Anthelmintic tolerance in free-living and facultative parasitic isolates of *Halicephalobus* (Panagrolaimidae)

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(*Received 22 March 2011; revised 21 November 2011, 23 February and 2 March 2012; first published online 13 April 2012*)

SUMMARY

Studies on anthelmintic resistance in equine parasites do not include facultative parasites. *Halicephalobus gingivalis* is a free-living bacterivorous nematode and a known facultative parasite of horses with a strong indication of some form of tolerance to common anthelmintic drugs. This research presents the results of an *in vitro* study on the anthelmintic tolerance of several isolates of *Halicephalobus* to thiabendazole and ivermectin using an adaptation of the Micro-Agar Larval Development Test hereby focusing on egg hatching and larval development. *Panagrellus redivivus* and *Panagrolaimus superbus* were included as a positive control. The results generally show that the anthelmintic tolerance of *Halicephalobus* to both thiabendazole and ivermectin was considerably higher than that of the closely related Panagrolaimidae and, compared to other studies, than that of obligatory equine parasites. Our results further reveal a remarkable trend of increasing tolerance from fully free-living isolates towards horse-associated isolates. *In vitro* anthelmintic testing with free-living and facultative parasitic nematodes offers the advantage of observing drug effect on the complete life cycle as opposed to obligatory parasites that can only be followed until the third larval stage. We therefore propose *Halicephalobus gingivalis* as an experimental tool to deepen our understanding of the biology of anthelmintic tolerance.

Key words: facultative parasitism, ivermectin, MALDT method, thiabendazole, model organism.

INTRODUCTION

To date, studies on anthelmintic resistance or tolerance in equine parasites only include obligatory parasites, not facultative parasites. *Halicephalobus gingivalis* (Stefánsky, 1954, Andrássy, 1984) also referred to as *H. deletrix* or as *Micronema deletrix* (Anderson *et al*. 1998), is a small (235–460 μm) free-living bacterivorous nematode (Panagrolaimidae) and a known facultative parasite of horses (Blunden *et al*. 1987; Nadler *et al*. 2003) and zebra (Isaza *et al*. 2000). In addition, 4 cases of human infection, all with a fatal outcome, have been described (Ondrejka *et al*. 2010). *H. gingivalis* has all the characteristics of a free-living nematode, only at the ultrastructural level can some potential adaptations to facultative parasitism be observed (Fonderie *et al*. 2009). Infection probably occurs through open wounds and oral or nasal cavities (Pearce *et al*. 2001). Subsequently, nematodes most likely invade the bloodstream and lymphatic system and thus reach different organs (e.g. kidneys, liver and brain) where the number of nematodes increases rapidly through parthenogenetic reproduction (Akagami *et al*. 2007). The clinical symptoms vary depending on which organs are infected (Blunden *et al*. 1987; Spalding *et al*. 1990; Rames *et al*. 1995; Johnson *et al*. 2001; Müller *et al*. 2008). A few cases have been described in which the infection was recognized in time and the horse was successfully treated (Dunn *et al*. 1993; Pearce *et al*. 2001; Müller *et al*. 2008). Still, most infections were only recognized post-mortem after a thorough autopsy. Most importantly, the clinical histories of all reported equine infections show that the horses had been regularly treated with common anthelmintics (e.g. Boswinkel *et al*. 2006, Ferguson *et al*. 2008). This strongly indicates that *H. gingivalis* either has a high tolerance or some form of resistance to these anthelmintic drugs.

The current paper presents the first research on anthelmintic tolerance of the facultative parasitic nematode *Halicephalobus gingivalis*. Several isolates were tested for tolerance to common anthelmintic drugs through *in vitro* experiments focusing on egg hatching and larval development. Both free-living and parasitic isolates were included to examine whether tolerance is restricted to parasitic isolates or whether it also holds true for free-living isolates. The results on the *H. gingivalis* isolates were compared with those on the closely related free-living nematode species *Panagrellus redivivus* and *Panagrolaimus superbus*, allowing us to discriminate species- or

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doi:10.1017/S0031182012000558

strain-specific tolerance from any more general tolerance in the free-living Panagrolaimidae.

MATERIALS AND METHODS

Maintenance of cultures

We selected 4 isolates for our experiments. They were all light-microscopically identified as the morpho-species *Halicephalobus gingivalis* using different identification keys (Geraert *et al*. 1988; Shokoohi *et al*. 2007). No males were observed confirming that this species is parthenogenetic (Stefansky, 1954; Andrássy, 1984; Akagami *et al*. 2007; Fonderie *et al*. 2009). The JB128 isolate was obtained from a vegetable compost heap in Riverside (California, USA). The WB0708 isolate was obtained from a large-scale compost heap (Steel *et al*. 2010) at the Institute for Agricultural and Fisheries Research in Merelbeke (Belgium). The WB0801 isolate was obtained from fresh horse droppings from an individual stall on a stable in the province of West-Flanders (Belgium). The SAN100 isolate is a clinical isolate originating from an infection in a horse (Guelph, Ontario, Canada) described by Anderson *et al*. (1998). SAN100 has been maintained in culture on plain bacteriological agar with a bacterial food source since its isolation. Hence, we used 2 compost isolates from horse-independent habitats and 2 isolates from horse-associated habitats including 1 parasitic isolate and 1 isolate found in the near vicinity of horses. Two closely related free-living species, *Panagrellus redivivus* PS1163 and *Panagrolaimus superbus* DF5050, were included in the experiments to discriminate species- or strain-specific tolerance from a possible more general tolerance in the otherwise free-living Panagrolaimidae.

