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ORIGINAL PAPER

A rapid colorimetric assay for the quantitation of the viability of free-living larvae of nematodes in vitro

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Abstract With increasing drug resistance in gastrointestinal parasites, identification of new anthelmintics is essential. The non-parasitic nematode *Caenorhabditis elegans* is used extensively as a model to identify drug targets and potential novel anthelmintics because it can be readily cultured in vitro. Traditionally, the assessment of worm viability has relied on labour-intensive developmental and behavioral assays. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-formazan (MTT-formazan) colorimetric assay uses metabolic activity as a marker of viability in mammalian cell culture systems and has been applied for use with filarial nematodes. In the present study, this assay has been optimized and validated to rapidly assess the viability of *C. elegans* after drug treatment. Living, but not dead, *C. elegans* take up MTT and reduce it to the blue formazan, providing visual, qualitative, and quantitative assessment of viability. MTT at a concentration of 5 mg/ml with 3 h incubation was optimal for detecting changes in viability with drug treatment. We have applied this assay to quantitate the effects of ivermectin and short-chain alcohols on the viability of *C. elegans*. This assay is also applicable to first-stage larvae of the parasitic nematode *Haemonchus contortus*. The advantage of this assay is the rapid quantitation in screening drugs to identify potential anthelmintics.

Introduction

In mammalian cell culture systems, the evaluation of cellular viability in vitro has been simplified by the use of biochemical tetrazolium-based dye reduction colorimetric assays, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The MTT-formazan assay involves the metabolic reduction of the tetrazolium dye, a pale yellow water-soluble quaternary ammonium compound, by cellular dehydrogenases of viable immobilized or suspended cells (Mosmann 1983). The production of the dark-blue water-insoluble formazan crystals by viable mammalian cells is directly proportional to the cell number over a wide range (Mosmann 1983). Formazan crystals can be either observed microscopically in the cell cytoplasm or extracted and dissolved with organic solvents such as dimethyl-sulfoxide (DMSO), allowing fast spectrophotometric quantification (Dias et al. 1999). Modifications of the MTT assay have been successfully applied not only to mammalian cells but also to other organisms including protozoans, *Tetrahymena pyriformis* (see Dias et al. 1999), and also filarial nematodes such as *Onchocerca* spp., *Brugia pahangi*, *Dipetalonema vitear*, *Acanthocheilonema viteae*, and *Litomosoides carinii* for the assessment of the viability of these organisms cultured with toxicants (Comley et al. 1989a, b).

The free-living nematode *Caenorhabditis elegans*, being a key model organism of eukaryotes, has important applications in numerous fields of study, including the understanding of mechanisms in embryonic and developmental regulation (Goldstein et al. 2006), aging and longevity (Fujii et al. 2005), and metabolic mechanisms in the development of degenerative diseases such as Parkinson's disease (Ved et al. 2005). Because the pioneering work of Simpkin and Coles (1981) demonstrating *C. elegans* was a useful model for detecting novel anthelmintics, this nematode has been valuable in basic research on anthelmintic pharmacology of human and agricultural parasites (Dengg and van Meel 2004); its rapid life cycle and easy maintenance makes this organism a very useful tool for such applications. Many

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studies using *C. elegans* require measurement of surviving and/or reproducing worms. Currently, a number of techniques are available for measuring *C. elegans* viability after treatment, such as larval development assays (LDAs, reproduction responses), or by the assessment of motility and behavior responses (Rand and Johnson 1995; Burglin et al. 1998; Anderson et al. 2004). These techniques are time consuming and often reliant on a subjective scoring system. A quantitative biochemical method for determining worm viability and reproduction would be a useful analytical tool for the rapid analysis of drug, toxicant, or other treatment causing damage to worms. This paper describes the application of an optimized MTT-formazan assay for the faster quantitative assessment of whole *C. elegans* for a number of applications, including viability after exposure to cytotoxic compounds.

