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A semi-fluid gellan gum medium improves nematode toxicity testing

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ABSTRACT

This study examined an alternative test medium for nematodes that use gellan gum as the gelling agent instead of agar. The semi-fluid consistency of the gel-like component nematode growth gellan gum (CNGG) supports three-dimensional distribution of the nematodes and food bacteria, but still allows free movement of the former. Moreover, flexible preparation of the medium and easy recovery of the test organisms are possible. Here, the effects of the nematicides ivermectin (pharmaceutical) and aldicarb (pesticide) and of the metal cadmium on the growth and reproduction of the free-living nematodes *Caenorhabditis elegans* and *Panagrolaimus* cf. *thienemanni* were studied in CNGG media. Results were compared to those obtained with the standard liquid test media in order to evaluate the applicability of CNGG for nematode toxicity testing. The sensitivity of *P.* cf. *thienemanni* to all three substances was found to be higher than that of *C. elegans*, but both nematodes showed the highest sensitivity to ivermectin exposure. This raises concerns about the risk posed by the pharmaceutical to non-target nematodes. In contrast to ivermectin bioassays carried out in CNGG medium, those conducted in liquid medium resulted in wide-ranging variability between and within replicates. Thus, CNGG seems to be particularly valuable for testing hydrophobic substances with a high sorption affinity as it favors their sorption to food bacteria and minimizes contact with the surfaces of the test vessels. However, the medium was less suitable for deriving toxicity thresholds for cadmium and may likewise not be an appropriate choice for testing other metals. The medium introduced herein was shown to be appropriate for sublethal nematode toxicity testing and likely provides a convenient environment for testing other nematode species. Besides improved testing of hydrophobic substances, CNGG also offers advantages for long-term studies, such as full life-cycle experiments, in which fresh medium is regularly needed. Moreover it may be beneficial for testing other poorly soluble or insoluble substances, such as nanoparticles.

1. Introduction

Nematodes are the most abundant multicellular organisms worldwide, with free-living representatives occurring in great diversity and at high densities in every type of sediment or soil (Bongers and Ferris, 1999; Traunspurger et al., 2006). Moreover, they fulfill an important role in benthic and soil food webs because they comprise various feeding types (Yeates et al., 1993; Traunspurger, 1997). Consequently, the impact of contaminants on free-living nematodes was studied in several aquatic and terrestrial environments (reviewed in Wilson and Kakouli-Duarte, 2009) and standard nematode bioassays were developed (ASTM, 2008; ISO, 2010). For risk characterization of potentially toxic substances, nematode toxicity tests are typically conducted in liquid medium, which allows for rapid screening of potential toxicities and provides information about the susceptibility of nematodes compared to other organisms (Williams and Dusenbery, 1990; Traunspurger et al., 1997; Höss et al., 2002, 2008; Boyd and Williams, 2003; Sochova et al., 2007). However, many pollutants are hydrophobic and thus difficult to test, because of their tendency to rapidly sorb onto the surfaces of test vessels, such that the desired test concentrations cannot be achieved or maintained with certainty (Breitholtz et al., 2006). Although several methods have been developed for assaying hydrophobic substances in liquid media, such as preconditioning with the test substance or surface treatment of the test vessels (Rufli et al., 1998), the reliability of the test results cannot be guaranteed especially for substances with a high sorption affinity (Breitholtz et al., 2006). Even the presence of a solvent may not prevent that these substances adsorb onto surfaces once they are inside an aqueous solution (Prasse et al., 2009). Moreover, hydrophobic substances partition onto food and other particles, but rather than contributing to a loss of the test substance this is, for many species,

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an important route of pollutant exposure (Chandler et al., 1994; Fliedner, 1997; Breitholtz and Wollenberger, 2003). Breitholtz et al. (2006) commented that this might be the major route of exposure of hydrophobic substances instead of the water pathway. However, this route of exposure can be also important for readily water-soluble substances, such as metals (Höss et al., 2001; Hook and Fisher, 2001; Yoo et al., 2004; Offermann et al., 2009).