Stock cultures of all species were maintained on 1% bacteriological agar (Oxoid, Basingstoke, UK) plates containing cholesterol (1 mg ml^{-1}) and *Escherichia coli* OP50 as a food source. The stock cultures were incubated at 20 °C and generally handled as described by Brenner (1974). To provide enough eggs to start the experiments, the *Halicephalobus* isolates were subcultured and incubated at 37 °C for 3 to 4 days. At this temperature the cultures grow fast and numerous eggs can be generated over a short period of time. The *P. redivivus* and *P. superbus* isolates were subcultured 2 weeks beforehand and incubated at 20 °C to yield a sufficient amount of eggs.

Anthelmintics

The anthelmintics used in the experiments were thiabendazole (TBZ; Sigma-Aldrich, Bornem, Belgium) and ivermectin (IVM) that represent members of 2 important anthelmintic groups, the benzimidazoles and the avermectin/mylbecins, respectively. They were selected because of the use of products of these groups on the location where the WB0801 isolate was found. TBZ is the most soluble member of the benzimidazole group that facilitates *in vitro* experiments. The IVM used in the experiments are dilutions of Ivomec® Injection (Merial, Brussels, Belgium), a commercially used form of the drug.

Experimental setup

The technique used was a modification of the micro-agar larval development test (MALDT) (Coles *et al*. 2006). The MALDT method was originally designed as a larval development test (LDT) on a solid instead of in a liquid medium. MALDT was chosen over LDT because of the ease of culturing *Halicephalobus* on solid medium and because this very small nematode is easier to discern on solid than in liquid medium. The main objective of any larval development test is to follow the development of nematode eggs onto third-stage larvae, which generally is the infective stage in obligatory animal parasites. Here we mainly focused on quantitative hatching data and development to the adult stage.

The experiments were performed on 24-well plates (Greiner Bio-One, Frickenhausen, Germany). The anthelmintics were dissolved in 100% dimethylsulfoxide (DMSO; Carl Roth GmbH, Karlsruhe, Germany). Five stock solutions of TBZ (100, 1000, 2000, 5000, 10 000 $\mu g\ ml^{-1}$) and 5 stock solutions of IVM (1, 10, 50, 100, 200 $\mu g\ ml^{-1}$) were prepared. Stock solutions were diluted 100x by adding 49·5 ml of 1% bacteriological agar at approximately 45 °C to 0·5 ml of drug solution in a 50 ml Falcon flask. The final solution was carefully homogenized before adding 3 ml to each well. The control consisted of 1% bacteriological agar with a final concentration of 1% DMSO. We chose to keep the concentration of DMSO in the final solutions at 1% to exclude any influence on the mortality rate as reported for *Caenorhabditis elegans* by Ura *et al*. (2002) for DMSO concentrations in excess of 5%. Final drug concentrations in the wells were 1, 10, 20, 50 and 100 $\mu g\ ml^{-1}$ for TBZ and 0·01, 0·1, 0·5, 1·5, and 2 $\mu g\ ml^{-1}$ for IVM. Initially, the *Halicephalobus* isolates were tested against a range of drug concentrations based on Várady *et al*. (2009). Because only small to no effects were noticed, higher concentrations were chosen in the present experiments.

The tests were performed in 3 replicates for each anthelmintic concentration with the zero concentration as a negative control. Approximately 50 nematode eggs were transferred into each well. The exact number of eggs was counted for each well. The plates were subsequently incubated at an optimal temperature for development, i.e. 30 °C for the *Halicephalobus* isolates and 20 °C for *P. redivivus* and *P. superbus*. Hatching was quantified at the time eggs normally develop into the adult stage, which is

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after 48 h incubation at 30 °C for the *Halicephalobus* isolates and after 7 days incubation at 20 °C for *P. redivivus* and *P. superbus*. In order to assess the reproducibility of our bioassay, we repeated the entire experiment 15 months after the first trial.

The hatching proportion (HP) is calculated for each well as follows: the number of hatched eggs and surviving larvae or adults is divided by the number of eggs originally transferred onto the agar. This proportion is determined at each concentration.

Moreover, in contrast to TBZ which prevents both embryonation and hatching of nematode eggs (Taylor *et al*. 2002), IVM mainly has an effect on the larval stage and only prevents hatching at very high concentrations (Patel, 1997). Therefore, an experiment was performed to verify whether larval stages surviving high IVM concentrations but initially not developing into the adult stage, can overcome the effect of drug treatment and resume development. To this end, 3 replicas of 40 eggs of each *Halicephalobus* isolate were transferred onto 1% bacteriological agar containing 1·5 $\mu g\,ml^{-1}$ IVM and were incubated at 30 °C. After 76 h, 20 surviving larvae of each replicate were transferred onto plain 1% bacteriological agar and observed for several days. Their recovery rate is defined as the number of transferred larvae that develop into the adult stage divided by the number of initially transferred larvae ×100.

Effect of pre-exposure on anthelmintic tolerance

Anthelmintic tolerance can be caused or increased by contact of the nematodes with the anti-parasitic drug in question. In order to verify the short-term effect of a prior anthelmintic treatment on the anthelmintic tolerance of *Halicephalobus* cf. *gingivalis*, all 4 isolates (JB128, WB0708, SAN100 and WB0801) were cultured for approximately 12 ± 2 generations at 30 °C on 1% bacteriological agar containing a low dose of anthelmintics, i.e. 10 $\mu g\,ml^{-1}$ TBZ or 0·01 $\mu g\,ml^{-1}$ IVM. After this period, the modification of the MALDT method was performed as described above, leaving out the lowest TBZ concentration of 1 $\mu g\,ml^{-1}$.