Materials and methods

Nematodes and culture conditions

The 'wild-type' Bristol N2 strain of *C. elegans* (gift from Dr. Carolyn Behm, ANU Canberra, Australia) was cultured on nematode growth medium (NGM) with OP50 *Escherichia coli* under standard conditions (Brenner 1974). An ivermectin-resistant strain developed in our laboratory from the N2 strain was cultured on NGM media with 10 ng/ml ivermectin (Sigma, St. Louis, MO, USA). Synchronous worm populations were prepared from cultures, washed with M9 buffer (1 M KH_2PO_4, 40 mM Na_2HPO_4, 80 mM NaCl, 1 mM $MgSO_4$) and scraped with a glass rod to remove eggs stuck to the medium. Worms and eggs were pelleted by gentle centrifugation (1,400 g) for 3 min, bleach treated with 1 ml of fresh hypochlorite solution (1.6 M NaOH, 60% bleach) to dissolve worms and leave eggs, and agitated vigorously for 90 s. Eggs were washed with M9 and bleach treated again. The eggs were washed in M9 buffer (6), resuspended in M9 with penicillin–streptomycin (Sigma), and allowed to hatch overnight at room temperature with gentle agitation. The resulting synchronised L1-stage worms were then centrifuged and resuspended in M9 buffer.

The McMaster strain of *Haemonchus contortus* was provided by Prof. N. Sangster, Sydney University, Australia. In brief, *H. contortus* was produced in helminth-free lambs, and larval stages were produced as described by Nikolaou et al. (2002). Faecal cultures were collected, and first-stage larvae (L1) were recovered after 1 day and infective third-stage larvae (L3) after 6–7 days incubation at 27°C. Worms were centrifuged at 1,400×*g* for 3 min and resuspended in phosphate-buffered saline.

MTT assay

Synchronised L1s in suspension (500 worms in 50 µl) were added to triplicate wells of a 96-well microtitre plate. Fifty microliters of MTT (10 mg/ml; Sigma) dissolved in filter-sterilized phosphate-buffered saline was added. The plates were incubated at 20°C for 3 h, then centrifuged to pellet worms at 800 g for 10 min, and the supernatant aspirated. Formazan production was determined 1 h after the addition of 100 µl DMSO (Sigma) by reading in a Bio-Tek PowerWave HT (BioTek, Winooski, VT, USA) microplate reader at 575 nm.

Drug cytotoxicity assay

Assays were performed in replicates of four wells in a final volume of 50 µl. Synchronous L1s were added to a 96-well microtitre plate (500 worms per well), followed by the addition of serial twofold dilutions of drug. MTT assay to determine viability was performed after 48 and 72 h incubation of ivermectin for the Bristol N2 and 10 ng/ml ivermectin-selected strain. Viability to methanol (Selby Biolabs, Melbourne, Australia), ethanol (Riedel-de Haen, Seelze, Germany), propanol (Riedel-de Haen), and 1-butanol (BDH, Poole, Dorset, UK) exposure was performed for the Bristol N2 strain for 24 h incubation at 20°C. All experiments were repeated at least three times and differences determined using the student *t* test. Significance was determined at $P<0.05$.

Results

Optimization of the MTT-formazan assay

Several MTT concentrations were assessed to define the optimal MTT concentration for the detection of the formazan crystals produced. Worms were incubated in concentrations from 0.625 to 5 mg/ml for 3 h, and then developed with DMSO. L1s of *C. elegans* were shown to form formazan in 3 h in which there was a significant increase in the amount of formazan detected in worms incubated in 5 mg/ml MTT compared with 1.25 mg/ml ($P<0.05$). The MTT concentration for formazan detection was selected for worms incubated in a final concentration of 5 mg/ml MTT, which gave higher A_{575} readings. Significant color was produced from 1–5 mg/ml at low numbers (<100). However, as 5 mg/ml gave higher absorbances, this concentration was chosen for further assays.

