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Standardization of the egg hatch test for the detection of benzimidazole resistance in parasitic nematodes

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Abstract The ability to reliably detect anthelmintic resistance is a crucial part of resistance management. If data between countries are to be compared, the same test should give the same results in each laboratory. As the egg hatch test for benzimidazole resistance is used for both research and surveys, the ability of different laboratories to obtain similar results was studied through testing of known isolates of cyathostomins, *Haemonchus contortus*, *Ostertagia ostertagi*, and *Cooperia oncophora* in programs supported by the EU (Cost B16 and FP6-PARASOL).

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Initial results showed difficulties in obtaining reproducible and similar data within and between laboratories. A series of ring tests, i.e., simultaneous and coordinated rounds of testing of nematode isolates in different laboratories was subsequently performed. By adopting identical protocols, especially the use of deionized water and making dilutions of thiabendazole in dimethyl sulfoxide in the final ring test, laboratories correctly identified both susceptible and resistant isolates. The protocols for the test and preparation of solutions of thiabendazole are described.

Introduction

The incubation of nematode eggs in solutions of thiabendazole (TBZ) to detect benzimidazole (BZ) resistance was first described as an egg hatch test (EHT) by Le Jambre (1976) and as an egg embryonation test by Coles and Simpkin (1977). More than 50 reports have been published using the EHT for the detection of either BZ or levamisole resistance in trichostrongylid nematodes of sheep and goats (e.g., Boersema et al. 1987; Dobson et al. 1986; Ghisi et al. 2006; Hoekstra et al. 1997; Hunt and Taylor 1989; Johansen and Waller 1989; Martin et al. 1989; Várady et al. 2006), cattle (e.g., Borgsteede et al. 1992; Jackson et al. 1987), horses (e.g., Ullrich et al. 1988; Ihler and Bjorn 1996; von Samson-Himmelstjerna et al. 2002b; Várady et al. 2000; Whitlock et al. 1980; Wirtherle et al. 2004), pigs (e.g., Várady et al. 1996), and humans (e.g., DeClerq et al. 1997; Albonico et al. 2005). However, to date no standardized EHT protocol for which data on the repeat-

ability of data generated within laboratories and reproducibility of results between laboratories has been published. Initial results obtained within a COST B16 action funded project indicated unexpected high levels of variation in EHT data generated by a group of laboratories. This prompted a large scale ring testing activity aiming at the development of a standardized EHT protocol for major nematode parasites of livestock. This process was started using an EHT protocol recommended as method for the detection of BZ resistance with cut off of >0.1 μg TBZ per ml for 50% egg hatch inhibition (EC$_{50}$; Coles et al. 1992). Issues envisaged as possibly contributing to data variation were the method of drug preparation, the sequence of sample preparation setup, or the storage of eggs post collection (Hunt and Taylor 1989). To try to eliminate protocol variations contributing to data inconsistencies in the EHT, susceptible and resistant isolates of gastrointestinal nematodes were tested in 18 laboratories in 11 European countries, and the results of these tests together with the improvements produced are reported here.

Materials and methods

From February 2003 to March 2006, EHT ring tests were performed in between five and 13 of the total of 18 participating laboratories (Table 1) and the results were discussed at meetings funded by the European Union (COST action B16). Fecal samples with eggs from either different *Haemonchus contortus* isolates or a BZ-resistant cyathostomin isolate were distributed under anaerobic