Statistical analysis

To test for differences in the response of the isolates towards each anthelmintic across the two trials, the data were modelled by means of a generalized linear mixed model (PROC GLIMMIX in SAS® v.9.3, SAS Institute Inc., Cary, NC, USA). As we were merely interested in the effect of 'isolate' and 'concentration' and their interaction, these factors were treated as fixed effects in the model. Yet, as the whole experiment was replicated in 2 trials, the fixed effects were assessed across both trials by including the factor 'trial' as random effect in the model. As the response variable includes the number of hatched or survived individuals on the total number of individuals, a binomial error distribution was assumed and a logit link was incorporated to relate the predictive part of the model to the mean response. Standard error and degrees-of-freedom were estimated according to the method described by Kenward and Roger (1997). Significance of the fixed effects and their interactions were tested by means of Type III tests. Differences in tolerance between the isolates were post-hoc tested by comparing the expected hatching success and survival probability at different concentrations (least square means), using a Tukey-Kramer-adjustment to correct for multiple testing.

Given that generalized linear mixed models are large sample tests (Agresti, 2002), we did not rely on this procedure to compare the effect of the anthelmintics between the *Halicephalobus* isolates and *P. redivivus* and *P. superbus* as their hatching proportion approached zero at higher concentrations. Therefore, a Fisher exact test procedure was used for these comparisons as implemented in StatXact® v.5.0 (Cytel Inc., Cambridge, MA, USA).

The effect of pre-exposure to anthelmintics was analysed using Statistica 7 (StatSoft Europe GmbH, Hamburg, Germany) for each *Halicephalobus* isolate separately using two-way analysis of variance (ANOVA) with the factors anthelmintic concentration and pre-exposure, followed by a post-hoc Tukey HSD test. The assumptions for ANOVA (normality and homogeneity of variances) were tested using a Kolmogorov-Smirnov test and a Bartlett test, respectively.

RESULTS

Thiabendazole (TBZ)

Figure 1 shows the mean hatching proportions (HPs) of the *Halicephalobus* isolates and of *P. redivivus* and *P. superbus* for both trials at different TBZ concentrations, with 0 $\mu g\,ml^{-1}$ being the negative control. The mean HPs of the negative controls were comparable for the parasite isolate, the droppings isolate, *P. redivivus* and *P. superbus*, with an average ranging from 0·96 to 0·99. However, the HPs of the negative controls of the compost isolates, with an average ranging from 0·91 to 0·93, were significantly (Tukey post-hoc, $P < 0{\cdot}0001$) lower than those of the parasite isolate and the droppings isolate.

The response of *P. redivivus* and *P. superbus* to TBZ concentration was similar in both trials; they had an initially high HP with an average ranging from 0·85 to 0·94 at 1 $\mu g\,ml^{-1}$ followed by a steep decrease towards zero hatching at 10 $\mu g\,ml^{-1}$ and higher TBZ concentrations. From 10 $\mu g\,ml^{-1}$ onwards, the HPs of both *P. redivivus* and *P. superbus* were significantly

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Table 1. Type III statistics of fixed effects generated by means of a generalized linear mixed model on the average hatching proportions (HPs)

	TBZ		IVM	
Effect	*F* value	*P*	*F* value	*P*
isolate	85	<0·0001	64	<0·0001
concentration	604	<0·0001	275	<0·0001
concentration*isolate	5·8	<0·001	7·3	<0·0001

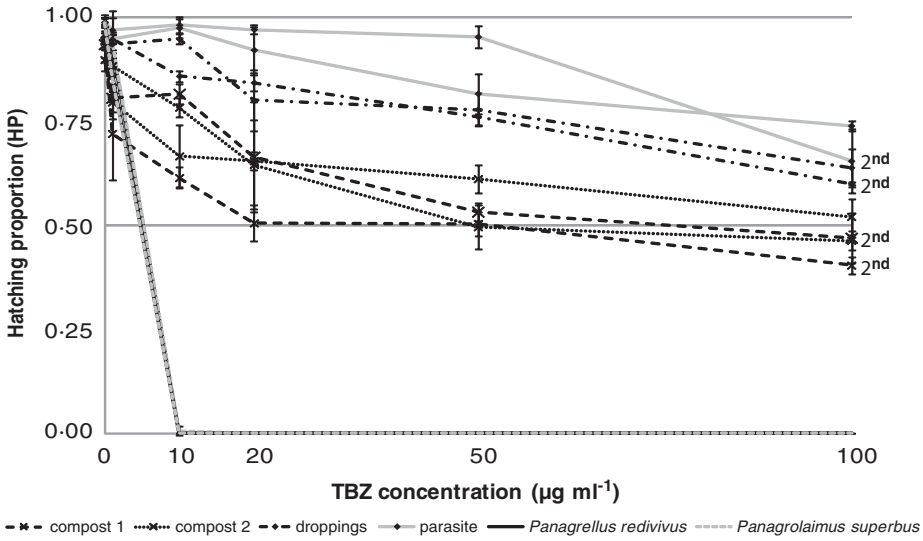

Fig. 1. The hatching proportion (HP) for all *Halicephalobus* cf. *gingivalis* isolates† and for *Panagrellus redivivus* (PS1163) and *Panagrolaimus superbus* (DF5050) at different thiabendazole (TBZ) concentrations ($\mu g\ ml^{-1}$). Data represent the mean of 3 replicates (±1 S.D.) for 2 independent and consecutive trials. Data from the second trial are indicated (2^{nd}). †Compost isolates, i.e. compost 1 (WB0708) and compost 2 (JB128); horse-associated isolates, i.e. parasite (SAN100) and droppings (WB0801).

lower than those of all *Halicephalobus* isolates (Fig. 1; Fisher's exact test: *P* all <0·0001).