In the present study, a gel-like test medium, referred to as component nematode growth gellan gum (CNGG), is proposed for nematode toxicity tests. The use of CNGG allows the adsorption of hydrophobic substances onto food bacteria to be exploited as a test advantage. Instead of agar, the medium's gelling agent is gellan gum, a highly purified polysaccharide initially used in bacteriological media (Shungu et al., 1983) but since then also described for use in nematode media (Ferris et al., 1995; Muschiol and Traunspurger, 2007; Muschiol et al., 2009). Unlike agar, gellan-gum-based media solidify in the presence of (divalent) cations, such as magnesium or calcium, and can be re-liquefied by the addition of ethylenediaminetetraacetic acid (EDTA). CNGG test medium is prepared by directly mixing the test substance into a gellan gum/food bacteria suspension before the medium solidifies following the addition of cations. Thus, the test substance is expected to adsorb to the food bacteria rather than to the test vessels. Moreover, contact of the test substance with the test-vessel surfaces may be further minimized by the rapid solidification of the medium, triggered by the added cations. In order to support handling and to minimize aqueous contact prior to testing, a solvent was still used for the hydrophobic test substance in the present study. However, studies using CNGG media that completely avoid a solvent for testing hydrophobic substances should be conducted in the future. Additional advantages of CNGG over liquid test media include its semi-fluid consistency, which prevents the settling of both food bacteria and test organisms to the bottom of the test vessels, while at the same time allowing the worms to move freely and three-dimensionally, just as they would in the sediments and soils of their natural environment.

Here, three types of substances were tested in experiments aimed at evaluating the applicability of CNGG in sublethal nematode toxicity testing: (1) the pharmaceutical ivermectin, representative of hydrophobic substances with a high sorption affinity ($\log K_{OW}$ 3.2, $\log K_{OC}$ 3.07–4.4 L/kg; Liebig et al., 2010); (2) the pesticide aldicarb, a hydrophilic organic compound ($\log K_{OW}$ 1.13, $\log K_{OC}$ 1.43–2.42 L/kg; Vink and van der Zee, 1997); and (3) cadmium, an inorganic strongly accumulative priority substance (European Parliament and Council, 2008). Ivermectin is frequently administered to livestock as an endo- and ectoparasiticide that protects against parasitic infections, such as by mites and nematodes (Omura, 2008). Aldicarb protects plants against pests, such as plant bugs and plant-parasitic nematodes (Moore et al., 2010). While both have a neurotoxic mode of action (Risher et al., 1987; Wolstenholme and Rogers, 2005) and are known to be toxic for several non-target organisms (Roberts and Dorough, 1984; Edwards et al., 2001; Liebig et al., 2010; Moore et al., 2010), little is known about their impact on non-target (i.e., non-parasitic) nematodes, which might be at high risk due to their close phylogenetic relationship with the target organisms. Cadmium, by contrast, has a rather unspecific mode of action, inducing oxidative stress and therefore oxidative deterioration of biological macromolecules (Stohs et al., 2001). Its toxicity is well established and has been extensively studied (Kammenga et al., 1994; Wright and Welbourn, 1994).

We investigated the effects of these three substances on the growth and reproduction of the most frequently tested free-living nematode, *Caenorhabditis elegans*. The results obtained in standard-liquid and CNGG test media were compared in order to assess the usefulness of CNGG in the assaying of hydrophobic substances with a high sorption affinity, as well as in nematode toxicity testing in general. Additionally, the suitability of CNGG for sublethal toxicity testing of a non-standard test nematode, in this case *Panagrolaimus* cf. *thienemanni*, was examined.

2. Methods

2.1. Test organisms and stock cultures

C. elegans (strain N2) was provided by the Caenorhabditis Genetics Center (Minneapolis, USA) and maintained on nematode growth medium (NGM) agar (17 g agar, 2.5 g peptone, and 3 g NaCl in 975 ml deionized water, with 1 ml 1 M $CaCl_2$, 1 ml 1 M $MgSO_4$, 25 ml 1 M KPO_4 buffer (pH 6), and 1 ml cholesterol solution (5 mg/ml in ethanol) added after autoclaving; Brenner, 1974) seeded with *Escherichia coli* (OP50). *P.* cf. *thienemanni* cultures were established from freshwater microbial mats of Movile Cave (Dobruja, Romania; Muschiol and Traunspurger, 2007) and maintained on nematode growth gelrite (NGG) medium (modified NGM with agar replaced by 1.5 g gellan gum (Gelrite®, Merck & Co. Inc., Kelco Division); Muschiol and Traunspurger, 2007) seeded with *E. coli* (OP50).