Table 1 List of participating laboratories and corresponding abbreviation

Investigator	Institution	Country	Abbreviation
Christian Bauer	Justus Liebig University Giessen	Germany	Ger3
Veli Çirak	Uludag University Bursa	Turkey	Tr
Gerald Coles	University of Bristol	UK	UK2
Pierre Dorny	Institute of Tropical Medicine, Antwerp	Belgium	B
Christian Epe	University of Veterinary Medicine Hannover	Germany	Ger1
Achim Harder	BayerHealthcare AG	Germany	Ger4
Johan Höglund	Swedish University of Agricultural Sciences	Sweden	Sw
Frank Jackson	Moredun Research Institute	UK	UK1
Ronald Kaminsky	Novartis Animal Health Inc.	Switzerland	Sz
Maurice McKoy	Disease Surveillance & Investigation Branch	Ireland	Ir
Dominique Kerboeuf	INRA Tours	France	F
Elias Papadopoulos	Aristotle University of Thessaloniki	Greece	Gr
Janez Posedi	University of Ljubljana	Slovenia	SL
Georg v. Samson-H.	University of Veterinary Medicine Hannover	Germany	Ger2
Marián Várady	Parasitological Institute of SAS	Slovakia	Sk
Jozef Vercruysse	University of Ghent	Belgium	B
Nicole Wirtherle	Free University Berlin	Germany	Ger5
Rowan Wood	VLA Aberystwyth	UK	UK3

conditions by courier either from the Moredun Research Institute, UK or the Institute for Parasitology, Hannover University of Veterinary Medicine, Germany to the other laboratories. Prior to ring testing, the nematode isolates used within this study had been repeatedly in vivo or in vitro tested in at least one anthelmintic resistance assay by the respective institutes initially providing the isolates (details not shown). Based on these data, the isolates were categorized as either BZ susceptible or resistant. Due to technical reasons, the time point of fecal sampling with respect to the phase of patency differed between the various ring tests. The specificity of all isolates was checked following coproculture microscopically or by molecular analysis (von Samson-Himmelstjerna et al. 2002a) at least on the genus level.

Under the EU-funded PARASOL (FOOD-CT-2005-022851) project, eggs of a susceptible isolate *Ostertagia ostertagi* and a susceptible isolate of *Cooperia oncophora*, both originating from the Central Veterinary Laboratory at Weybridge, UK, were also tested with the EHT during 2006 and 2008. The feces were collected rectally from infected calves and circulated under anaerobic conditions (Hunt and Taylor 1989) to the participating laboratories.

Preparation and analysis of TBZ test solutions

Two protocols to prepare solutions from the same batch of TBZ (Sigma) were compared in the different laboratories. The major difference between the two protocols was the use of either water/HCl or dimethyl sulfoxide (DMSO) for dissolving the drug. Thus, for preparation of stock solution A 50-mg TBZ were either dissolved in 5 ml water by adding 300 µl 1 N HCl or in 5 ml of DMSO. Subsequent dilutions were made in water or in DMSO. Stock solution B was obtained by adding 1 ml of stock solution A to 9 ml water or DMSO. Between 100 and 1,000 µl of stock solution B were added up to 10 ml of water or DMSO. Of these working solutions, 10 µl was added to 1,990 µl egg suspension to obtain final working concentrations between 0.01 and 0.5 µg TBZ per ml (Table 2), thus always containing the same DMSO concentration of 0.5%. Following the third round of tests, the ready prepared solutions B of TBZ were always supplied by the Moredun Research Institute. In this institute, the actual TBZ concentration in the supernatant of the different working solutions prepared according to the two protocols was analyzed over a time course of 96 days. At each time point, solutions were mixed thoroughly and 200 µl was diluted with 800 µl of methanol in a vial for high pressure liquid chromatography (HPLC; see below in the section "Chemical analysis of TBZ solutions"). All supernatants were identically diluted and peak areas were compared to a standard curve by linear regression.

Table 2 Preparation of solutions of thiabendazole

Volume B (µl)	Volume DMSO (ml)	TBZ concentration in working solution (µg/ml)	Final TBZ concentration in well (µg/ml)
20	9.98	2	0.01
50	9.95	5	0.025
100	9.9	10	0.05
200	9.8	20	0.1
400	9.6	40	0.2
600	9.4	60	0.3
1,000	9	100	0.5

Steps: (1) Weigh out 50 mg thiabendazole (TBZ) and dissolve in 5 ml DMSO—solution A (10 mg TBZ per ml). (2) Add 1 ml solution A to 9 ml DMSO – solution B (1 mg TBZ per ml). (3) Make up working solutions for adding to plates as shown in the table