Besides an overall significant negative effect on the HPs of all the *Halicephalobus* isolates, significant differences were observed in their mean HP ('Isolate' effect), as well as in their response towards TBZ concentration across trials (Isolate*concentration effect) (Table 1). Comparison of the mean HPs across trials revealed that the parasite isolate had the highest average HPs, which were significantly different from the average HPs of the droppings isolate (Tukey post-hoc, *P* all <0·05) and from both compost isolates at all TBZ concentrations (*P* all <0·0001). The droppings isolate had the second highest average HPs, which were also significantly higher than the average HPs of both compost isolates at all drug concentrations (*P* all <0·0001). Compost isolates 1 and 2 had the overall lowest average HPs of the *Halicephalobus* isolates, without significant (*P* all >0·07) mutual HP differences.

Ivermectin (IVM)

The mean HPs of the *Halicephalobus* isolates and of *P. redivivus* and *P. superbus* of both trials at the different IVM concentrations are shown in Fig. 2, with $0\ \mu g\ ml^{-1}$ being the negative control. The average HPs of the negative controls were similar for all *Halicephalobus* isolates and for *P. redivivus* and *P. superbus* with an average HP ranging from 0·92 to 1.

As for TBZ, the HPs of both *P. redivivus* and *P. superbus* soon dropped significanty lower (at $0{\cdot}1\ \mu g\ ml^{-1}$) compared to those of the *Halicephalobus isolates* (Fisher's exact test: *P* all <0·0001). As the HPs for both *P. redivivus* and *P. superbus* even approached zero for IVM concentrations that were higher than $0{\cdot}5\ \mu g\ ml^{-1}$, they were significantly lower compared to the HPs of the *Halicephalobus* isolates for all the remaining IVM concentrations (Fisher's exact test: *P* all <0·0001).

Also for IVM, within the *Halicephalobus* isolates significant differences were observed in both the mean hatching rate and in their response to IVM concentration across both trials (Table 1). The average HPs of the parasite isolate were also the highest at all IVM concentrations, followed by those of the droppings isolate and subsequently those of compost isolate 1. Although they were not clearly separated, they were significantly different from each

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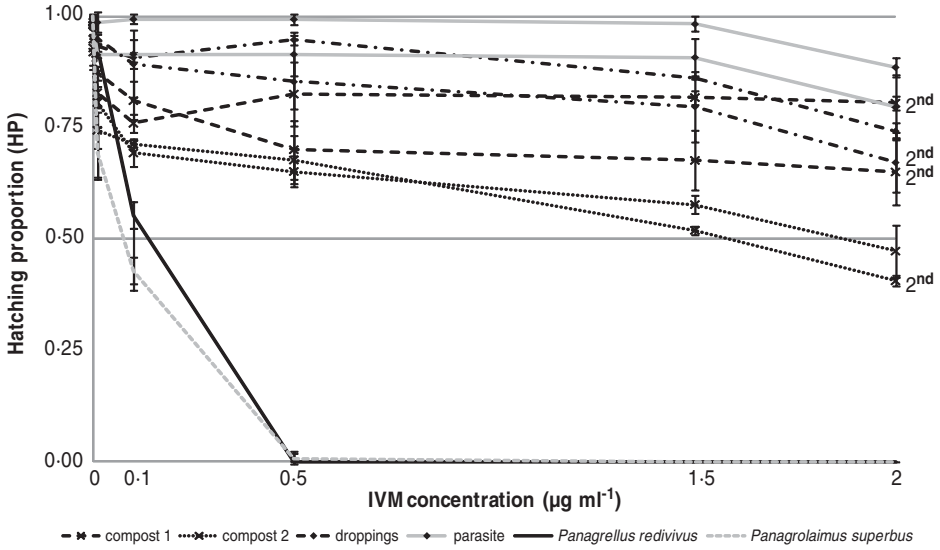

Fig. 2. The hatching proportion (HP) for all *Halicephalobus* cf. *gingivalis* isolates† and for *Panagrellus redivivus* (PS1163) and *Panagrolaimus superbus* (DF5050) at different ivermectin (IVM) concentrations (μg ml^{-1}). Data represent the mean of 3 replicates (±1 S.D.) for 2 independent and consecutive trials. Data from the second trial are indicated (2^{nd}). †Compost isolates, i.e. compost 1 (WB0708) and compost 2 (JB128); horse-associated isolates, i.e. parasite (SAN100) and droppings (WB0801).

other (P all <0·02), except for the average HPs of the droppings isolate and compost isolate 1 from 1·55 μg ml^{-1} onwards (P all >0·06). Compost isolate 2 had the overall lowest average HPs, which were significantly lower than the average HPs of the other *Halicephalobus* isolates at all IVM concentrations (Tukey post-hoc, P all <0·0001).