2.2. Liquid test media

According to the standard methods (ISO, 2010), M9-medium (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 86 mM NaCl, and 1 mM $MgSO_4$) was used as test medium for the liquid bioassays with ivermectin and aldicarb. However, as metals are likely to precipitate in media containing phosphate buffer (Donkin and Williams, 1995), K-medium (3.1 mg/L NaCl; 2.4 mg/L KCl; Williams and Dusenbery, 1990) was used for cadmium testing. The measured pH values of these media were 6.9 and 6.8, respectively. As food source, *E. coli* (OP50) was suspended, depending on the tested substance, in either M9- or K-medium according to the standard methods (ISO, 2010; turbidity 1000 FAU). The final test medium consisted of one part food bacteria and one part test solution (test substance dissolved in either M9- or K-medium), resulting in a final bacterial density of 500 FAU (approximately 1.07×10^9 cells/ml).

2.3. Component nematode growth gellan gum (CNGG) test media

Two CNGG media differing in their compositions and referred to as CNGG-1 and CNGG-2 were used. Neither contained nutrients, such as peptone, that might bind the test substance and increase oxygen demand. CNGG-1 is a less rich medium containing only a minimum of salts and no phosphate buffer whereas CNGG-2 is similar to media used for nematode cultivation. The measured pH values of CNGG-1 and CNGG-2 were 6.9 and 6.1, respectively. Aldicarb was tested in CNGG-2 but cadmium was tested with CNGG-1, for the same reasons K-medium was used for liquid testing (see Section 2.2). In ivermectin experiments, CNGG-1 was also used as the test medium.

For CNGG-1, the following components were prepared and autoclaved separately: (1) gellan gum solution (1.9 g/L Gelrite®, Merck & Co. Inc., Kelco Division), with 1.25 ml/L cholesterol solution (5 mg/ml in ethanol) added after autoclaving and (2) salt solution (10 mM $MgSO_4$; 10 mM $CaCl_2$). As food bacteria, *E. coli* (OP50) was suspended in sterile K-medium. The suspension was adjusted to a cell density of 6.25×10^9 cells/ml (final medium density: approximately 5×10^9 cells/ml) and then centrifuged (20 min, 2000*g*). The supernatant was discarded and the pellet resuspended in gellan gum solution of the same volume. The final test medium was prepared by mixing eight parts gellan gum/bacterial suspension with one part test solution (test substance dissolved in water) followed by the addition of one part salt solution, as the final step. The test medium was thoroughly mixed between every step.

The composition of CNGG-2 was similar to that of NGG, and thus NGM, except for the lack of peptone and a reduced NaCl content (1/10). Thus, a different salt solution (0.1 M NaCl, 20 mM $MgSO_4$, and 20 mM $CaCl_2$) than the one used for CNGG-1 and, additionally, a buffer solution (0.5 M KPO_4 buffer, pH 6) were prepared. The gellan gum/bacterial suspension was identical to the one described for CNGG-1. The final test medium was prepared by mixing 16 parts gellan gum/bacterial suspension with two parts test solution (test substance dissolved in water) followed by the addition of one part buffer solution and then one part salt solution, as the final step. The medium was thoroughly mixed between every step.

2.4. Preparation of stock solutions and chemical analysis

Stock solutions of ivermectin (Sigma, CAS 70288-86-7) dissolved in acetone were prepared and analyzed. The determined ivermectin concentrations were 53, 98, 154, 219, 424, 663, 920, and 1373 μg/L. Additionally, a high-concentration stock solution of approximately 1830 μg/L was prepared. Chemical analysis of