anthelmintic activity (Fig. 2). Simonetta and Golombek (2007) developed a "worm tracking" system to assess the locomotion of *Caenorhabditiselegans* using infrared microbeam scattering. The system measured the interference of an infrared light beam by worm movement, and recorded "activity counts" as a measure of motility. This system was the basis for the development of a new, automated, instrument called "WMicroTracker" to measure worm motility. This improved system has been applied to parasitic helminths including *Haemonchus contortus*, *Ostertagia ostertagi*, *Teladorsagia circumcincta* and *Cooperia oncophora* (see Liu et al., 2019).

Recently, Taki et al. (2021a, 2021b) adapted WMicroTracker to develop a low-cost, high throughput assay to screen compounds against *Caenorhabditiselegans* and *Haemonchus contortus*. A critical appraisal of the literature and understanding of technical aspects of this device identified that a change of a setting in the instrument allowed the throughput of the assay to be substantially increased (Taki et al., 2021a, 2021b). The authors showed that the use of "Mode 1" setting allowed the capture of high "activity counts" with a marked reduction in time required for data acquisition (15 min) compared with much longer acquisition times in the previous studies (i.e. ~ 3 h). This assay was critically evaluated by screening 14,400 small molecules from the "HitFinder" library (Maybridge) against *Caenorhabditiselegans* (see Taki et al., 2021a), and 80,500 compounds from the Jump-stARter library against *Haemonchus contortus* (see Taki et al., 2021b). This enhancement in performance of the WMicroTracker now allows ~10,000 compounds to be screened per week in a 384-well format (Taki et al., 2021a, 2021b).

5.2. Heat flow or impedance-based techniques

Assays based on the measurement of heat production or impedance have been developed to assess different phenotypic characters in helminths (Fig. 2). Recent advances in microcalorimetry have enabled the establishment of new, multi-channel isothermal microcalorimeters (IMC), which can quantitatively assess parasite motility indirectly by measuring changes in heat production during parasite movement. Assays based on isothermal microcalorimetry have been developed to measure compound/drug-induced changes of motility in schistosomula and adults of the blood fluke *Schistosoma mansoni* (see Manneck et al., 2011), as well as juveniles and adults of the liver fluke *Fasciola hepatica* (see Kirchhofer et al., 2011; Keiser et al., 2013). These assays use an IMC with 48 channels. However, through recent advances, calorimeters on chips (i.e., microfluidics) and well-plate calorimeters (https://symcel.com) have been developed. Therefore, there is potential for developing high-throughput platforms for helminths employing these commercially available instruments.

An impedance-based, real-time cell monitoring device, called xCELLigence, was used to measure egg hatching and motility of parasitic worms in an automated, high-throughput format. The xCELLigence system comprises microelectrodes that measure changes in electrical impedance as worms move across the electrodes. This system was applied to the nematodes *Haemonchus contortus*, *Ancylostoma caninum, Ancylostoma ceylanicum, Strongyloides ratti*, *Trichuris muris* and to the blood fluke *Schistosoma mansoni* to measure egg hatching and/or motility (Smout et al., 2010; Silbereisen et al., 2011; Tritten et al., 2012). Rinaldi et al. (2015) improved the xCELLigence assay to become a xCELLigence worm real-time motility assay (xWORM) by using diverse frequency settings. Published evidence indicates that both isothermal microcalorimetry and impedance-based assays are reliable, although neither of these techniques was able to measure sufficient signals when L3s of *Ancylostoma ceylanicum* (see Tritten et al., 2012) and L4s of *Trichuris muris* (see Silbereisen et al., 2011) were tested. Furthermore, both these techniques require extensive analysis time, and a new batch of compounds cannot be screened until the prior analysis is completed. Impedance-based platforms have also been coupled to microfluidic systems. Such impedance-based microfluidic platforms were developed to analyse size and motility of larvae, such as newly transformed schistosomula of *Schistosoma mansoni* (see Chawla et al., 2018; Ravaynia et al., 2020).