Recovery capacity after drug treatment

TBZ treatment showed a dose-related inhibitory effect on egg hatching for the *Halicephalobus* isolates and for *P. redivivus* and *P. superbus*. However, the eggs that hatched show no discernible delay in developmental rate at all TBZ concentrations, and almost all the hatched larvae developed into the adult stage. In contrast, for the tested IVM concentrations the inhibitory effect on egg hatching was less explicit. Only little influence on hatching was observed compared to the negative controls and the developmental rate of the eggs at the different IVM concentrations was the same. However, there was a dose-related effect on the survival of the juveniles and at higher IVM concentrations there was a noticeable delay in the development of the juveniles into the adult stage. At 0·01 μg ml^{-1} IVM concentration the adult stage of all *H. gingivalis* isolates and *P. redivivus* and *P. superbus* was attained with a delay of 24 to 48 h. At 0·5–2 μg ml^{-1} IVM, the larvae of the *Halicephalobus* isolates survived, with a reduced motility, but did not develop into the adult stage. However, this negative effect on larval development was found to be reversible for the parasite isolate, the droppings isolate and compost isolate 1. Hatched larvae incubated for 76 h on wells containing 1·5 μg ml^{-1} IVM had a survival rate of 85·4 ± 4·5% (mean ±1 S.D.) for the parasite isolate, 71·6 ± 2·5% for the droppings isolate and 75·8 ± 4·6% for compost isolate 1. Surviving larvae were subsequently transferred onto 1% plain bacteriological agar whereupon they reached the adult stage after 3 days. The recovery rate was 94·1 ± 2·3% (mean ±1 S.D.) for the parasite isolate, 77·9 ± 5·7% for the droppings isolate and 91·1 ± 5·1% for the compost isolate 1. Compost isolate 2 only had 16·5 ± 4·5% surviving larvae, which did not develop into the adult stage after transfer onto plain 1% bacteriological agar. Finally, this recovery capacity could not be tested for *P. redivivus* and *P. superbus* at 1·5 μg ml^{-1} IVM since there were no surviving larvae at higher IVM concentrations.

Influence of pre-exposure on anthelmintic tolerance

The effect of pre-exposure with anthelmintics for approximately 12 generations was very similar for all isolates and is therefore only illustrated for compost isolate 1 (Fig. 3). For both anthelmintics, the HPs of all *Halicephalobus* isolates in the control treatment were significantly lower (Tukey post-hoc, P < 0·001) upon pre-exposure as opposed to the HPs of eggs deposited by nematodes which had not been pre-exposed, i.e. 10–20% and 37–47% lower for IVM and TBZ, respectively. The dose-response upon pre-exposure was nevertheless different between TBZ and IVM.

Upon pre-exposure to TBZ, the HPs exhibited limited (compost isolate 2) to no (compost isolate 1, parasite isolate, droppings isolate) concentration dependence, which was demonstrated by the lack of significant differences (Tukey post-hoc, P all >0·05)

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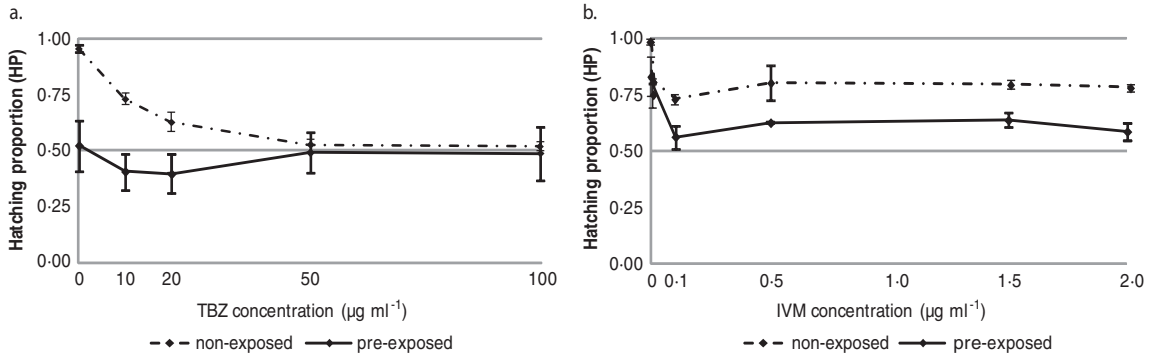

Fig. 3. The mean hatching proportion (HP) of pre-exposed eggs (dotted line) is compared with the mean HPs of non-exposed eggs (full line) for *Halicephalobus* compost isolate 1 (WB0708) at different TBZ (a) and IVM (b) concentrations (μg ml^{-1}). Data represent the mean of 3 replicates (±1 S.D.).

between the HPs at different drug concentrations, including the control.

Upon pre-exposure to IVM the HPs still showed a similar concentration dependence as opposed to non-pre-exposure. However, for most isolates (compost isolate 1, parasite isolate, droppings isolate) the HPs at all concentrations were significantly lower (P all <0·05) than in the original experiment, except the HPs of compost isolate 2 that showed no significant difference (P>0·05) at all concentrations.

DISCUSSION

Methodological considerations

Several methodological aspects may bear upon the results of dose-effect studies like the present one. We are, however, convinced that the methods used here allow an accurate assessment of the effect of the anthelmintics used in this study. The MALDT method has been proven earlier to give reliable results concerning the detection of benzimidazole resistance (Várady *et al*. 2009), and by using this agar-based method the insolubility problem of IVM is eliminated (Lacey *et al*. 1991). Moreover, former studies have revealed that the activity of IVM incorporated in agar is higher than in aqueous solutions (Várady *et al*. 2009). Secondly, our negative controls of the *Halicephalobus* isolates, *P. redivivus* and of *P. superbus* have HPs close to 100%. Therefore it can be assumed that the incubation conditions used here are adequate. Thirdly, the steep decrease of the HPs of *P. redivivus* and *P. superbus* compared to the relatively high HPs of the *Halicephalobus* isolates at higher concentrations of both TBZ and IVM confirms that effective drug treatment is detectable using this method. Finally, the highly concordant results of 2 independent experimental trials demonstrate the reproducibility of our bioassay.

