Utilization of computer processed high definition video imaging for measuring motility of microscopic nematode stages on a quantitative scale: "The Worminator"

Bob Storey [a,*], Chris Marcellino [c,d], Melissa Miller [a], Mary Maclean [a], Eman Mostafa [a,b], Sue Howell [a], Judy Sakanari [c], Adrian Wolstenholme [a,e], Ray Kaplan [a]

[a] Department of Infectious Diseases, University of Georgia, Athens, GA, USA
[b] Parasitology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt
[c] Center for Discovery and Innovation in Parasitic Diseases, University of California San Francisco, San Francisco, CA, USA
[d] Case Western Reserve University School of Medicine, Cleveland, OH, USA
[e] Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, GA, USA

ARTICLE INFO

ABSTRACT

A major hindrance to evaluating nematode populations for anthelmintic resistance, as well as for screening existing drugs, new compounds, or bioactive plant extracts for anthelmintic properties, is the lack of an efficient, objective, and reproducible in vitro assay that is adaptable to multiple life stages and parasite genera. To address this need we have developed the "Worminator" system, which objectively and quantitatively measures the motility of microscopic stages of parasitic nematodes. The system is built around the computer application "WormAssay", developed at the Center for Discovery and Innovation in Parasitic Diseases at the University of California, San Francisco. WormAssay was designed to assess motility of macroscopic parasites for the purpose of high throughput screening of potential anthelmintic compounds, utilizing high definition video as an input to assess motion of adult stage (macroscopic) parasites (e.g. Brugia malayi). We adapted this assay for use with microscopic parasites by modifying the software to support a full frame analysis mode that applies the motion algorithm to the entire video frame. Thus, the motility of all parasites in a given well are recorded and measured simultaneously. Assays performed on third-stage larvae (L3) of the bovine intestinal nematode Cooperia spp., as well as microfilariae (mf) of the filarioid nematodes B. malayi and Dirofilaria immitis, yielded reproducible dose responses using the macrocyclic lactones ivermectin, doramectin, and moxidectin, as well as the nicotinic agonists, pyrantel, oxantel, morantel, and tribendimidine. This new computer based-assay is simple to use, requires minimal new investment in equipment, is robust across nematode genera and developmental stage, and does not require subjective scoring of motility by an observer. Thus, the "Worminator" provides a relatively low-cost platform for developing genera- and stage-specific assays with high efficiency and reproducibility, low labor input, and yields objective motility data that is not subject to scorer bias.

© 2014 The Authors. Published by Elsevier Ltd. on behalf of Australian Society for Parasitology Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

1. Introduction

Anthelmintic resistance of gastrointestinal nematode (GIN) parasites constitutes a major problem for livestock health and productivity around the world (Fleming et al., 2006; Kaplan and Vidyashankar, 2012). The common practice of intensive farming methods coupled with heavy reliance on anthelmintics has resulted in a serious escalation in the prevalence, distribution, and scope of AR in many of the most important GIN parasite species. In sheep and goats the situation is the most severe, with increasing number of farms around the world experiencing resistance to all classes of available anthelmintics (Howell et al., 2008; Almeida et al., 2010; Sczesny-Moraes et al., 2010). Multiple anthelmintic resistance in GIN parasites of cattle (Sutherland and Leathwick, 2011) and horses (von Samson-Himmelstjerna, 2012; Canever et al., 2013) is also being increasingly reported raising the level of concern in these hosts. In contrast to the situation with GIN, until recently the filarioid parasites, such as Dirofilaria immitis, were considered to be at a low risk for developing AR (Prichard, 2005). However, reports of resistance to several currently available

macrocyclic lactone compounds (Bourguinat et al., 2011; Geary et al., 2011) have proven these predictions wrong. Though AR is not yet a problem in human nematodes, sub-optimal responses of ivermectin against *Onchocerca volvulus* (Osei-Atweneboana et al., 2007; Churcher et al., 2009), and the potential for heavy drug selection pressures on populations of soil transmitted helminths (STH) due to scale up of Mass Drug Administration (MDA) programs are raising serious concerns (Churcher et al., 2010; Keenan et al., 2013).

In vitro assays are the most efficient and cost effective means of diagnosing and characterizing anthelmintic resistance in nematode populations. Additionally, *in vitro* assays are an essential component of new anthelmintic compound testing/discovery. Highly sophisticated instrumentation has been developed to quantitatively measure motility of microscopic stages of parasites (e.g. Lemnatec Scanalyzer™ HTS, Aachen Germany) with high throughput, but these instruments are extremely expensive to purchase, costing several hundred thousand dollars. Additionally they are extremely expensive to maintain, making them impractical and unavailable to most all but the well-financed laboratories of the pharmaceutical industry.

Currently, there are several low-throughput *in vitro* assays available for screening new compounds for anthelmintic activity and for diagnosing anthelmintic resistance in nematode populations; these are the egg hatch assay (EHA) (LeJambre, 1976), the larval development assay (LDA) (Taylor, 1990; Coles et al., 2006), the larval migration inhibition assay (LMIA) (Wagland et al., 1992), and the larval motility assay (LMA) (Martin and Le Jambre, 1979). All of these assays were originally developed for diagnosing anthelmintic resistance in strongylid nematode parasites of sheep, and a few have been modified for use in related nematode species of other livestock hosts. The LMA has been adapted for sensitivity testing of anthelmintics for hookworm and *Strongyloides* spp. (Kotze et al., 2004, 2005), both being human STH. Validation of this assay for the detection of resistance has not been possible due to the lack of confirmed resistant isolates (this is also true for all in vitro assays relating to human STH at the time of this writing).

The LMIA and LMA utilize motility as an indicator of larval health and drug effectiveness and thus have the least limitations with regard to stage and species. However, to date these assays have not been well standardized across laboratories, and despite numerous publications that describe the LMIA, no consistent protocol has been established (Matthews et al., 2012). However, in one study, inter-lab variability was shown to be low when standardized protocols for LMIA were used on the same parasite isolates (Demeler et al., 2010a). The LMIA is based on the ability of larvae to migrate through a fine mesh screen, with larvae that are negatively affected by the test compound less able to migrate through the mesh. A major limiting factor to this assay is differing sizes of larval stages of different parasite species requiring custom selection of mesh size for every species; (Kotze et al., 2006; Demeler et al., 2010a; Evans et al., 2013). While this issue is not important in research laboratories where mono-specific samples are available and assays can be customized for the species being tested, it prevents this assay from being useful in diagnostic samples containing multiple species. Additionally, only one time point for migration can be tested in a single assay, thus numerous assays must be done to optimize incubation and migration periods for each nematode species and drug. The LMA requires an observer to assign a motility score for each larva on a 0–3 scale, or to classify it as motile (thrashing) or non-motile (moving in a restricted manner or still) (Gill et al., 1991). Consequently, the LMA is highly subjective, vulnerable to reader bias, poorly quantitative, and extremely low throughput.

The first use of technology to improve the measurement and analysis of nematode motility was the micromotility meter (Bennett and Pax, 1986). This instrument uses photo-detectors to measure changes in light refraction due to motility of parasites. The micromotility meter technology is covered under US Patent #4,603,977 (Bennett et al., 1986) and was sold through B & P Instruments (Mason, MI, USA). The inventors demonstrated the utility of this instrument by measuring the motility of a number of different helminths (including both nematodes and trematodes) and stages (Bennett and Pax, 1987). Subsequently, several labs have attempted to develop in vitro assays to detect anthelmintic resistance using the micromotility meter, with mixed results (Folz et al., 1987; Coles et al., 1989; Várady et al., 1998; Demeler et al., 2010b). Thus, despite offering an improvement in technology, this instrument has not gained wide usage, and has not led to new validated assays of importance.

Recent technological developments have facilitated the adaptation of computer image processing coupled with high definition video for studying and characterizing the motion of nematodes (Krajacic et al., 2012). WormAssay is a specific application of this type of technology developed at the University of California, San Francisco (UCSF). The WormAssay application utilizes high definition (HD) video as an input to assess motility of macro-parasites (i.e. visible to the naked eye) in 12,24,48, and 96 well cell culture plates for the purpose of high throughput screening of potential anthelmintic drug compounds (Marcellino et al., 2012). The program analyzes differences in worm position from successive video frames to determine the rate of movement using the Lucas–Kanade Optical flow algorithm. The lower the movement, the lower the motility number and more effective the compound is against the parasite.