5.3. Imaging techniques

Advances in image acquisition techniques, image analysis software and automated systems enabled the development of high-throughput screening platforms (Sepúlveda-Crespo et al., 2020; Fig. 2). Some imaging platforms were developed using bright-field microscopy coupled to high-resolution cameras for image/video recording, with analyses performed using computer-based analysis software. Such platforms were developed to measure phenotypic characters such as body morphology and/or motility of the nematodes *Caenorhabditis elegans*, *Haemonchus contortus*, *Strongyloides ratti and Strongyloides venezuelensis*, and the flatworms *Schistosoma mansoni* and *Echinococcus multilocularis* (see Satou et al., 2001; Buckingham and Sattelle, 2009; Paveley et al., 2012; Preston et al., 2015; Ritler et al., 2017; Chen et al., 2020). Some assays have used high-content approaches employing bright-field or fluorescence microscopy, with enhanced sensitivity and accuracy for measuring phenotypic changes (Paveley et al., 2012; Chen et al., 2020; Jacob et al., 2021).

"WormAssay" is open-source computer software, developed by Marcellino et al. (2012) at the Centre for Discovery and Innovation in

Parasitic Diseases at the University of California, San Francisco, to assess the motility of adults of *Brugia malayi*. This assay was based on a visual imaging system, in which the videos of 24-well plates with treated parasites, and relevant controls, were recorded using a video camera positioned under the plate, with videos being subsequently analysed using an analysis software (Marcellino et al., 2012). Storey et al. (2014) later adapted this assay and modified the software to develop a platform called "Worminator" to assess the motility of L3s of *Cooperia* spp., microfilariae of *Brugia malayi* and *Dirofilaria immitis* (see Storey et al., 2014) and adults of *Brugia pahangi* (see Abongwa et al., 2021). The "Worminator" platform allowed medium- to high-throughput in 96- or 384-well plates (Storey et al., 2014). Partridge et al. (2018) also implemented an "Invertebrate automated phenotyping platform" (INVAPP), coupled to an algorithm called "Paragon". This INVAPP/Paragon system consisted of a high-resolution camera/lens, and a panel of light-emitting diodes to measure motility and growth of *Caenorhabditis elegans*, *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichuris muris* by analysing the videos using MATLAB scripts (Partridge et al., 2018).

The "WormScan" platform developed by (Mathew et al., 2012) provided an automated, quantitative assessment of four phenotypes (i.e., death, fecundity, motility and size) of *Caenorhabditis elegans* grown on Petri dishes. This system utilised a light stimulus from a flat-bed scanner to induce worm motility, and the scanned images were analysed using a software (Mathew et al., 2012). This assay was developed to study the effects of toxins, but Stroustrup et al. (2013) later adapted it to measure *C. elegans* lifespan (on Petri dishes). Mathew et al. (2016) further increased the throughput of this assay by adapting it to screen four 96-well plates at a time. Puckering et al. (2019) also adapted the initial assay to develop an "automated Wormscan", which employed a hardware to enumerate worms, compared to a machine learning-based system used in the initial assay (Mathew et al., 2012). This system can scan up to 12 Petri dishes simultaneously, followed by an immediate second scan (Puckering et al., 2019). It has a limited computational demand, as it does not require software training for phenotypic detection. It also has the potential to enhance throughput further, as there is no limit in the number of scanners that can be run simultaneously from a single desktop PC (Puckering et al., 2019).

High-content and/or high-throughput image/video capturing platforms have also been coupled to microfluidic chips to obtain detailed, real time measurements of the effects of compounds on worms. Most of these chips were developed to measure worm motility and/or growth of *Caenorhabditis elegans* in detail, usually on a small scale (Carr et al., 2011; Aubry and Lu, 2017; Ding et al., 2017; Atakan et al., 2019; Ge et al., 2019).