EHT procedure

The test procedure followed that recommended by the WAAVP (Coles et al. 1992). Fresh fecal samples containing at least 100 eggs per gram (epg) from sheep, cattle, or horses were homogenized in tap water and used to completely fill 50 ml tubes or 100 ml bottles rendering the samples anaerobic. Samples were despatched by courier and eggs were extracted on arrival (usually within 48 h post sampling but not later than 6 days post sampling) at the different laboratories. Eggs were extracted by sieving, centrifugation, and flotation in saturated sodium chloride as described in Coles et al. (1992). Eggs were washed and suspended in deionized or tap water at a concentration of 100 per ml and eggs were inspected microscopically to record if embryonation had not begun. Each sample was at least tested in duplicate and at least two negative control samples were used (sample but no drug).

Egg suspension (1.99 ml) was placed in wells (24-well tissue culture test plates, TPP, Switzerland) and then 10 µl of TBZ solutions added. Plates were sealed to prevent drying out and incubated for 48 h at 25°C before being stopped by addition of one drop of Gram's iodine (1 g of iodine and 2 g of potassium iodide in 100 ml distilled water) to each well. At least 100 eggs and larvae were counted from each well. To fit the achieved dose–response data by non-linear regression, a four-parameter logistic equation with a variable slope was chosen, using the computer program GraphPad Prism 5.0 for Windows. All analyses were performed after transforming the drug concentrations into its logarithm ($X = \log X$) and defining the bottom value 0. The EC_{50} and, except for the initial ring test rounds, also the EC_{95} values, the 95% confidence intervals, and R^2 values were calculated.

Chemical analysis of TBZ solutions

Modifications to this protocol were made during the ring testing with specific reference to the preparation of TBZ working solutions, the sequence of some of the EHT steps, and the quality of the water used for suspending the eggs. The latter was studied by chemical analysis using 1 ml of the supernatant from egg hatch tests conducted with water from different sources and degree of ionization. Without addition of any solvents, the samples were centrifuged at 1,500 rpm for 5 min to pellet any eggs or debris. Following centrifugation, 0.5 ml of the supernatant as well as the working solution samples (see above) was used for HPLC analyses using the method described by Sanchez et al. (2002) with modification. Briefly, HPLC analyses were carried out on an integrated chromatographic system with a manually controlled vacuum solvent degasser unit (Thermo Separation Products). A C18 nucleoside column (150× 3 mm) was employed with the solvent profile for HPLC analyses consisting of 55% NH_4 acetate (1 g in 500 ml of H_2O) and 45% acetonitrile run in the mobile phase at 1 ml per minute. An injection volume of 25 µl was used with a total run time of 3 min and fluorescent detector set at emission wavelength of 340 nm. The system functioned under the control of a customized software package (TSP Chrom Quest Version 2.51).

Results

The first ring test performed within this standardization process was undertaken by six laboratories (Fig. 1) using eggs from a BZ-resistant cyathostomin isolate (Drogemuller et al. 2004). This nematode isolate had previously been shown to be BZ-resistant by failure of treatment with fecal
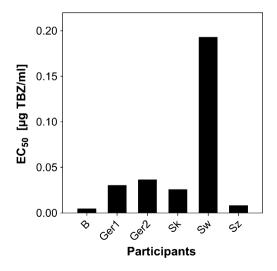
egg count reduction (FECR) less than 50% and an EHT EC_{50} above 0.2 µg per ml TBZ. Surprisingly, only one laboratory diagnosed it as resistant with $EC_{50}>0.1$ µg per ml TBZ (Fig. 1). At this stage, each laboratory prepared the solutions of TBZ independently using the solid TBZ provided which was dissolved either in water/HCl or DMSO. The same equine isolate was tested in the second round of the ring test. This time, every laboratory prepared solutions of TBZ following both methods, dissolving in DMSO and dissolving in water/HCl. Again, using water/HCl dissolved TBZ one laboratory (Ger1) diagnosed resistance (Fig. 2). However, one consistent finding was that all tests performed with TBZ dissolved in DMSO gave higher EC_{50} than those with TBZ dissolved in water/HCl. Therefore, the TBZ concentrations over time were determined using HPLC. Initially, in both cases the concentration of TBZ measured in working solutions prepared using double distilled water to dilute the original water/HCl or DMSO stock solutions declined by as much as 60% over 48 h following their preparation. Importantly, when further examined until 96 days post preparation, the measured TBZ concentrations remained relatively stable only if the drug was dissolved in DMSO and continuously decreased when dissolved in water/HCl (J. Small, personal communication, data not shown).