Four MTT incubation times were tested for worms incubated in 5 mg/ml MTT for 0.5–3 h. Formazan production increased with time, with a statistically significant increase in

the signal detection between 2- and 3-h incubations (P=0.02). The effect of the length of DMSO development was also optimized using three times. The optimal signal obtained with 1-h DMSO development, with a significant increase in A_{575} between 15-min development and 1 h (P<0.05; results not shown).

These conditions were then used to determine the relationship between worm number, and the amount of formazan produced was investigated to test the linearity of formazan production. Figure 1 shows the absorbance at 575 nm, indicative of formazan production, linearly correlated to the number of worms, with a Pearson correlation coefficient of 0.9783 (P=0.0001). The linearity extends over the range of worm concentrations tested, from 0 to 1,600 worms.

Extending from the assessment in *C. elegans*, the same conditions were employed to determine the MTT uptake in *H. contortus* larvae. Figure 1 shows that the uptake of MTT by L1s was linear, consistent but significantly lower than the uptake in *C. elegans*. Furthermore *C. elegans* L3 larvae took up MTT; however, *H. contortus*-infective L3 larvae did not (results not shown).

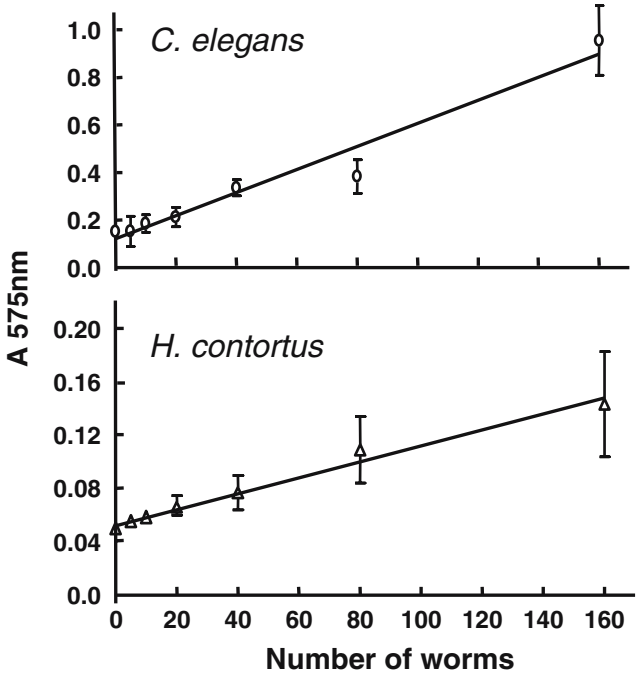

Fig. 1 Formazan production by *C. elegans* and *H. contortus*. Synchronized L1-stage *C. elegans* and L1-stage *H. contortus* were serially twofold diluted, and MTT added to a final concentration of 5 mg/ml. Plates were incubated at 20°C for 3 h and centrifuged, and the supernatant aspirated. Formazan production was determined 1 h after the addition of 200 µl DMSO. *Points* are the mean, and the *error bars* show the standard deviation of four replicates from two independent assays. The *straight line plotted* is the best-fit line calculated using all points

Application of the MTT assay to evaluate the viability of *C. elegans* after toxicant exposure

To investigate the viability of *C. elegans* cultured with ivermectin, synchronous L1s were cultured in 96-well microtitre plates as described in "Materials and methods." Ivermectin was added, and worms cultured for 48 and 72 h, the maximal time the negative control L1-larvae could survive without *E. coli*. Figure 2 shows that the ivermectin-resistant strain of *C. elegans* is more resistant to ivermectin than wild-type Bristol N2 strain at both 48- and 72-h time points with LD_{50} values of 9.9 and >50 ng/ml ivermectin for 48-h incubation and 1.6 and 32 ng/ml for 72-h incubation for the Bristol N2 strain and the 10 ng/ml ivermectin-cultured strains, respectively. These LD_{50} values are