Our objective was to create a system functionally equivalent to that described by Marcellino et al. (2012) that can be used with microscopic stages of nematode parasites of human and veterinary importance. The successful modification and validation of this image analysis system, which we call the "Worminator", provides a new tool for quantifying the motility of microscopic nematode stages. The Worminator provides a relatively low-cost, easy to assemble platform for developing genera- and stage-specific assays for measuring sensitivity to existing anthelmintics, as well as screening new compounds for anthelmintic properties. The Worminator assays developed to date have high efficiency and reproducibility, low labor input, and yield objective data that is not subject to scorer bias.

2. Materials and methods

2.1. "Worminator" system

The open source "WormAssay" computer application described by (Marcellino et al., 2012) is the core around which the "Worminator": system is built. The WormAssay application executes on an Apple Mac desktop or laptop computer running Mac OS X. Modifications to the WormAssay program were made to enable the analysis of a single well (full video frame) containing multiple organisms, while retaining the original multi-well (single organism per well) full plate assay capability. Additionally, support was added for HDMI input via Apple's Thunderbolt I/O port using a BlackMagic Design's (Freemont, CA, USA) "Intensity Extreme" video capture unit to deliver the HDMI stream via Thunderbolt. All of the aforementioned changes are contained in WormAssay revision 1.4, whose source and executable code can be found at https://code.google.com/p/wormassay. WormAssay version 1.4 requires Mac OS X 10.9 or later. A Canon Vixia HF M52 video camera (Canon, Inc.) was used as the HD video capture input for both micro and macroscopic assays. Macroscopic, full plate assays were accomplished using a dark field illuminator and plate holder

constructed in a similar fashion to the one built and described by Marcellino. Microscopic parasite stage assays utilized an Olympus IX51 inverted microscope with the Canon Vixia HF M52 attached via a Martin Microscope adapter, model MM99-58 (http://www.martinmicroscope.com), and a Diagnostic Instruments DBX 1.0X C-mount adapter (http://www.spotimaging.com). An Olympus PLAN-N 2x objective is used for L3 (~700 microns) assays and an Olympus PLANF-N 4x objective is used for mf (~275 microns) assays. An Olympus Long Distance phase contrast condenser is used as a light source.

WormAssay's "Assay Analyzer" option was set to "Consensus Voting Luminance Difference (dArea)" and the "Plate Orientation" option was set to "No Plate Mode" for analyzing the motility of mf and L3 stages. The "Assay Analyzer" option was set to "Lucas-Kanade Optical Flow (Velocity, 1 organism per well)" with the "Plate Orientation" set to "Bottom-Read" for the *Brugia malayi* adult parasites (Marcellino et al., 2012)

2.2. Parasites

B. malayi mf and adults, as well as the *D. immitis* mf were sourced from the NIH/NIAID Filariasis Research Reagent Resource Center (FR3; College of Veterinary Medicine, University of Georgia, Athens, GA, USA). The *D. immitis* used in this study is a macrocyclic lactone susceptible strain (2005 Missouri strain) maintained and passaged in beagle dogs at the University of Georgia (Athens, GA).

A mixed population of L3 stage cattle GIN, was obtained from a commercial beef operation in South Georgia. Cocultures were performed on pooled calf feces, and larvae were harvested after a 2 week incubation period using a Baermann apparatus (Dinaburg, 1942). These L3 were then used to infect a parasite free calf. Feces from this calf were collected on days 14 through 18 post infection and the resulting coproculture produced a mono-specific culture of *Cooperia punctata*. These L3 were then used to infect another parasite free calf to produce a mono-specific infection of *Cooperia punctata*. Purity of the resulting infection was confirmed by microscopic examination of a random sample of several hundred L3 (Ministry of Agriculture, 1977).

2.3. Brugia malayi microfilariae assay (Nicotinic Agonists)

Peritoneally derived mf of *B. malayi* (~124 mf/well) were assayed in RPMI-1640 with L-Glutamine (RPMI) (BioWhittaker® #12-702F) containing 1% Penicillin/Streptomycin (P/S) (Sigma–Aldrich #P4333) using 384-well, black with optically clear bottom, tissue culture plates (Nunc, Inc., #142761).

10 mM stock solutions of pyrantel tartrate (PYR) (1-Methyl-2-(2-[2-thienyl]ethenyl)-1,4,5,6 tetrahydropyrimidine, Sigma #P7674), oxantel pamoate (OXA) (1-methyl-2-(3 hydroxyphenylethenyl)-1,4,5,6-tetrahydropyrimidine, Sigma–Aldrich #O4755) and morantel citrate salt (MOR) (1,4,5,6-tetrahydro-1-methyl-2-(2-[3-methyl-2-thienyl]ethenyl)pyrimidine, Sigma–Aldrich #M5404) were prepared using 100% dimethyl sulfoxide (DMSO) (Sigma–Aldrich #D41639) and then serially diluted using RPMI + P/S to create working solution concentrations of 300, 200, 140, 100, 60, 20, 6, and 2 μM, with a DMSO concentration of 2%. DMSO was used as the negative control in concentration of 2% (v/v) in RPMI + P/S. A stock solution (10 mM) of Tribendimidine (TBDN) (Shandong Xinhua Pharmaceutical CO, Ltd) was prepared with absolute ethanol (ETOH) (Sigma–Aldrich #E7023) and serially diluted with RPMI + P/S into working concentrations of 300, 200, 140, 100, 60, 20, 6, and 2 μM with an ETOH concentration of 2%. ETOH (2% (v/v)) in RPMI + P/S was used as a negative control. All drug concentrations and controls were run in triplicate. A volume of working solution equal to the volume of RPMI and P/S containing the *B. malayi* mf were added the corresponding plate wells to achieve final drug concentrations of 150,

100, 70, 50, 30, 10, 3, and 1 μM, with 1% (v/v) ETOH in RPMI + P/S as a negative control. The plates were maintained at 37 °C at 5% CO_2. Motility readings using the Worminator were taken 1 h, 2 h, and 24 h after addition of the drug solution to the mf. Approximately 30 s of video was recorded and analyzed for each well at each reading.

2.4. Brugia malayi microfilariae assay (macrocyclic lactones)

10 mM stock solutions of ivermectin (IVM) (22,23-dihydroavermectin B1, Sigma–Aldrich # I8898), eprinomectin (EPR) ((4″R)-4″-(Acetylamino)-4″-deoxy-avermectin B₁, Sigma–Aldrich #32526), doramectin (DOR) (25-Cyclohexyl-5-O-demethyl-25-de(1-methylpropyl)avermectin, Sigma–Aldrich #33993), and moxidectin (MOX) (VETRANAL™ analytical standard Sigma–Aldrich #33746) were prepared in both DMSO and in propylene glycol (PG) (Sigma–Aldrich #PHR1051) and diluted in RPMI + 1% P/S to create working solutions of 0.002, 0.02, 0.2, 2, 4, 12, 20, 40 and 100 μM. Negative controls with 2% (v/v) DMSO in RPMI + P/S, and 2% PG(v/v) in RPMI + P/S were also prepared. Working solutions were dispensed into 0.5 ml micro-centrifuge tubes, with *B. malayi* mf added in equal volumes for final concentrations of 0.001, 0.01, 0.1, 1, 2, 6, 10, 20 and 50 μM with 1% (v/v) DMSO and PG controls. Tubes were mixed using a vortex mixer (Fisher Scientific #02215365) for 5–7 s at medium speed setting (5.5) (for each well) and then pipetted immediately into the 384 well plate (NUNC black with optically clear bottom #142761) for a total of 50 μl per well containing approximately 100 mf each. Wells surrounding those containing parasites and drug were filled with 50 μl RPMI 1640 containing 1% Pen/Strep to reduce evaporation during incubation. Plates were kept in a 37 °C incubator with 5% CO_2 for 24 h and then read using the Worminator system scanning each well for approximately 30 s.

2.5. Brugia malayi adult assay

Stock solutions of 10 mM ivermectin and 10 mM moxidectin serially diluted using 100% DMSO to yield stock working solution concentrations of 10.0, 5.00, 2.50, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, 0.0195, and 0.0097 mM in 100% DMSO. These working stock solutions were each further diluted 50 fold using incubation medium (described below) in order to create a final set of working solution with 2% DMSO and drug concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.781, 0.391, and 0.195 μM of each drug.

One adult female *B. malayi* was added to an individual well of a 24 well cell culture plate (Corning Costar #3524) in 1 mL of medium comprised of 83% RPMI-1640, 10% heat inactivated Fetal Bovine Serum (Sigma–Aldrich #F4135), 5% Penicillin/Streptomycin (Sigma–Aldrich #P4333), and 2% Gentamicin Sulfate Salt (Sigma–Aldrich #G12).