Further studies were undertaken using susceptible and resistant isolates of *H. contortus* with TBZ dissolved in DMSO prepared at the Moredun Institute. A total of four ring tests using different *H. contortus* isolates were run. The EC_{50} value (see Figs. 3 and 4) as well as the corresponding 95% confidence interval results (data not shown) obtained by the participating laboratories during the first two rounds of ring tests was highly variable. For these ring test rounds, namely to illustrate the degree of variability and the process
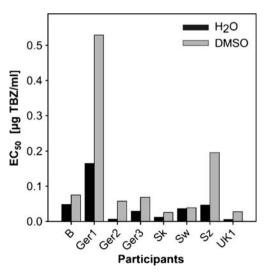

Fig. 1 Calculated EC_{50} values of a German BZ-resistant cyathosto-min isolate tested in six different laboratories in February 2003

Fig. 2 Calculated EC_{50} values of a German BZ-resistant cyathosto-min isolate tested in eight different laboratories in November 2003 using either dimethyl sulfoxide (DMSO) or distilled water/HCl (H_2O) to set up the thiabendazole concentrations
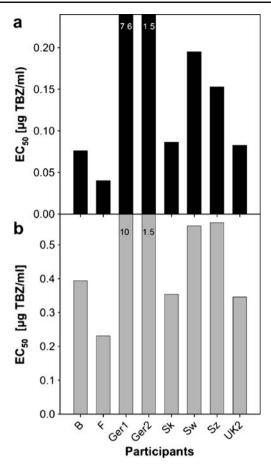
Fig. 3 Calculated EC_{50}-values of a German BZ-susceptible (**a**, MHco9) and a South African BZ-resistant (**b**, MHco4) *H. contortus* isolate tested in eight different laboratories in May 2004. For EC_{50} values exceeding the *y*-axis scale, the data are given in the respective columns

Fig. 4 Calculated EC_{50} values of a Dutch BZ-susceptible (**a**, MHco3) and a resistant (**b**, MHco5) *H. contortus* isolate tested in 11 different laboratories in February 2005