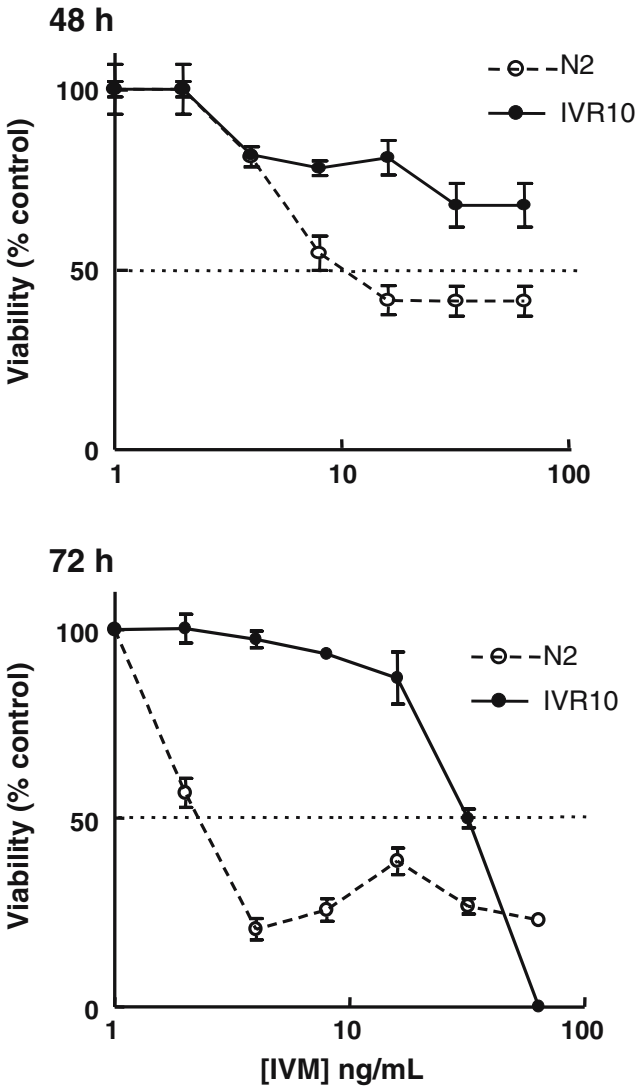

Fig. 2 Effect of ivermectin exposure on the viability of *C. elegans*. L1-stage *C. elegans* in M9 buffer were incubated with serial twofold dilutions of ivermectin. Viability was determined by the MTT assay after 48 and 72 h incubation. *Points* show the mean and standard deviation of triplicate wells

consistent with their ability to grow at these concentrations of ivermectin.

Recently, toxicity of short-chain alcohols to *C. elegans* was evaluated using several different endpoint assays (Thompson and de Pomerai 2005). To validate the MTT assay, the effects of these short chain alcohols on *C. elegans* viability were investigated. Worms were cultured with 0–10% methanol, ethanol, *iso*-propanol, or 1-butanol and incubated overnight. Figure 3 shows the viability of Bristol N2 *C. elegans* after overnight exposure to these short-chain alcohols. The LD_{50} concentrations were 7.5, 3.9, 6.2, and 3.4% for methanol, ethanol, *iso*-propanol, and 1-butanol, respectively.