One mL of each final working solution was added to a pair of wells in order to further achieve a final DMSO concentration of 1% and final anthelmintic concentrations of 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.781, 0.391, 0.195, and 0.097 μM. Negative control wells contained 1% DMSO. The prepared plate was then incubated at 37 °C and 5% CO_2 for 48 h. Worminator readings were taken prior to addition of the drug, immediately following addition of the drug, and at 1, 12, 18, 24, 36, and 48 h post addition of the drug.

2.6. Dirofilaria immitis assays

Stock solutions of ivermectin, doramectin, and moxidectin were prepared in DMSO and PG and diluted in RPMI with 1% P/S to create working solutions of 0.002, 0.02, 0.2, 2, 4, 8, 12, 16, 20, 40, 100 μM and controls of 2% (v/v) DMSO and PG. *D. immitis* mf were

isolated and purified from freshly collected canine blood. Microfilariae were added to wells along with drug yielding final drug concentrations of 0.001, 0.01, 0.1, 1, 2, 4, 6, 8, 10, 20, and 50 μM with 1% (v/v) DMSO and PG controls. Plates were then incubated at 37 °C with 5% CO_2 for 24 h. Worminator readings were taken once 24 h after addition of the drug.

2.7. Cooperia spp. L3 assays

Initial stock solutions of 10 mM ivermectin and 10 mM moxidectin were prepared using 100% DMSO as a solvent. Initial stock solutions were then diluted in 100% DMSO to yield 1.0, 0.50, 0.25, 0.125, 0.0625, 0.0313, 0.0156, and 0.0078 mM working stock solutions for each drug. These stock solutions were each diluted 50 fold in deionized water yielding working concentrations of 20, 10, 5, 2.5, 1.25, 0.625, 0.313, and 0.156 μM.

Approximately 50 L3 were added to each well of a 96-well, flat bottom, non-treated, black with clear bottom, non-sterile plate (Corning Costar #3631) in 150 μL of deionized water. 150 μL of each drug solution was then added to respective wells to arrive at a final DMSO concentration of1% and final anthelmintic concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, and 0.78 μM. Negative control wells containing 1% DMSO in deionized water were also formulated. Assay plates were placed in an incubator at 25 °C for 48 h and Worminator readings were taken immediately prior to adding drug, immediately after adding drug, and at 1, 12, 18, 24, 36, and 48 h post addition of drugs.

2.8. Data and analysis

The Worminator output is written to two comma separated value (CSV) files; one contains the average of the motion detected in the individual well for the analyzed period for each well or plate tested, and a second file contains the underlying raw values used in determining the aforementioned averages. The average motility scores for the three technical replicates of each drug concentration were used in the analysis. The average motility for the control wells was calculated by averaging the average motility results of the three negative control wells on each plate. The results for the drug containing wells were analyzed in terms of percent inhibition of motility at each concentration as compared to the control wells, with a higher percentage motility unit inhibition interpreted as a higher level of drug activity (effectiveness).

% Motility inhibition

$$= \left(\frac{(\text{Average control motility units} - \text{Average treated motility units})}{\text{Average control motility units}} \right) \times 100$$

These values were calculated across drug concentrations and time periods using Microsoft® Excel® (Microsoft, 2013).

Dose response analysis was performed with GraphPad Prism version 6.00 using a variable slope nonlinear regression model (GraphPad Software, La Jolla, California, USA, http://www.graphpad.com). Drug concentrations were log_{10} transformed prior to analysis. The "log (inhibitor) vs response (four parameters) logistic equation" output provided IC_{50} values, as well as dose response curves for each drug and time point tested.

3. Results

3.1. Worminator

Worm assay version 1.01 was modified to support motility analysis of microscopic parasites, as well as support for current production computer and video equipment (see Section 2.1). The

resulting WormAssay version 1.4 was used in the Worminator system for the data presented in this paper (Fig. 1). Version 1.4 and the listed equipment are capable of processing 5–12 video frames per second, which provides more than adequate sensitivity for accurate motility analysis of adult, larval, and mf stages of nematode parasites.

Several types of microscopes were tested for use in the Worminator system, but we found that an inverted microscope (Olympus IX51) provided the best depth of field and eliminated the meniscus-induced optical distortion encountered with compound or dissection microscopes. An additional benefit of the inverted microscope is the X–Y positioning stage, which facilitates easy and smooth movement from plate well to plate well. Tissue culture plates having optically clear and flat bottoms must be used. Additionally, nematodes tend to sink to the bottom of the well, thereby placing more of them in the same plane of focus near the bottom of the well. Objective lenses were selected such that a full well field of view was delivered to the video camera (2× objective for 96 well plates and 4× objective for 384 well plates). Video camera zoom was then adjusted such that the well occupied ~90% of the camera's LCD view screen (Fig. 2). WormAssay shows in real time the recorded mean movement unit (mmu) and standard deviation for the well currently being scanned and analyzed. Numerous experiments were carried out to determine an optimal scanning time period that would provide an accurate motility measurement, while minimizing excessive scanning time. Readings were determined to have stabilized when standard deviation of mmu decreased to less than 2 mmu. In most cases, this occurred after 15–20 s of scanning, depending on the species and size plate being used. Based on these observations and the inclusion of a "safety margin", a standard scan time of 30 seconds was established for all assays.

Assays performed on macroscopic parasites (e.g. B. malayi adults) can be performed in 6, 12, 24, or 48 well plates (supported by WormAssay's automatic plate detection feature), depending on the physical size of the parasite species. Clear, flat bottom, non-treated, polystyrene plates provided very good results. Assays on B. malayi adults (50 mm × 150 μm) worked best in 24 well plates (Corning, Costar #3738).

Assays performed on microscopic parasites require 96 or 384 well plates, depending on the size/life stage of the parasite. The physical size of B. malayi and Cooperia spp. L3 permitted Worminator assays to be performed using 96 well plates. However, 96 well plates were unsuccessful with B. malayi and D. immitis mf due to this life stage being very small in size; the higher magnification
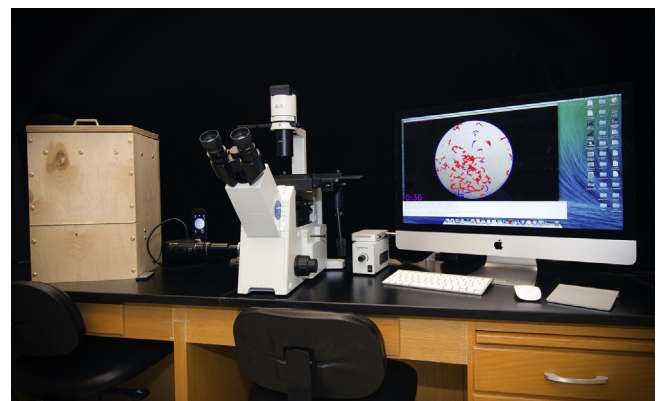
Fig. 1. Worminator system (left to right) Dark field illuminator/plate holder, inverted microscope with camera attached, microscope light controller with BlackMagic "Intensity Extreme" video converter positioned on top, and 27" iMac with WormAssay user interface window.
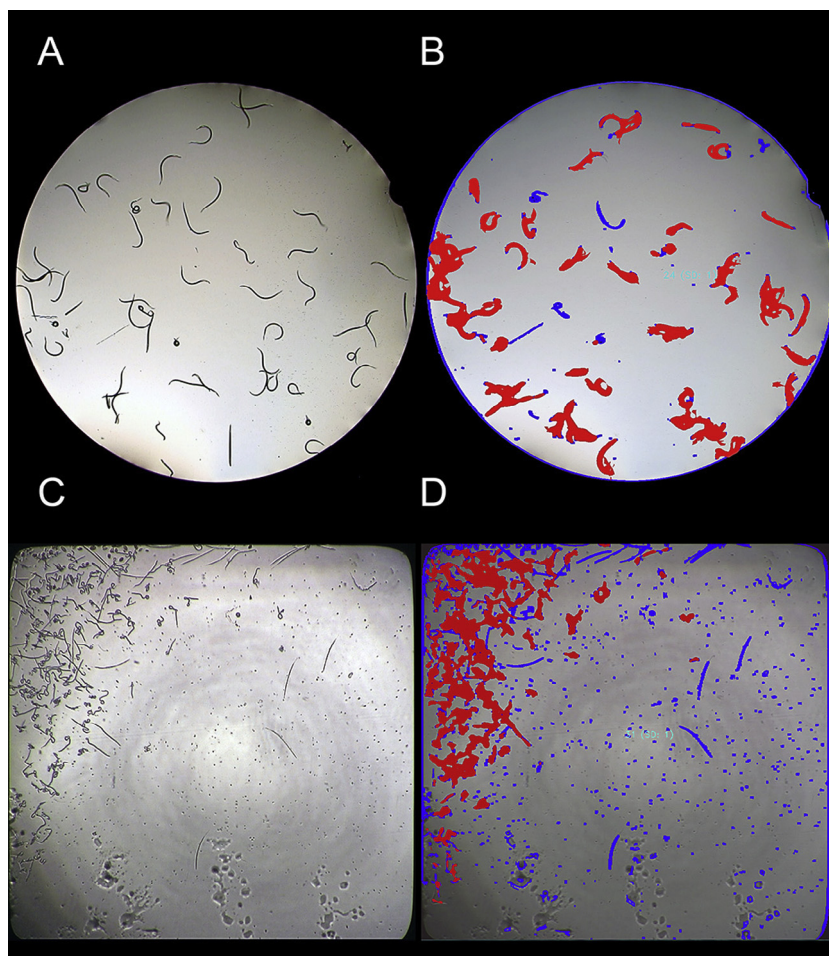
Fig. 2. Worminator system video input for 96 well and 384 well plates. (A) is the raw video input to WormAssay of *Brugia malayi* L3 in a 96 well plate. (B) is the real time display from WormAssay of *Brugia malayi* L3 in 2A (red shading indicates movement, blue shading indicates lack of movement). (C) is the raw video input for *Brugia malayi* microfilariae in a 384 well plate. (D) is the real time display from WormAssay. (Image obtained from 27″ iMac display screen capture using Apple OS X 10 native screen capture function. Image collage created in Adobe® Photoshop® CS6, version 13.0.1 × 64 (Adobe Systems, Inc. San Jose, CA). Post processing limited to Photoshop® auto white balance correction).

