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Short communication

An *in vitro* larval migration assay for assessing anthelmintic activity of different drug classes against *Ascaris suum*

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ABSTRACT

In vitro methods have been developed for the detection of anthelmintic resistance in a range of nematode species. However, the life cycle of *Ascaris suum* renders the commonly used egg hatch assay and larval development assay unusable. In this study we developed a combined multi-well culture and agar gel larval migration assay to test the effect of benzimidazole and tetrahydropyrimidin/imidazothiazole anthelmintics against nine isolates of *A. suum* collected from locations in China and Denmark. Drugs tested were thiabendazole, fenbendazole, mebendazole, levamisole, and pyrantel. The percentages of larvae that migrated to the surface of each treated and control well were used to calculate the drug concentration which inhibits 50% of the larvae migration (EC_{50}). The values of EC_{50} of thiabendazole, fenbendazole, mebendazole, levamisole, and pyrantel against *A. suum* isolates ranged 74–150, 4.9–13.9, 2.3–4.3, 358–1150 and 1100–4000 nM, respectively. This combined multi-well culture and agar gel larval migration assay was a sensitive bioassay for anthelmintic activity and could serve as an *in vitro* method to detect for lowered drug efficacy against *A. suum* or possibly to screen for anthelmintic drug candidates.

1. Introduction

Gastrointestinal nematodes (GIN) are amongst the most prevalent pathogens in livestock throughout the world, causing significant economic losses for farmers. (Charlier et al., 2014; Fitzpatrick, 2013). Current strategies for control of GIN in livestock are almost entirely dependent on regular treatment with broad-spectrum anthelmintic drugs. Anthelmintic chemotherapy is largely limited to the major chemical classes: the benzimidazoles, tetrahydropymidin/imidazothiazoles and macrocyclic lactones, and more recently: the spiroindoles, cyclic octadepsipeptides and amino-acetonitrile derivatives (Vercruysse and Claerebout, 2014; Wolstenholme, 2011). However, anthelmintic resistance is an undeniably catastrophic problem in some segments of the animal health market, especially in small ruminants (Conder and Campbell, 1995; Waller, 1997), but may be an emerging issue in other livestock such as pigs. To date, there is evidence of drug resistance in the swine strongyle nematode *Oesophagostomum* spp. against pyrantel, levamisole + pyrantel, benzimidazoles, and ivermectin, confirmed under controlled conditions (Bauer and Gerwert, 2002; Bjorn et al., 1990; Roepstorff et al., 1987; Varady et al., 1996) and in a limited number of on-farm surveys using fecal egg count reduction test (FECRT) and *in vitro* assays (Dangolla et al., 1994; Gerwert et al., 2002; Kagira et al., 2003). At present, there is no credible evidence of drug resistance in any other porcine nematode species, e.g. *Ascaris suum* and *Trichuris suis* (Hansen et al., 2014). In contrast, there are

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Table 1
Different isolates examined in the assay. Female worms isolated from the same pig were considered as one isolate. The number of individual females tested separately in each isolate is given in brackets.

Locations collected	Isolates
Qinghai, China	QH1 (3), QH2 (4)
Xingjiang, China	XJ1 (3), XJ2 (3)
Beijing, China	BJ1 (3), BJ2 (3)
Three farms in Denmark	DA (3), DB (2), DD (3)

some indications of reduced efficacy in isolates of related nematode species regarding infection stages, e.g. *A. lumbricoides* (Vercruysse et al., 2011) and *T. trichiura* (Keiser and Utzinger, 2008) in humans.

A. suum is a large nematode that resides in the small intestine of pigs (Stewart and Hale, 1988; Thamsborg et al., 2013). *A. suum* is highly prevalent worldwide, both in intensive indoor systems and outdoor free-range systems (Han et al., 1995; Haugegaard, 2010) and may have a substantial impact on production (Jankowska-Makosa and Knecht, 2015; Vlaminck et al., 2015). While *in vitro* methods have been developed for the detection of anthelmintic resistance in other GIN species, the life cycle of *A. suum* renders the commonly used egg hatch and larval development assays unusable. Rew at al., used an *in vitro* cultivation of *A. suum* larvae in different concentration of anthelmintic drugs to evaluate anthelmintic activity (Rew et al., 1986). They found changes in the development of third to fourth larval stage (L3–L4) and motility of the larvae after exposure to various anthelmintic drugs. However, their methods were unable to estimate the EC_{50} of tested anthelmintic drugs accurately. Consequently, a fast, effective, accurate assay for comparison of multiple classes of anthelmintic drugs against different *A. suum* isolates is still warranted.

In this paper, we adaptated and evaluated an agar gel larval migration bioassay, previously reported (Han et al., 2000), to determine the quantitative effects of different drugs on migration and survival of *A. suum* larvae. This method may be used to detect isolates of *A. suum* with reduced drug efficacy and potentially drug resistance.