6. Concluding remarks

The animal and human health industries urgently need new anthelmintics. However, the consolidation of the industry has reduced the overall effort in this area, and the assays used in industry are generally held as proprietary, and were, thus, not a topic pursued in the present analysis. Some efforts have been directed toward discovery of new antifilarials (macrofilaricides) with funding from the Bill & Melinda Gates Foundation, among others, particularly for onchocerciasis (including Anti-*Wolbachia* Consortium; https://awol.lstmed.ac.uk/), but these efforts have recently matured into field trials, and de novo discovery efforts are now minimal. The role of academic laboratories in this enterprise is, thus, more important than ever, and - considering the rather limited financial support available for such efforts – requires coordination and development of 'best practices' for optimal utilisation of these resources.

This review summarises whole-worm phenotypic screening techniques that have been employed in academic laboratories over the years 1977–2021 to screen compounds (synthetic or natural products), with an emphasis on the discovery of nematocides. It discusses recent advances in semi-automated and quantitative platforms with medium- to high-throughput. Some platforms, such as xCELLigence, Wormscan and WMicroTracker, can be established using commercially available instruments/equipment. For instance, the WMicroTracker system, which measures infrared light interference, is available through Phylumtech (Argentina) and can be set up in various constellations (either partially or fully automated) at relatively low cost (tens to hundreds to thousands of dollars) compared with robotic systems in industry (millions of dollars), and achieves favourable performance and throughput in an academic context (e.g., 5000 to 10,000 compounds per week; employing 96- or 384-well plates, depending on worm size and the throughput required). While most high- throughput screening methods yield quantitative, univariate phenotypic data, advanced high-content methods (e.g., Paveley et al., 2012; Aulner et al., 2019; Chen et al., 2020) employ high-resolution microscopy combined with image capture and analysis, and produce multivariate data, allowing for deeper quantitative assessments of compound-induced phenotypic alterations with improved specificity and sensitivity. Such deeper evaluations are pertinent, given the observation that some anthelmintics that work against actual worm infections in vivo do not induce obvious phenotypes on the same worms assayed in vitro (Zamanian and Chan, 2021). Therefore, the ability to discern subtle whole-animal phenotypes, rather than obvious ones (e.g., lethality), might be surprisingly relevant to detecting effective anthelmintic compounds. Clearly, a range of high-content, high-throughput platforms, such as Opera, Operetta (Perkin Elmer), IN Cell Analyzer 2000 (GE Healthcare), Acumen eX3 (TTP LabTech), CellWoRx (Thermo Scientific) and Cellomics ArrayScan VTI (Thermo Scientific), are available commercially, but they are usually unaffordable for academic research groups, but may be accessible via core/joint facilities. Although not routinely applied to parasitic worms in the academic context, use of such high-content platforms would substantially accelerate and enhance the accuracy of high-throughput screening for early phase anthelmintic discovery.

Based on recent published evidence, it is anticipated that integrative 'omic, chemoinformatic and/or advanced bioinformatics (e.g., machine and deep learning) approaches (Lo et al., 2018; Ma et al., 2020; Elbadawi et al., 2021) will enable the prediction and prioritisation of drug targets (Campos et al., 2020a, 2020b), annotation and structural modelling of such targets (Alphafold; Jumper et al., 2021; Varadi et al., 2021), the selection of small molecule candidates (Agamah et al., 2020) and molecular simulation of binding of small molecules to predicted targets (Bagherian et al., 2021) in silico prior to detailed laboratory experimentation. Such in silico, biology-guided approaches, once validated, might be able to guide the selection of chemical entities/groups to be screened on particular parasite species and/or targets, with the potential of increasing efficiency, reducing costs and accelerating the drug discovery/development process overall. These are exciting prospects, but much work needs to be done to find the best combination of in silico tools and tailor them to support/enhance early phase anthelmintic drug discovery and development, as we work toward achieving some of the SDGs, particularly in relation to reducing poverty and disease, increasing food production and enhancing the health and wellbeing of animals and people.

Authorship

All authors participated in the planning, design, analysis and/or interpretation of the data, the drafting and/or editing of the manuscript for important intellectual content, and approved the final manuscript.

Declaration of Competing Interest

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter described in the manuscript.

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