Tolerance versus resistance

It is very important to distinguish between an original low, or lack of, effectiveness of an anthelmintic drug to a population and the presence of actual resistance to that same anthelmintic (Brady and Nichols, 2009). The lack of effectiveness can be seen as an existing natural tolerance to an anthelmintic even before the parasite has come in contact with the drug (Fallon *et al*. 1996; Coles, 2006), whereas acquired resistance is the conversion within a species of a low or absent tolerance towards a higher tolerance which is initiated by contact with the anthelmintic (James *et al*. 2009). The overall high hatching proportions of the *Halicephalobus* isolates at all concentrations tested in this study suggest the presence of some kind of natural tolerance to IVM and TBZ. This tolerance appears specific for the facultative parasitic genus *Halicephalobus*, since in at least some Panagrolaimidae (*P. redivivus* and *P. superbus*) no tolerance was observed. The stunning tolerance of the *Halicephalobus* isolates to TBZ and IVM is further confirmed by the considerably higher concentrations (roughly 75 times the maximum dose used for TBZ and roughly 45 times the maximum dose used for IVM) used in the present study as compared to TBZ and IVM concentrations used in another *in vitro* study using the MALDT method for testing anthelmintic resistance of the obligatory parasite *Haemonchus contortus* (Várady *et al*. 2009).

Our results reveal that the horse-associated *Halicephalobus* isolates are highly tolerant for both tested anthelmintic drugs and that the *Halicephalobus* compost isolates show an anthelmintic tolerance that is generally lower. Thus, our results also reveal a remarkable trend of increasing tolerance from fully free-living isolates towards horse-associated isolates, which is especially true for TBZ. This difference in tolerance to anthelmintics between the *Halicephalobus* isolates may be associated with earlier contact to these anti-parasitic drugs. However, none of the *Halicephalobus* strains have been found to be fully susceptible to either anthelmintic. Since there is no fully susceptible strain available, no actual acquired resistance can be proven (Brady and Nichols, 2009). In addition, the pre-exposure experiments did not show a decreased susceptibility to the

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tested anthelmintics. The average HPs of all pre-exposed isolates in the control treatment (no anthelmintic added) were considerably lower (by 10–47%) than those of non-pre-exposed nematodes, which indicates that the fitness of all isolates is negatively affected by prolonged exposure to the anthelmintics, resulting in a lower egg viability. This type of negative effect of a chemical compound on nematode egg viability has been shown earlier for e.g. tannins on gastro-intestinal parasites (Min and Hart, 2003). Further, the HPs under anthelmintic exposure exhibited only limited (compost isolate 2) to no (the other isolates) concentration dependence for TBZ and similar (compost isolate 2) or generally lower HPs (the other isolates) for IVM. This is contrary to the idea that the high tolerances observed in short exposure experiments and the differences between the horse-associated and the other isolates would be due to a true resistance. Moreover, differences in the D2D3 expansion segment of the LSU rDNA region (data not shown) shows a remarkable interpopulation variation. However, phylogenetic analyses, including GenBank (Benson *et al*. 2008) sequences, appointed our *Halicephalobus* isolates (WB0801, GenBank HQ697251 and WB0708, GenBank JF706244) within an internally unresolved *H. gingivalis* clade (data not shown). Light microscopically, they are not discernible from other *H. gingivalis* isolates (JB128 and SAN100) and should therefore be referred to as *Halicephalobus* cf. *gingivalis*. Most likely, biological differences including anthelmintic tolerance between the *Halicephalobus* isolates are associated with different evolutionary lineages or cryptic species as indicated by these D2D3 sequence differences.

Finally, since even the very high anthelmintic concentrations used in the present study appear ineffective to control the *Halicephalobus* isolates and since IVM administered to horses at the recommended dosage has a maximum plasma persistence of 4 to 62 ng ml^{-1} (Gokbulut *et al*. 2010), it is very unlikely that *in vivo* anthelmintic treatments are effective for infections with this facultative parasite. This is supported by the medical history of horses that suffered lethal infections of this nematode species in spite of regular treatment with common anthelmintics (e.g. Boswinkel *et al*. 2006, Ferguson *et al*. 2008).

Halicephalobus *as a model organism*

In research on the effects of anti-parasitic drugs, the use of free-living nematodes for *in vitro* experiments has the advantage of allowing observations on their complete life cycle, including survival and (delayed) development. In contrast, obligatory animal parasites can only be followed until the infective stage. *Caenorhabditis elegans* has been used as a model for studies on the development of anthelmintic resistance and the testing of the efficiency of new drugs (e.g. Simpkin and Coles, 1980; Sangster *et al*. 2002; James and Davey, 2009). Since its complete genome is known, *C. elegans* is especially suitable for studying the effects of anthelmintics at the gene level (Holden-Dye and Walker, 2007). However, the usefulness of *C. elegans* as a model for parasitic nematodes has been questioned (Geary and Thompson, 2001), among other reasons simply because it is not capable of parasitism in its natural environment. *Halicephalobus gingivalis* shares several of the advantages of *C. elegans* as a model organism: it is amenable to culture under laboratory conditions; it has a very short generation time (2 days at 30 °C), produces a lot of offspring, can be cultured in liquid (monoxenic as well as axenic) as well as on solid media (Fonderie *et al*. 2009) and at temperatures ranging from 4 °C to more than 40 °C (*personal observations, unpublished*). Additionally, *H. gingivalis* is capable of parasitism in its natural environment. Moreover, since complete genome sequencing is nowadays relatively fast and easy (Elsworth *et al*. 2011), the 'genetic barrier' can easily be overcome. Although the lack of a susceptible isolate is a drawback to the use of *Halicephalobus* cf. *gingivalis* as a model organism for testing new anthelmintics, the presence and ease of cultivation of susceptible close relatives such as *Panagrellus* and *Panagrolaimus* provides great potential as an experimental tool for testing the effects of various drugs on a model system encompassing a range of tolerances and including an organism with a life-history intermediate between that of obligatory parasites and of fully free-living nematodes.