2. Materials and methods

2.1. Preparation of A. suum larvae for culture

A. suum females were collected from different locations in China and Denmark. *A. suum* eggs from each individual female were incubated separately. Isolates QH1 and QH2 were from a pig in a village and a small pig farm, respectively, in Qinghai province, China; XJ1 and XJ2 were from two different small pig farms in Xinjiang Uygur autonomous region, China; BJ1 and BJ2 were from two different pig farms in Beijing area; and the remaining 3 isolates (designated DA, DB and DD), were from 3 different farms in Denmark. Isolates in Denmark were obtained in the abattoir during processing of intestines and others were obtained from individual pigs with *A. suum* egg positive in feces. (Table 1). Eggs (Batch 1) for a pre-test were isolated from ~50 *A. suum* females collected in a Danish abattoir. Eggs of all isolates were dissected from the lower parts of uteri of female *A. suum* worms, decoated in 3% sodium-hypochlorite, washed three times with phosphate buffered saline (PBS) and stored for at least 3 months at room temperature in 0.1N sulphuric acid at a concentration of 25 eggs/μl to allow embryonation to the infective stage (L3) (Oksanen et al., 1990). *A. suum* eggs were hatched using a glass beads method (Han et al., 2000; Urban and Douvres, 1981) with a slight modification by adding 5% bovine bile in the hatching buffer. After hatching, free larvae were separated and cleaned as previously described (Han et al., 2000). After that they were washed once in Dulbecco's Modified Eagle's Medium plus 2 mM L-Glutamine, 50 μg/ml gentamycin and 10 μg/ml Amphotericin B, and were re-suspended in the same culture medium and adjusted to around 200 l3/ml medium. This procedure yielded a clean larval suspension, which was then used for the anthelmintic activity assay.

2.2. Pre-incubation of A. suum larvae with anthelminthic drugs

A variable number of L3 (50–300 L3) larvae were incubated with anthelminthic drugs in 48 well plates (Nunc, Denmark) with 0.59 ml medium (Dulbecco's modified Eagle's medium plus 2 mM L-Glutamin, 50 μg/ml gentamycin and 10 μg/ml Amphotericin B) in each well for 1 day at 38 °C in an atmosphere containing 5% CO_2. The drugs used were thiabendazole (TBZ), fenbendazole (FBZ), mebendazole (MBZ), pyrantel citrate (PYR), and levamisole hydrochloride (LEV) (Sigma, St Louis, MO, USA). All drugs were dissolved in DMSO. Each drug stock solution was serially diluted by two-fold using DMSO at a maximum of 1.7%, and then transferred 10 μl diluted drug to each corresponding well through the plate giving a final concentration range of 160–2000 nM for TBZ, 0.39–50 nM for FBZ and MBZ, 39–5000 nM for LEV, and 240–3000 nM for PYR based on a pilot test for determining concentration range for each individual anthelmintic drug. In the pilot test, we identified each anthelmintic concentration for 100% larval migration inhibition by testing $1 \times 10^{(-1to4)}$ nM of all tested anthelmintic drugs. We also tested the DMSO effect on larval migration and found 2% DMSO did not significantly inhibit the migration. Therefore, control wells contained 10 μl DMSO (1.7% v/v) without any drug in the medium.

2.3. Agar gel larval migration assay

After 1 day incubation an equivalent volume of 1.5% agar at 52 °C (0.6 ml) was added to each well. The agar was allowed to set prior to the addition of 0.3 ml of PBS to each well and the plates were then incubated at 38 °C overnight in an atmosphere containing 5% CO_2. The numbers of larvae that migrated onto the top/outside of the agar gel were counted by an inverse microscope.

2.4. Data analysis

Larvae produced from each individual female were tested separately and all tests were conducted at least in triplicate. In each isolate, larvae from 2 to 4 different females were tested in the assay (Table 1). Logit (Dobson et al., 1987) was used to calculate the concentration that inhibited 50% of the larvae from migrating outside of the agar gel (EC_{50}) compared with the control. Triplicates were averaged to one EC_{50} per female worm, which was used to calculate the mean of EC_{50} of different females in each isolate. Values of EC_{50} for each drug were analyzed for significant difference between different isolates ($n = 9$) and between different locations ($n = 4$) using one-way ANOVA analyses and $P < 0.05$ is considered a significant difference between isolates.

3. Results

3.1. Egg hatching

The hatching method with glass beads and bile (5%) resulted in around 70–90% hatching for all isolates. Very small numbers of larvae were damaged during hatching by the glass beads. During one day incubation, we did not observe any signs of bacteria or fungi growth in the culture. In some wells with high concentration of anthelmintic drugs added, we observed high percentage of larval death and sluggish movement of surviving larvae.