Discussion

In the present study, we extend the use of the MTT assay (Marks et al. 1992) to L1 stages of both the free-living nematode *C. elegans* and the parasitic nematode *H. contortus*. This provides rapid analysis for cytotoxicity testing and allows simple analysis of multiple samples and drugs. While *C. elegans* provides a useful tool for many analyses, a major impediment to its use in toxicological testing is the time consuming assays of viability. While the vital staining of some filarial nematodes has been described previously (Comley et al. 1989a, b), limited knowledge is available in the application of this method to *C. elegans*. Conventional toxicological assay techniques designed for *C. elegans* are arduous and involve manually counting surviving worms or describing the level of motility after drug treatment (Anderson et al. 2001; Boyd et al. 2003). These techniques are slow, labour intensive, and somewhat impractical to assay many compounds and/or multiple drug concentrations. Colorimetric, fluorometric, and luminescence-based assays provide a faster end-point method for determining not only viability but also proliferation measurements and have been widely used in mammalian systems. The metabolism of MTT as a quantitative measure of living cells has been well validated (Mosmann 1983). We optimized the MTT-formazan assay for use with *C. elegans* due to this organism's simplicity in culturing synchronous life stages of the worm. These life stages have previously been shown to have consistent cell numbers, with adult hermaphrodites containing 959 cells (Hope 1999). This reproducibility makes this nematode an ideal candidate for use with the MTT-formazan assay.

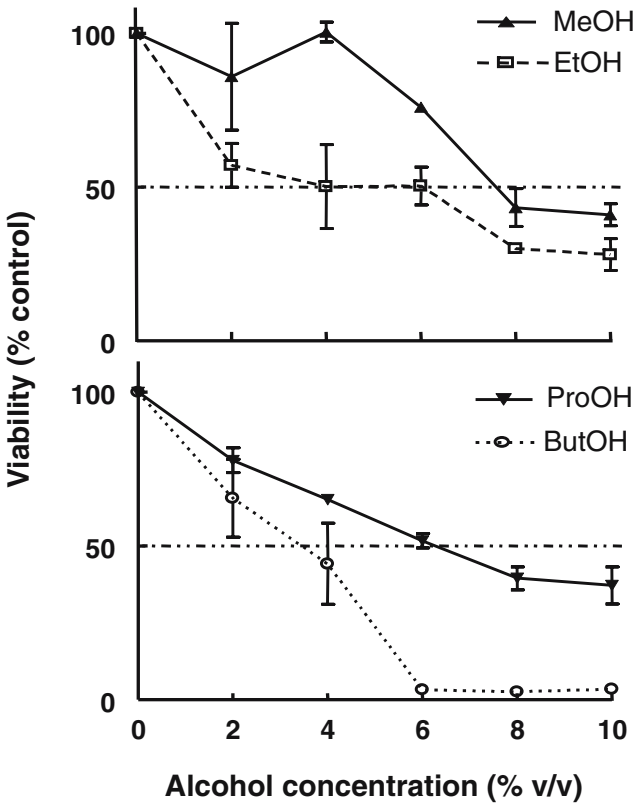

Fig. 3 Viability of *C. elegans* after exposure to methanol, ethanol, 1-butanol, or *iso*-propanol. Worms were cultured in 96-well microtitre plates with 0–10% (*v*/*v*) of each alcohol for 24 h. Viability of the worms was assessed using the MTT assay. *Points* are the mean, and the *error bars* show the standard deviation of four replicate wells

A further advantage of the application of this assay to *C. elegans* is the speed at which samples can be processed. The optimized MTT-formazan assay is a useful quantitative tool for the rapid determination of drug efficacy in *C. elegans*. Metabolically active larvae are able to take up and reduce MTT dye to produce formazan, while dead worms do not. Formazan detection is dependant on the concentration and time of MTT incubation and that formazan production is linearly correlated to the number of worms in the sample. For the use with *C. elegans*, 5 mg/ml MTT for 3 h gave reproducible and reliable determinations. Recently, SYTOX green was used for the rapid assessment of *C. elegans* viability (Gill et al. 2003). This assay is dependent of membrane integrity because the dye is not able to penetrate uncompromised cell membranes. While efficiently assessing viability, this assay is technically more unreliable, as each worm needs to be scanned for fluorescence. The MTT assay, as it solubilizes the dye, overcomes potential problems of uneven scanning of wells. It also overcomes the more expensive requirement for a fluorescence detector and the COPAS Biosort worm sorter. In addition to the application of this assay as a means to quantitate in vitro anthelmintic and other toxicant effects on worm viability, there are many other potential applications of this assay. In *O. volvulus*, the MTT-formazan assay was utilized to optimize cryopreservation techniques (Comley et al. 1989b). The application of the MTT-formazan assay in *C. elegans* may extend not only to anthelmintic studies, but have application in the fields of longevity studies,