ACKNOWLEDGEMENTS

We kindly thank J. Baldwin and S. Nadler for providing the JB128 and SAN100 *H. gingivalis* isolates, respectively. We further thank A. Vierstraete for sequencing and E. Dens for practical support in the realization of the experiments.

FINANCIAL SUPPORT

This research was supported by funding from the Foundation of Scientific Research, Flanders (FWO, grant number G003306N).

REFERENCES

Agresti, A. (2002). *Categorical Data Analysis* 2nd Edn. Wiley-Interscience, New Jersey, USA.

Akagami, M., Shibahara, T., Yoshiga, T., Tanak, N., Yaguchi, Y., Onuki, T., Kondo, T., Yamanaka, T., and Kubo, M. (2007). Granulomatous nephritis and meningoencephalomyelitis caused by *Halicephalobus gingivalis* in a pony gelding. *Journal of Veterinary Medical Science* **69**, 1187–1190.

Anderson, R. C., Linder, K. E. and Peregrine, A. S. (1998). *Halicephalobus gingivalis* (Stefansky, 1954) from a fatal infection in a horse in Ontario, Canada with comments on the validity of *H. gingivalis* and a review of the genus. *Parasite* **5**, 255–261.

Andrássy, I. (1984). *Klasse Nematoda (Ordnungen Monhysterida, Desmoscolecida, Araeolaimidae, Chromadorida, Rhabditida)*. Gustav Fischer Verlag, Stuttgart, Germany.

https://doi.org/10.1017/S0031182012000558 Published online by Cambridge University Press

Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J. and Wheeler, D. (2008). GenBank. *Nucleic Acids Research* **36**, D25-D30. doi: 10.1093/nar/gkm929

Blunden, A. S., Khalil, L. F. and Webbon, P. M. (1987). *Halicephalobus deletrix* infection in a horse. *Equine Veterinary Journal* **19**, 255–260.

Boswinkel, M., Neyens, I. J. S. and Sloet van Oldruitenborgh-Oosterbaan, M. M. (2006). *Halicephalobus gingivalis* infection in a 5-year old Tinker gelding. *Tijdschrift voor Diergeneeskunde* **131**, 74–80.

Brady, H. A. and Nichols, W. T. (2009). Drug resistance in equine parasites: an emerging global problem. *Journal of Equine Veterinary Science* **29**, 285–295.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71–94.

Coles, G. C. (2006). Drug resistance or drug tolerance in parasites. *Trends in Parasitology* **22**, 348.

Coles, G. C., Jackson, F., Pomroy, W. E., Prichard, R. K., von Samson-Himmelstjerna, G., Silvestre, A., Taylor, M. A. and Vercruysse, J. (2006). The detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology* **136**, 167–185.

Dunn, D. G., Gardiner, C. H., Dralle, K. R. and Thilsted, J. P. (1993). Nodular granulomatous posthitis caused by *Halicephalobus* (syn. *Micronema*) sp. in a horse. *Veterinary Pathology* **30**, 586–589.

Elsworth, B., Wasmuth, J. and Blaxter, M. (2011). NEMBASE4: The nematode transcriptome resource. *International Journal for Parasitology* **41**, 881–894.

Fallon, P. G., Tao, L. F., Ismail, M. M. and Bennett, J. L. (1996). Schistosome resistance to praziquantel: Fact or artifact? *Parasitology Today* **12**, 316–320.

Ferguson, R., van Dreumel, T., Keystone, J. S., Manning, A., Malatestinic, A., Caswell, J. L. and Peregrine, A. S. (2008). Unsuccessful treatment of a horse with mandibular graulomatous osteomyelitis due to *Halicephalobus gingivalis*. *Canadian Veterinary Journal* **49**, 1099–1103.

Fonderie, P., Willems, M., Bert, W., Houthoofd, W., Steel, H., Claeys, M. and Borgonie, G. (2009). Intestine ultrastructure of the facultative parasite *Halicephalobus gingivalis* (Nematoda: Panagrolaimidae). *Nematology* **11**, 859–868.

Geary, T. G. and Thompson, D. P. (2001). *Caenorhabditis elegans*: How good a model for veterinary parasites? *Veterinary Parasitology* **101**, 371–386.

Geraert, E., Sudhaus, W., Lenaerts, L. and Bosmans, E. (1988). *Halicephalobus laticauda* sp. n., a nematode found in a Belgian coal mine (Nematoda, Rhabditida). *Annales de la Société Royale Zoologique de Belgique* **118**, 5–12.

Gokbulut, C., Cirak, V. Y., Senlik, B., Aksit, D., Durmaz, M. and McKellar, Q. A. (2010). Comparative plasma disposition, bioavailability and efficacy of ivermectin following oral and pour-on administrations in horses. *Veterinary Parasitology* **170**, 120–126.