prevented the entire well from being visualized. This issue was overcome by using 384 well plates, which corralled the mf in an area that permitted adequate magnification as well as a full well view to be analyzed by WormAssay. Additionally, black with optically clear bottom 96 well and 384 well plates eliminated errant readings resulting from stray reflections and/or shadows.

3.2. Brugia malayi adult assay

B malayi female adult worms were assayed using the Worminator system and the macrocyclic lactone anthelmintics ivermectin and moxidectin. Dose responses were calculated using both parasite mean motility units (mmu) and percent change (%Δ) in parasite motility relative to negative controls vs log drug concentration (Table 1, Fig. 3). Hyper-motility, where the motility of the treated parasites increased relative to the controls was evident at the lower ivermectin concentrations. This produced a negative inhibition, which is seen in the % inhibition vs log concentration of ivermectin dose response curve (Fig. 3).

3.3. Brugia malayi microfilariae assay

B. malayi mf were assayed with the Worminator system using two different classes of anthelmintics: macrocyclic lactones and nicotinic agonists. The nicotinic agonist drugs utilized were

levamisole, pyrantel, morantel, oxantel, and tribendimidine. Results from the 24 h Worminator readings are summarized in Table 1 and Fig. 4, with the exception of levamisole. We found that the paralytic effect of levamisole was very short-lived (<60 min); this prevented the gathering of consistent well-to-well dose response data.

Three macrocyclic lactone anthelmintics were tested against *B. malayi* mf; ivermectin, moxidectin, and doramectin. We found that moxidectin dissolved using PG was more effective at inhibiting motility than moxidectin dissolved DMSO. Data are presented in Table 1 and Fig. 5.

3.4. Cooperia spp. L3 assays

Cooperia spp. L3 were tested using the macrocyclic lactone anthelmintics ivermectin and moxidectin. Dose responses were calculated using both parasite mean motility units (mmu) and percent change (%Δ) in parasite motility relative to negative controls vs log drug concentration (Fig. 6). Dose response curves and IC$_{50}$ data are presented in Fig. 6 and Table 1.

3.5. Dirofilaria immitis microfilariae assays

D immitis mf were assayed using the macrocyclic lactone drugs ivermectin, moxidectin, and doramectin. Results from ivermectin

Table 1
Dose response analysis of Worminator output for all parasite species and drug combinations tested. A *Variable slope nonlinear regression model analysis* using GraphPad Prism 6 was performed using mean motility units (mmu) vs log concentration (column heading IC_{50} mmu) and per cent change mmu vs control mmu vs log concentration (column heading IC_{50} %Δ).

Parasite species	Stage	Drug	Time (h)	IC_{50} mmu (μM)	SEM	R^2	IC_{50} %Δ (μM)	SEM	R^2
B. malayi	Adult	IVM	24/48	2.22/1.37	1.11/1.12	0.92/0.89	2.24/1.28	1.21/1.10	0.79/0.93
B. malayi	Adult	MOX	24/48	2.85/1.26	NA/1.23	0.75/0.79	3.01/1.19	NA /1.20	0.61/0.83
B. malayi	mf	OXA	24	47.63	1.09	0.92	50.49	1.15	0.82
B. malayi	mf	MOR	24	30.85	1.09	0.96	42.38	1.15	0.92
B. malayi	mf	PYR	24	40.50	1.11	0.89	41.44	1.05	0.97
B. malayi	mf	TBDN	24	48.76	1.18	0.70	46.13	1.05	0.87
B. malayi	mf	IVM	24	6.136	1.11	0.92	5.884	1.16	0.89
B. malayi	mf	MOX-DMSO	24	5.759	NA	0.1	1.453	2.11	0.19
B. malayi	mf	MOX-PG	24	*12.02*	9.74	0.67	*11.75*	3.61	0.65
B. malayi	mf	DOR	24	8.095	5.15	0.92	7.162	1.91	0.96
D. immitis	mf	IVM	24	43.03	NA	0.27	28.2	2.94	0.59
D. immitis	mf	MOX	24	9.288	2.64	0.16	9.008	1.79	0.26
D. immitis	mf	DOR	24	2.778	1.62	0.81	2.765	1.52	0.82
Cooperia spp.	L3	IVM	48	1.69	1.29	0.81	1.75	1.31	0.82
Cooperia spp.	L3	MOX	48	1.52	1.34	0.68	1.45	1.34	0.66

Key to abbreviations: Standard error of mean (SEM), microfilariae (mf), ivermectin (IVM), moxidectin (MOX), oxantel pamoate (OXA), doramectin (DOR), tribendimidine (TBDN), pyrantel pamoate (PYR), morantel tartrate (MOR), propylene glycol (PG), Not applicable (NA) e.g. model did not converge.
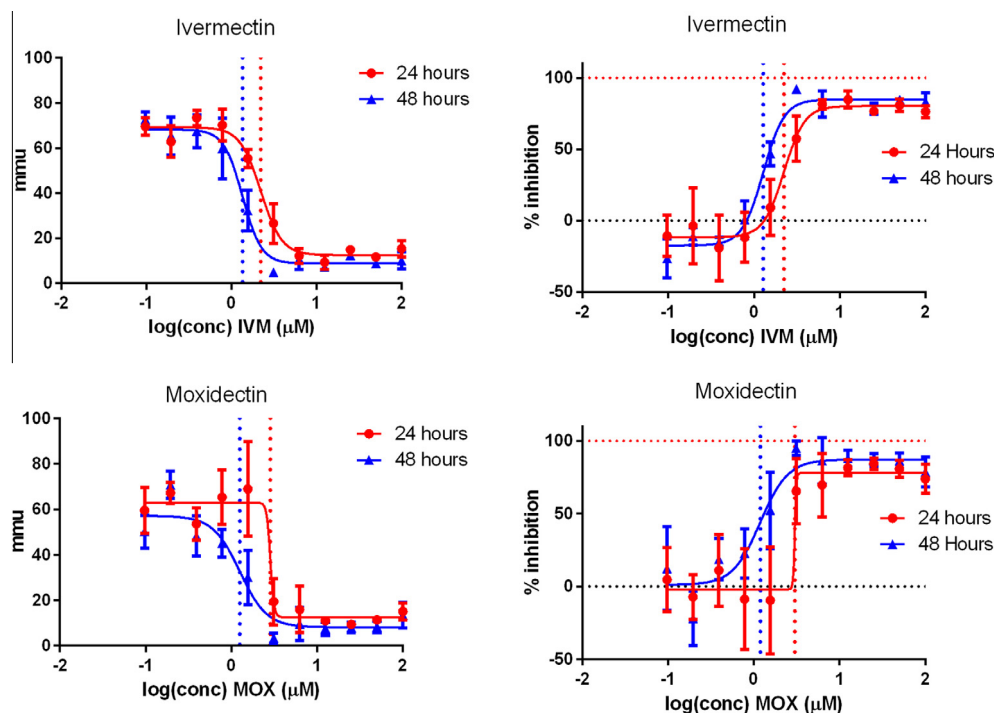
Fig. 3. *Brugia malayi* adult female worm mean motility unit (mmu) and dose response curves for ivermectin and moxidectin. Curves were generated applying the variable slope nonlinear regression model analysis contained in GraphPad Prism 6. Broken vertical lines indicate IC_{50} values.

and moxidectin with *D. immitis* mf were quite different from those obtained using *B. malayi* mf, and we were unable to generate good fits for the dose–response data generated using these drugs (Fig. 7). In contrast, we were able to obtain reliable fits to the data using doramectin; the R^2 values for these two curves for doramectin were 0.818 and 0.8112, whereas the R^2 values for ivermectin and moxidectin ranged from 0.16 to 0.59.