reproduction, and embryonic development. The assay may also be applied to L1 stage of the closely related parasitic nematode *H. contortus*. However, unlike *C. elegans*, the uptake of MTT was not observed in the infective L3 stage larvae of *H. contortus*. This inability to take up MTT is likely due to the protective sheath of the L3 larvae, preventing the diffusion of MTT into the worm in *H. contortus* but not *C. elegans*. Success with L1 but not L3 stage larvae reflects the limited success of RNAi in L1, but not L3 *H. contortus* (Geldhof et al. 2006). This also suggests that MTT is taken up by diffusion rather than by feeding. If MTT was ingested, *H. contortus* L3 larvae would also take up MTT. This is supported by the microscopic appearance of the stained *C. elegans* showing an even distribution of the dye throughout the body. The assay was used to determine the toxicity of the anthelmintic ivermectin and short-chain alcohols to *C. elegans.* The ivermectin cytotoxicity results reflect each strain's ability to grow on those concentrations of ivermectin, with longer exposure to ivermectin at 72 h, killing a greater percentage of worms than 48 h exposure as expected. The ability of this assay to detect anthelmintics is dependent on nematode viability after 72 h incubation in the presence of drug, as this is the maximal time that the control untreated *C. elegans* are viable. Interestingly, ivermectin paralyses pharyngeal pumping, starving the worms and causing death (Avery and Horvitz 1990). As these nematodes are not fed during drug treatment, it is unlikely that lack of food is the cause of death. However, this assay clearly detects the difference between the sensitive *C. elegans* and drug resistant worms, suggesting that ivermectin has other toxic effects on *C. elegans*. Many important anthelmintics are neurotoxins, and detection will be dependent on the effect of the drug on cell viability. However, it is likely that most drugs would have an effect on viability in this time and so be detected in this screening assay.

The results on the cytotoxicity of short-chain alcohols demonstrated the different results obtained from viability assays. Previously, feeding inhibition, gravid scoring, and induction of *hsp16-1::lacZ* reporter activity in the transgenic PC161 strain were compared to score effects of alcohol on worm viability. All provided different orders of toxicity for the alcohols. Similar to the MTT assay, most assays found that ethanol was more toxic than methanol (Fig. 3). However, the reporter assay showed higher toxicity for methanol than ethanol. The lethality assay provided LD_{50} concentrations of 9.5–10.8% for ethanol, 12% for methanol, *iso*-butanol 2.6%, and 6.6% for *iso*-propanol after consideration of all effects measured (Thompson and de Pomerai 2005). The MTT LD_{50} values for the Bristol N2 strain do not significantly differ from the PC161 strain results, with the following order of toxicity: 1-butanol>ethanol>*iso*-propanol>methanol; LD_{50} values of methanol, ethanol *iso*-propanol, or 1-butanol are 7.5, 3.9, 6.2, and 3.4%, respectively. The previous study also highlighted inconsistencies between scoring different measurements of growth or feeding and proposed the use of *hsp16-1::lacZ* reporter activity as a rapid alternative to other assays. However, the reporter assay looks at the induction response to toxicant treatment and still requires treated worms to be frozen before processing for microscopic analysis of color production. The LD_{50} values reported from the *hsp16-1::lacZ* reporter assay for *iso*-butanol and *iso*-propanol were 6 and 8%, respectively, much higher than obtained for the lethality assay results of 2.6 and 6.6%, respectively. The MTT viability results more closely mirror the result of the lethality assay with values of 3.4 and 6.2% for 1-butanol and *iso*-propanol, respectively. Overall the MTT assay provides a simple method for viability testing in drug screening assays for *C. elegans* and may also be useful for L1-stage *H. contortus*.

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