Holden-Dye, L. and Walker, R. J. (2007).Anthelmintic drugs In *WormBook* (ed. The *C. elegans* Research Community, WormBook) doi/10.1895/wormbook.1.7.1, http://www.wormbook.org.

Isaza, R., Schiller, C. A., Stover, J., Smith, P. J. and Greiner, E. C. (2000). *Halicephalobus gingivalis* (Nematoda) infection in a Grevy's zebra (*Equis grevyi*). *Journal of Zoo and Wildlife Medicine* **31**, 77–81.

James, C. E. and Davey, M. W. (2009). Increased expression of ABC transport proteins is associated with ivermectin resistance in the model nematode *Caenorhabditis elegans*. *International Journal for Parasitology* **39**, 213–220.

James, C. E., Hudson, A. L. and Davey, M. W. (2009). Drug resistance mechanisms in helminths: is it survival of the fittest? *Trends in Parasitology* **25**, 328–335.

Johnson, J. S., Hibler, C. P., Tillotson, K. M. and Mason, G. L. (2001). Radiculomeningomyelitis due to *Halicephalobus gingivalis* in a horse. *Veterinary Pathology* **38**, 559–561.

Kenward, M. G. and Roger, J. H. (1997). Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics* **53**, 983–997.

Lacey, E., Redwin, J. M., Gill, G. H., Demargheriti, V. M. and Waller, P. J. (1991). A larval development assay for the simultaneous detection of broad spectrum anthelmintic resistance. In *Resistance of Parasites to Anti-parasitic Drugs* (ed. Boray, J. C., Martin, P. J. and Roush, R. T.), pp. 177–184. MSD AGVET, Rahway, New Jersey, USA.

Min, B. R. and Hart, S. P. (2003). Tannins for suppression of internal parasites. *Journal of Animal Science* **81**, E102–E109.

Müller, S., Grzybowski, M., Sager, H., Bornand, V. and Brehm, W. (2008). A nodular granulomatous posthitis caused by *Halicephalobus* sp. in a horse. *Veterinary Dermatology* **19**, 44–48.

Nadler, S. A., Carreno, R. A., Adams, B. J., Kinde, H., Baldwin, J. G. and Mundo-Ocampo, M. (2003). Molecular genetics and diagnosis of soil and clinical isolates of *Halicephalobus gingivalis* (Nematoda: Cephalobina: Panagrolaimoidea), an opportunistic pathogen of horses. *International Journal for Parasitology* **33**, 1115–1125.

Ondrejka, S. L., Procop, G. W., Lai, K. K. and Prayson, R. A. (2010). Fatal parasitic meningoencephalomyelitis caused by *Halicephalobus deletrix*. A case report and review of the literature. *Archives of Pathology and Laboratory Medicine* **134**, 625–629.

Patel, M. R. (1997). Effects of ivermectin on eggs and first-stage larvae of nematodes. *Bios* **68**, 152–162.

Pearce, S. G., Bouré, L. P., Taylor, J. A. and Peregrine, A. S. (2001). Treatment of a granuloma caused by *Halicephalobus gingivalis* in a horse. *Journal of the American Veterinary Medical Association* **219**, 1735–1738.

Rames, D. S., Miller, D. K., Barthel, R., Craig, T. M., Dziezyc, J., Helman, R. G. and Mealey, R. (1995). Ocular *Halicephalobus* (syn. *Micronema*) *deletrix* in a horse. *Veterinary Pathology* **32**, 540–542.

Sangster, N. C., Batterham, P., Chapman, H. D., Duraisingh, M., Le Jambre, L., Shirley, M., Upcroft, J. and Upcroft, P. (2002). Resistance to antiparasitic drugs: the role of molecular diagnosis. *International Journal for Parasitology* **32**, 637–653.

Shokoohi, E., Abolafia, J. and Zad, J. (2007). Nematodes of the order Rhabditida from Tehran province, Iran. The family Panagrolaimidae with description of *Halicephalobus persicus* sp. n. and a key to species of *Halicephalobus* Timm, 1956. *Nematology* **9**, 693–711.

Simpkin, K. G. and Coles, G. C. (1980). The use of *Caenorhabditis elegans* for anthelmintic screening. *Journal of Chemical Technology and BioTechnology* **31**, 66–69.

Spalding, M. G., Greiner, E. C. and Green, S. L. (1990). *Halicephalobus* (*Micronema*) *deletrix* infection in two half-sibling foals. *Journal of the American Veterinary Medical Association* **196**, 1127–1129.

Steel, H., de la Peña, E., Fonderie, P., Willekens, K., Borgonie, G. and Bert, W. (2010). Nematode succession during composting and the potential of the nematode community as an indicator of compost maturity. *Pedobiologia* **53**, 181–190.

Stefansky, W. (1954). *Rhabditis gingivalis* sp. n. parasite trouvé dans un granulome de la gencive chez un cheval. *Acta Parasitologica* **1**, 329–334.

Taylor, M. A., Hunt, K. R. and Goodyear, K. L. (2002). Anthelmintic resistance detection methods. *Veterinary Parasitology* **103**, 183–194.

Ura, K., Kai, T., Sakata, S., Iguchi, T. and Arizono, K. (2002). Aquatic acute toxicity testing using the nematode *Caenorhabditis elegans*. *Journal of Health Science* **48**, 583–586.

Várady, M., Čorba, J., Letková, V. and Kováč, G. (2009). Comparison of two versions of larval development test to detect anthelmintic resistance in *Haemonchus contortus*. *Veterinary Parasitology* **160**, 267–271.