4. Discussion

The "Worminator" system is a novel application of real time, computer analyzed, HD video that permits the detection, measurement and characterization of anthelmintic effects on motility of microscopic life stages of parasitic nematodes. This system uses

open source software and reasonably priced off the shelf computer and video imaging equipment, making it practical for almost any laboratory. The Worminator addresses the important need for practical, cost-efficient, and robust *in vitro* assays to screen compounds for anthelmintic activity, as well as for characterization of anthelmintic sensitivity in nematode populations. (Boatin et al., 2012; Vercruysse et al., 2012).

To create the Worminator System we adapted and integrated current technology to function in concert with the WormAssay computer application. Our goal was for a simple and relatively inexpensive general purpose assay that is accurate and repeatable while also able to support analysis of most species of parasitic nematodes in their various life stages. The ability of the Worminator system to assess in vitro anthelmintic sensitivity in a variety of parasite species and life stages ranging from mf (∼200 um) to L3
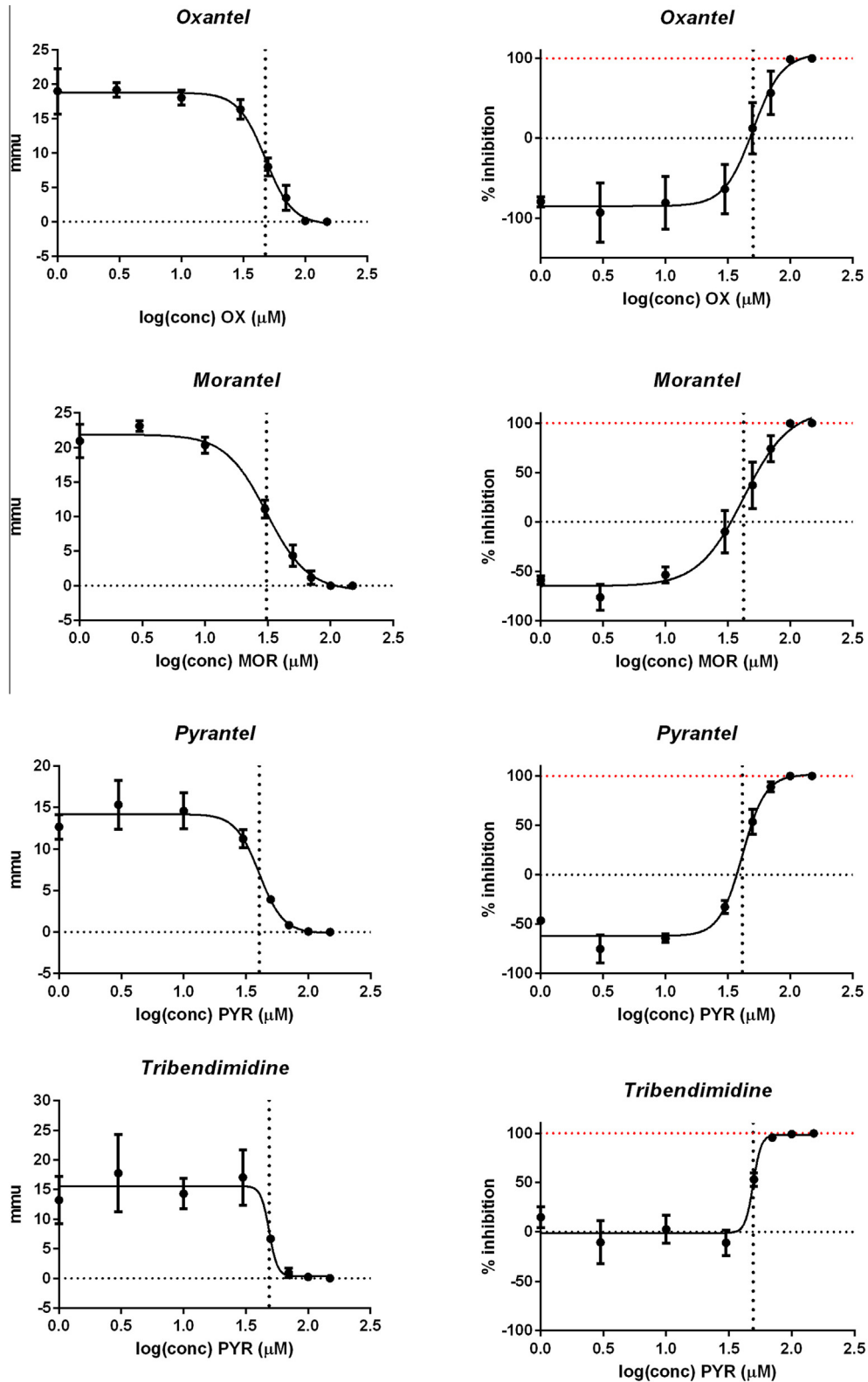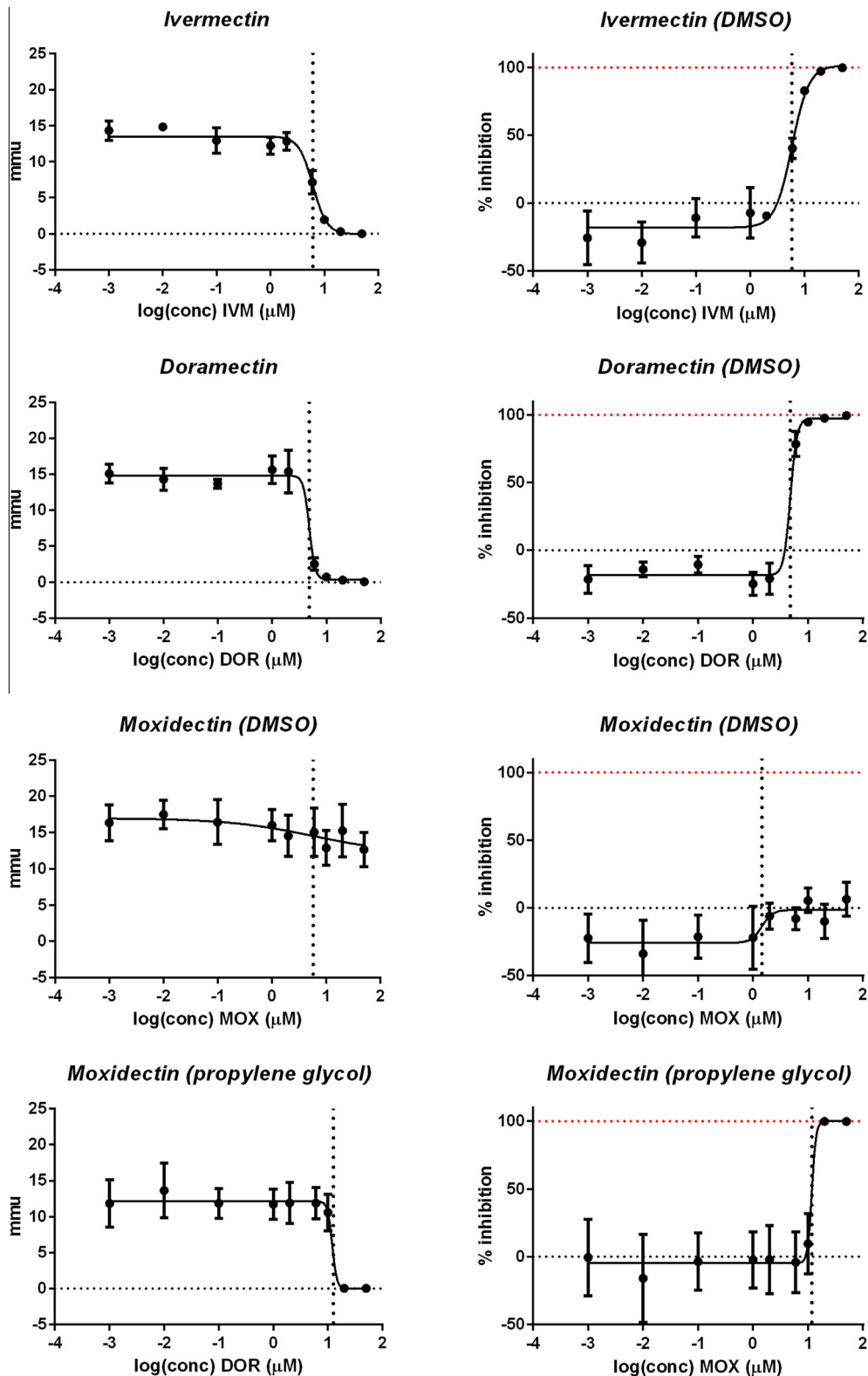
Fig. 4. *Brugia malayi* microfilaria mean motility unit (mmu) and dose response curves for nicotinic class anthelmintics. Curves were generated applying a variable slope nonlinear regression model analysis contained in GraphPad Prism 6. Broken vertical lines indicate IC_{50} values.

(~800 um) all the way to adult (~50 mm) is demonstrated in the data presented here. Additionally, the real-time quantitative data capture of the Worminator system enables new observations that would be extremely difficult to make with other available *in vitro* assays. One such observation is that all species and stages of

parasites tested with macrocyclic lactone anthelmintics demonstrated an increase in motility at low concentrations as compared to the no-drug control. Though our laboratory has a great deal of experience using the larval migration inhibition assay with macrocyclic lactone drugs in multiple parasite species (Evans et al.,
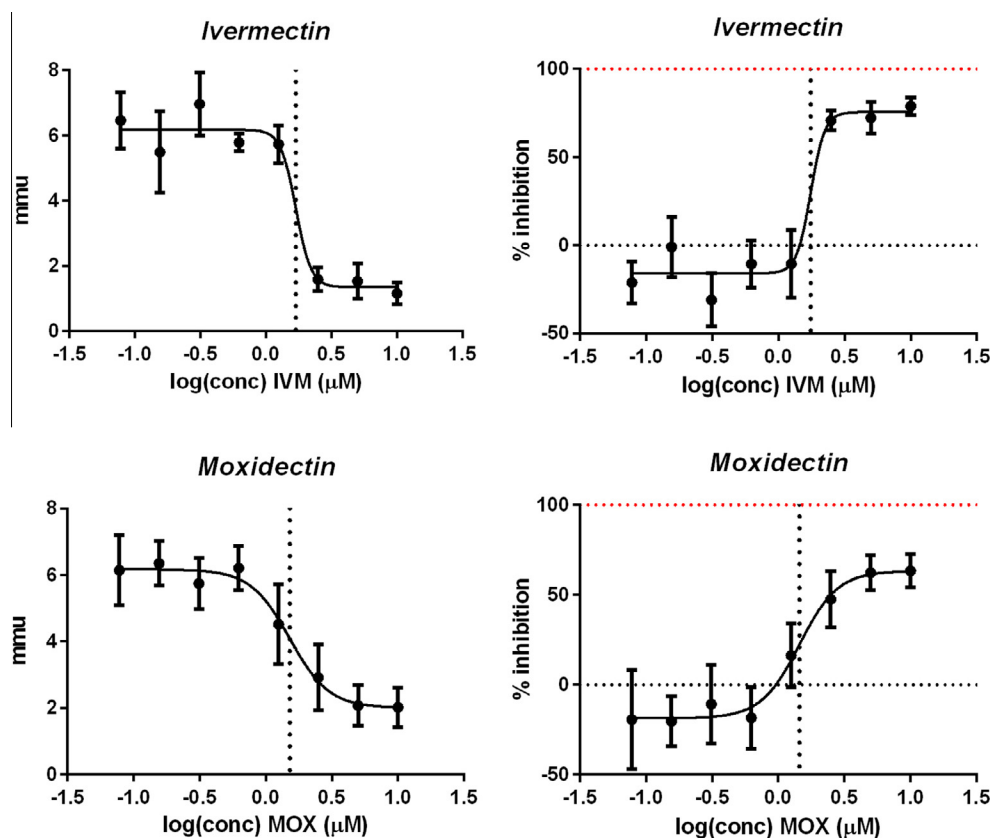
Fig. 5. *Brugia malayi* microfilaria mean motility unit (mmu) and dose response curves for macrocyclic lactone class anthelmintics. Curves were generated applying a Variable slope nonlinear regression model analysis contained in GraphPad Prism 6. Note differences in the moxidectin dissolved using DMSO as compared to moxidectin dissolved in PG. Broken vertical lines indicate IC_{50} values.

2013), this observation was never made previously. Examples of this "hyper-motility" can be seen in the dose response curves contained in Figs. 4–6.

The Worminator System provided informative dose response data with both *B. malayi* mf and adults. The greatest sensitivity

for *B. malayi* adults when tested against ivermectin and moxidectin was seen at the 48 h time point (Fig. 3). The IC_{50} results obtained are similar to those reported by Marcellino et al. (2012) for *B. malayi* adults with ivermectin. In contrast to the adult *B. malayi* worms, the mf stage demonstrated a different dose–response
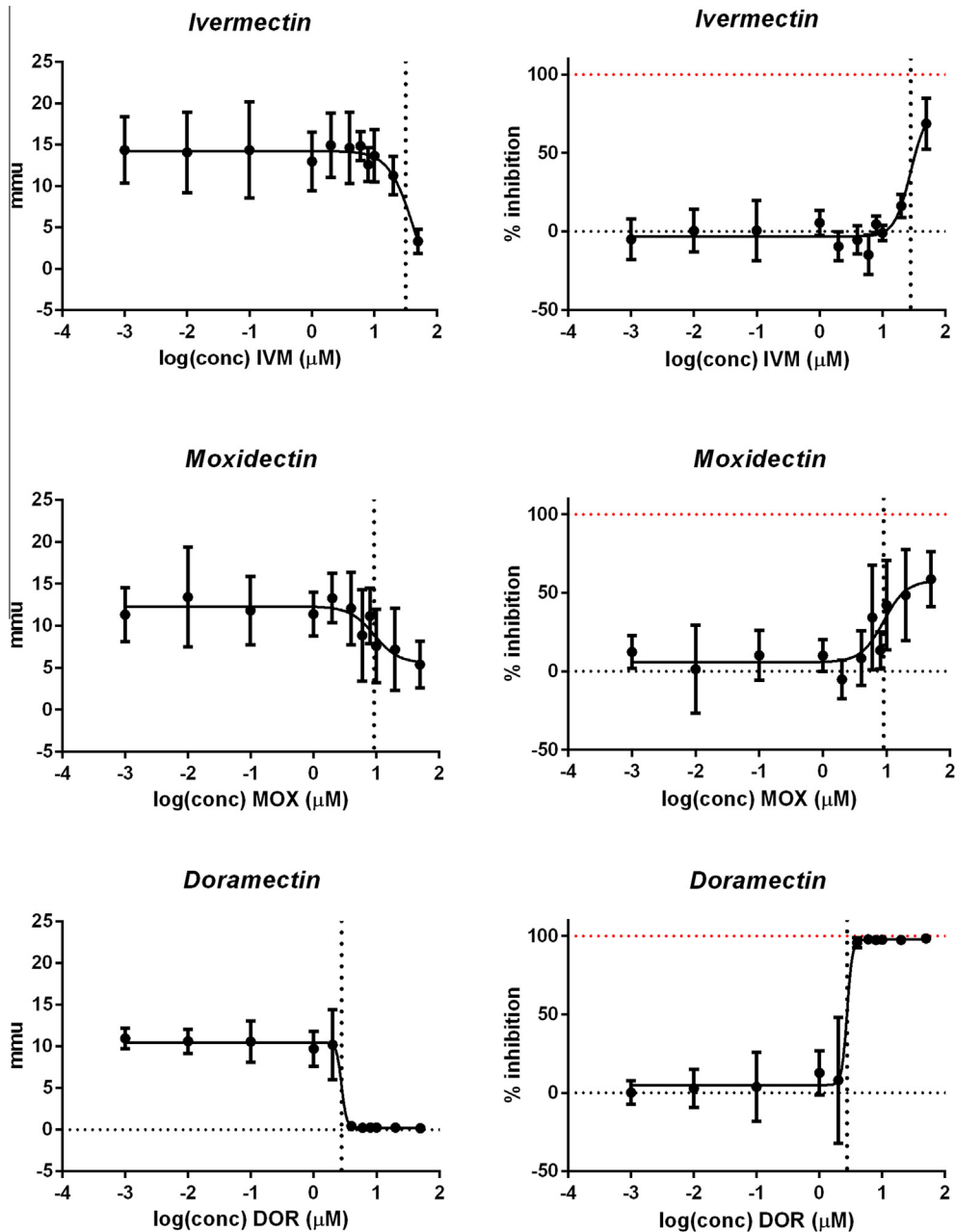
Fig. 6. *Cooperia* spp. L3 larvae mean motility unit (mmu) and dose response curves for ivermectin and moxidectin. Curves were generated applying the variable slope nonlinear regression model analysis contained in GraphPad Prism 6. Broken vertical lines indicate IC_{50} values.

phenotype. *B. malayi* mf when tested against a panel of macrocyclic lactone drugs demonstrated a threshold effect where there was little decrease in motility over a wide range of concentrations, and then a rapid decrease in motility over a narrow range of relatively high concentrations (Fig. 5). A similar *in vitro* dose–response phenotype was also observed in a previous study, which used visual scoring of *B. malayi* mf exposed to ivermectin and moxidectin (Tompkins et al., 2010). The nature of this dose response phenotype yielded a high level of variability between assays, resulting in high S.E.M. values for the IC_{50}. We also noted a very poor dose response of *B. malayi* mf to moxidectin when dissolved in DMSO; however, when the moxidectin was dissolved in PG, the response was similar to the other macrocyclic lactone drugs tested (Fig. 5). We speculate that this is attributable to the poor aqueous solubility of moxidectin, and that the PG increased the solubility of moxidectin. Future experiments will investigate further the differences in dose–response for macrocyclic lactone drugs dissolved in DMSO vs PG using a variety of different parasite species and stages.

In contrast to the macrocyclic lactone drugs, dose–response data for *B. malayi* mf with nicotinic agonists were very consistent across all four drugs tested (OXA, PYR, MOR, and TBDN), as indicated by similar IC_{50} values, small IC_{50} S.E.M. values and high R^2 values for the nonlinear curve fit. To our knowledge there are no similar published data to compare these results to, however, these data are consistent with electrophysiological responses of muscle from *B. malayi* adults, which shows that paralysis is the primary mode of action of nicotinic agonist anthelmintics (Robertson et al., 2013). It is also widely accepted that a primary mechanism of action for the macrocyclic lactone anthelmintics is paralysis of somatic musculature, due to irreversible binding at glutamate gated chloride channels that produces a long-lasting hyperpolarization of the neuron or muscle cell, therefore blocking further

function (Wolstenholme and Rogers, 2005; Kotze et al., 2012). Consistent with this belief, in vitro measurements of muscle contraction and motility-based assays such as the LMIA can be used to detect resistance to the macrocyclic lactones in strongylid species (Demeler et al., 2010a,b, 2014). However, when results of the *B. malayi* mf nicotinic agonist assays are compared with results of the macrocyclic lactone assays, a distinct contrast is apparent. The IC_{50} S.E.M values for the macrocyclic lactones are much greater, and the slopes of the dose–response curves for the macrocyclic lactone drugs are much steeper. Additionally, the macrocyclic lactone concentrations required to induce paralysis are thousands of times higher than tissue levels achieved following a therapeutic dose (Evans et al., 2013). Taken together, these data support suggestions that inhibition of motility may not be a primary mechanism of action for the macrocyclic lactone drugs in filarioid nematodes (Geary et al., 1993; Moreno et al., 2010; Vatta et al., in press), and thus would not be a good phenotypic measure for diagnosing resistance to these drugs.

The dose–response data obtained for *D. immitis* mf using the Worminator system were the least intuitive of any of the parasite species/life stage tested. Only doramectin produced a sigmoidal dose response, though the slope was very steep. With ivermectin and moxidectin there was only a minimal decline in motility over a wide range of concentrations, with only a modest decline even at the highest concentrations tested. With both of these drugs, inhibition of motility never exceeded 75%, even at maximal achievable in vitro concentrations (using DMSO as a solvent) (Fig. 7). Given this dose–response phenotype to ivermectin and moxidectin in a drug-susceptible isolate (Missouri 2005), it seems highly unlikely that motility of mf could ever be a useful *in vitro* assay for detecting resistance to macrocyclic lactone drugs in *D. immitis*, when using these drugs. However, using other macrocyclic lactone drugs, such

Fig. 7. *Dirofilaria immitis* microfilaria mean motility unit (mmu) and dose response curves for macrocyclic lactone class anthelmintics. Curves were generated applying a Variable slope nonlinear regression model analysis contained in GraphPad Prism 6. Broken vertical lines indicate IC$_{50}$ values.

as doramectin, further assay optimization might overcome this obstacle.

We have presented data demonstrating the ability of the Worminator System to quantify parasite motility for three different nematode parasites from two different phylogenetic clades, using three different life stages and seven different anthelmintics. These data demonstrate the utility of the Worminator System as a practical, efficient and robust tool for measuring the in vitro sensitivity of various nematode parasite species and stages to anthelmintics. Though only drug-susceptible parasite isolates were used in these initial studies, future work will compare these with drug-resistant isolates, with the goal of optimizing diagnostic assays for detecting drug resistance in nematode populations. In conclusion, the Worminator system provides a computer based quantitative platform that is simple and efficient to use, provides improved sensitivity and objectivity over previously used motility assays, is agnostic to parasite life stage, and can be setup in any

laboratory at a reasonable cost. Given these advantages compared to other available *in vitro* diagnostic systems for measuring susceptibility of nematodes to anthelmintic compounds, the Worminator should permit the development of multiple species- and drug-specific assays that will advance discovery of new anthelmintic compounds, as well as improve the monitoring of nematode populations for changes in their sensitivity to existing anthelmintic compounds.

Funding

Conflict of interest

The authors declared that there is no conflict of interest.

Acknowledgements

References

Almeida, F.A., Garcia, K.C.O.D., Torgerson, P.R., Amarante, A.F.T., 2010. Multiple resistance to anthelmintics by Haemonchus contortus and Trichostrongylus colubriformis in sheep in Brazil. Parasitol. Int. 59, 622–625.

Bennett, J., Pax, R., 1986. Micromotility meter: an instrument designed to evaluate the action of drugs on motility of larval and adult nematodes. Parasitol. Today 93, 341–346.

Bennett, J.L., Pax, R.A., 1987. Micromotility meter: instrumentation to analyse helminth motility. Parasitol. Today 3, 159–160.

Bennett, J.L., Haslett, R.A., Pax, M., 1986. micromotility meter, in: Patent, U.S. (Ed.). Research Corporation, New York, United States, p. 8.

Boatin, B.A., Basáñez, M.-G., Prichard, R.K., Awadzi, K., Barakat, R.M., García, H.H., Gazzinelli, A., Grant, W.N., McCarthy, J.S., N'Goran, E.K., Osei-Atweneboana, M.Y., Sripa, B., Guo-Jing, Y., Lustigman, S., 2012. A research agenda for helminth diseases of humans: towards control and elimination. PLoS Negl. Trop. Dis. 6, 1–10.

Bourguinat, C., Keller, K., Bhan, A., Peregrine, A., Geary, T., Prichard, R., 2011. Macrocyclic lactone resistance in Dirofilaria immitis. Vet. Parasitol. 181, 388–392.

Canever, R.J., Braga, P.R.C., Boeckh, A., Grycajuck, M., Bier, D., Molento, M.B., 2013. Lack of Cyathostomin sp. reduction after anthelmintic treatment in horses in Brazil. Vet. Parasitol. 194, 35–39.

Churcher, T.S., Pion, S.D.S., Osei-Atweneboana, M.Y., Prichard, R.K., Awadzi, K., Boussinesq, M., Collins, R.C., Whitworth, J.A., Basanaz, M.G., 2009. Identifying sub-optimal responses to ivermectin in the treatment of River Blindness. Proc. Natl. Acad. Sci. U.S.A. 106, 16716–16721.

Churcher, T.S., Kaplan, R.M., Ardelli, B.F., Schwenkenbecher, J.M., Basáñez, M.G., Lammie, P.L., 2010. Mass treatment of parasitic disease: implications for the development and spread of anthelmintic resistance. In: Webber, J.T. (Ed.), Antimicrobial Resistance – Beyond the Breakpoint. Issues in Infectious Diseases, Karger, Basel, pp. 120–137.

Coles, G.C., Folz, S.D., Tritschler II, J.P., 1989. Motility response of levamisole benzimidazole-resistant Haemonchus contortus larvae. Vet. Parasitol. 31, 253–257.

Coles, G.C., Jackson, F., Pomroy, W.E., Prichard, R.K., Samson-Himmelstjerna, G.V., Silvestre, A., Taylor, M.A., Vercruysse, J., 2006. The detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 136, 167–185.

Demeler, J., Küttler, U., El-Abdellati, A., Stafford, K., Rydzik, A., Varady, M., Kenyon, F., Coles, G., Höglund, J., Jackson, F., Vercruysse, J., von Samson-Himmelstjerna, G., 2010a. Standardization of the larval migration inhibition test for the detection of resistance to ivermectin in gastro intestinal nematodes of ruminants. Vet. Parasitol. 174, 58–64.

Demeler, J., Küttler, U., von Samson-Himmelstjerna, G., 2010b. Adaptation and evaluation of three different in vitro tests for the detection of resistance to anthelmintics in gastro intestinal nematodes of cattle. Vet. Parasitol. 170, 61–70.

Demeler, J., Samson-Himmelstjerna, G.V., Sangster, N.C., 2014. Measuring the effect of avermectins and milbemycins on somatic muscle contraction of adult Haemonchus contortus and on motility of Ostertagia circumcincta in vitro. Parasitology 141, 948–956.

Dinaburg, A.G., 1942. The efficiency of the Baermann apparatus in the recovery of larvae of haemonchus contortus. J. Parasitol. 28, 433–440.

Evans, C.C., Moorhead, A.R., Storey, B.E., Wolstenholme, A.J., Kaplan, R.M., 2013. Development of an in vitro bioassay for measuring susceptibility to macrocyclic lactone anthelmintics in Dirofilaria immitis. Int. J. Parasitol.: Drugs Drug Resist. 3, 102–108.

Fleming, S.A., Craig, T., Kaplan, R.M., Miller, J.E., Navarre, C., Rings, M., 2006. Anthelmintic resistance of gastrointestinal parasites in small ruminants. J. Vet. Intern. Med. 20, 435–444.

Folz, S.D., Pax, R.A., Thomas, E.M., Bennett, J.L., Lee, B.L., Conder, G.A., 1987. Detecting in vitro anthelmintic effects with a micromotility meter. Vet. Parasitol. 24, 241–250.

Geary, T.G., Sims, S.M., Thomas, E.M., Vanover, L., Davis, J.P., Winterrowd, C.A., Klein, R.D., Ho, N.F.H., Thompson, D.P., 1993. Haemonchus contortus: ivermectin-induced paralysis of the pharynx. Exp. Parasitol. 77, 88–96.

Geary, T.G., Bourguinat, C., Prichard, R.K., 2011. Evidence for macrocyclic lactone anthelmintic resistance in Dirofilaria immitis. Top. Companion Anim. Med. 26, 186–192.

Gill, J.H., Redwin, J.M., Van Wyk, J.A., Lacey, E., 1991. Detection of resistance to ivermectin in haemonchus contortus. Int. J. Parasitol. 21, 771–776.

Howell, S.B., Burke, J.M., Miller, J.E., Terrill, T.H., Valencia, E., Williams, M.J., Williamson, L.H., Zajac, A.M., Kaplan, R.M., 2008. Prevalence of anthelmintic resistance on sheep and goat farms in the southeastern United States. J. Am. Vet. Med. Assoc. 233, 1913–1919.

Kaplan, R.M., Vidyashankar, A.N., 2012. An inconvenient truth: global worming and anthelmintic resistance. Vet. Parasitol. 186, 70–78.

Keenan, J.D., Hotez, P.J., Amza, A., Stoller, N.E., Gaynor, B.D., Porco, T.C., Lietman, T.M., 2013. Elimination and eradication of neglected tropical diseases with mass drug administrations: a survey of experts. PLoS Negl. Trop. Dis. 7, 1–8.

Kotze, A.C., Clifford, S., O'Grady, J., Behnke, J.M., McCarthy, J.S., 2004. An in vitro larval motility assay to determine anthelmintic sensitivity for human hookworm and Strongyloides species. Am. J. Trop. Med. Hyg. 71, 608–616.

Kotze, A.C., Coleman, G.T., Mai, A., McCarthy, J.S., 2005. Field evaluation of anthelmintic drug sensitivity using in vitro egg hatch and larval motility assays with Necator americanus recovered from human clinical isolates. Int. J. Parasitol. 35, 445–453.

Kotze, A.C., LeJambre, L.F., O'Grady, J., 2006. A modified larval migration assay for detection of resistance to macrocyclic lactones in Haemonchus contortus, and drug screening with Trichostrongylidae parasites. Vet. Parasitol. 137, 294–305.

Kotze, A.C., Hines, B.M., Ruffell, A.P., 2012. A reappraisal of the relative sensitivity of nematode pharyngeal and somatic musculature to macrocyclic lactone drugs. Int. J. Parasitol.: Drugs Drug Resist. 2, 29–35.

Krajacic, P., Shen, X.N., Purohit, P.K., Arratia, P., Lamitina, T., 2012. Biomechanical profiling of Caenorhabditis elegans motility. Genetics 191, 1015–1021.

LeJambre, L., 1976. Egg hatch as an in vitro assay of thiabendazole resistance in nematodes. Vet. Parasitol. 2, 385–391.

Marcellino, C., Gut, J., Lim, K.C., Singh, R., McKerrow, J., Sakanari, J., 2012. WormAssay: a novel computer application for whole-plate motion-based screening of macroscopic parasites. PLoS Negl. Trop. Dis. 6, 1–8.

Martin, P.J., Le Jambre, L.F., 1979. Larval paralysis as an in vitro assay of levamisole and morantel tartrate resistance in Ostertagia. Vet. Sci. Commun. 3, 159–164.

Matthews, J.B., McArthur, C., Robinson, A., Jackson, F., 2012. The in vitro diagnosis of anthelmintic resistance in cyathostomins. Vet. Parasitol. 185, 25–31.

Microsoft, 2013. Microsoft Excel, 2013 ed. Microsoft, Redmond, Washington.

Ministry of Agriculture, F., and Food, 1977. Manual of Veterinary Parasitological Laboratory Techniques, London.

Moreno, Y., Nabhan, J.F., Solomon, J., Mackenzie, C.D., Geary, T.G., 2010. Ivermectin disrupts the function of the excretory-secretory apparatus in microfilariae of Brugia malayi. Proc. Natl. Acad. Sci. 107, 20120–20125.

Osei-Atweneboana, M.Y., Eng, J.L., Boakye, D.A., Gyapong, J.O., Prichard, R.K., 2007. Phenotypic evidence of emerging ivermectin resistance in some population of Onchocerca volvulus, the causative agent of onchocercasis. Lancet 369, 2021–2029.

Prichard, R.K., 2005. Is anthelmintic resistance a concern for heartworm control?: what can we learn from the human filariasis control programs? Vet. Parasitol. 133, 243–253.

Robertson, A.P., Buxton, S.K., Martin, R.J., 2013. Whole-cell patch-clamp recording of nicotinic acetylcholine receptors in adult Brugia malayi muscle. Parasitol. Int. 62, 616–618.

Sczesny-Moraes, E.A., Bianchin, I., da Silva, K.F., Catto, J.B., Honer, M.R., Paiva, F., 2010. Anthelmintic resistance of gastrointestinal nematodes in sheep, Mato Grosso do Sul, Brazil. Pesquisa Vet Brasil 30, 229–236.

Sutherland, I.A., Leathwick, D.M., 2011. Anthelmintic resistance in nematode parasites of cattle: a global issue? Trends Parasitol. 27, 176–181.

Taylor, M.A., 1990. A larval development test for the detection of anthelmintic resistance in nematodes of sheep. Res. Vet. Sci. 49, 198–202.

Tompkins, J.B., Stitt, L.E., Ardelli, B.F., 2010. Brugia malayi: in vitro effects of ivermectin and moxidectin on adults and microfilariae. Exp. Parasitol. 124, 394–402.

Várady, M., Čorba, J., Hrčková, G., 1998. The in vitro motility response to various anthelmintics of third-stage larvae of Oesophagostomum spp. from pigs. Vet. Res. Commun. 22, 299–304.

Vatta, A.F., Dzimianski, M., Storey, B.E., Camus, M.S., Moorhead, A.R., Kaplan, R.M., Wolstenholme, A.J., in press. Ivermectin-dependent attachment of neutrophils and peripheral blood mononuclear cells to Dirofilaria immitis microfilariae in vitro. Vet. Parasitol.

Vercruysse, J., Levecke, B., Prichard, R., 2012. Human soil-transmitted helminths: implications of mass drug administration. Curr. Opin. Infect. Dis. 25, 703–708.

von Samson-Himmelstjerna, G., 2012. Anthelmintic resistance in equine parasites – detection, potential clinical relevance and implications for control. Vet. Parasitol. 185, 2–8.

Wagland, B.M., Jones, W.O., Hribar, L., Bendixsen, T., Emery, D.L., 1992. A new simplified assay for larval migration inhibition. Int. J. Parasitol. 22, 1183–1185.

Wolstenholme, A.J., Rogers, A.T., 2005. Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics. Parasitology 131, S85–S95.