of harmonization accomplished during test standardization, only the calculated EC_{50} values are depicted. In the initial ring tests, EC_{50} values were above the 0.1-µg TBZ per ml threshold for the susceptible isolate in four out of eight laboratories (Fig. 3a). However, at least most laboratories obtained higher EC_{50} values for the resistant isolate (Fig. 3b). Two German laboratories (Ger1 and 2), both from the Institute for Parasitology, University of Veterinary Medicine Hannover, found no inhibition of hatching in either isolate. Both laboratories had been using tap water to suspend the nematode eggs but most other laboratories had been using distilled/deionized water. To resolve possible factors of water quality as contributors to inter-lab variation, batches of tap water from different geographical origin were analyzed at the Moredun Institute. Furthermore, the TBZ concentrations in the EHT samples over time were investigated. The availability of TBZ in the sample supernatants from EHTs declined over the 48 period of the test. The actual TBZ concentrations (microgram per milliliter) measured in the egg hatch test supernatants at the end of the
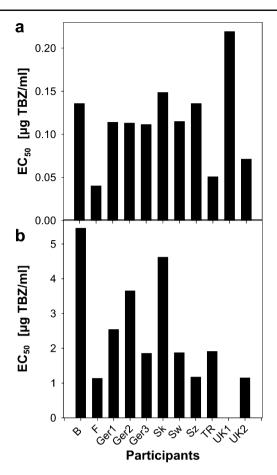
test period were on average about 40% of those seen in freshly prepared solutions. Table 3 shows the calculated EC_{50} values from TBZ susceptible (MHco3, syn. ISE) and TBZ resistant (MHco5, syn. IRE) isolates of *H. contortus* conducted in various tap and laboratory waters. Table 3 also shows the calcium, phosphorus, magnesium, and pH values for these same waters, demonstrating more than tenfold differences in calcium and magnesium concentrations. The tests performed with tap water from Clipston, showing the highest magnesium concentrations, resulted in the second highest EC_{50} values. The highest values were obtained by using the calcium-rich Hannover tap water. The EC_{50} values for tests conducted in Hannover water using TBZ-susceptible and -resistant *Haemonchus* isolates were more than 12 and five times higher, respectively, than those conducted using deionized water. Supernatants collected from EHTs run using Hannover water had as expected much lower TBZ concentrations averaging 13.8% of the expected TBZ concentration compared to more than 53.4% for deionized water.

When all laboratories used deionized water, the degree of variation was substantially reduced. However, other than

Table 3 Concentrations of calcium (Ca), magnesium (Mg), phosphorus (P) (millimole per liter), and pH together with corresponding calculated EC_{50} values from egg hatch tests conducted using *H.* *contortus* eggs from a susceptible (MHco3) and a BZ-resistant (MHco5) isolate in either tap waters from three English, two Scottish, and one German sources or double distilled or deionized waters

Country	Sample source City/town	Sample	Concentration (mmol/l)			pH	MHco3 (ISE) susceptible	MHco5 (IRE) resistant
			Ca	Mg	P			
England	Nottingham	Tap water	0.73	0.03	0.04	7.7	0.049	0.774
	Clipston	Tap water	1.20	0.84	0.01	8.1	0.090	0.849
	Newcastle	Tap water	0.73	0.03	0.04	7.7	0.055	0.773
Scotland	Onich	Tap water	0.12	<0.01	0.02	8.3	0.052	0.752
	Torry	Tap water	0.29	0.07	0.05	7.9	0.050	0.675
Germany	Hannover	Tap water	2.09	0.17	0.02	8.3	0.559	3.410
Scotland	Edinburgh	Double distilled	0.00	0.06	<0.01	7.5	0.046	0.668
	Edinburgh	Deionized	0.00	0.05	0.04	7.6	0.044	0.688

expected for the susceptible isolates (Fig. 4a), most of the EC_{50} values still ranged above the 0.1-μg TBZ per ml threshold, whereas as expected all laboratories obtained above threshold values for the resistant isolate (Fig. 4b). Therefore, it was agreed to avoid possible continued larval development by examination of all samples shortly post addition of iodine for further ring tests.

Finally, five susceptible (Fig. 5) and three (Fig. 6) resistant *H. contortus* isolates were compared (Table 4). Only data from experiments with at least 70% hatch rate in the control and R^2 values >0.8 were included. Usually a hatch rate >85% was recorded, and accordingly, only few tests had to be withdrawn from the analysis. Only one experiment (Ger5, MHco3) during the ring test performed in July 2005 was excluded since the R^2 criteria was not met. In all except one case, the susceptible isolates were identified as susceptible (Fig. 5). In the Sk laboratory, an EC_{50} value just over 0.1 μg TBZ per ml was obtained for the MHco8 isolate otherwise considered to be susceptible.
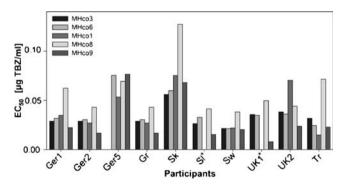
Here, the upper limit of the EC_{50} value 95% confidence interval was 0.135, whereas otherwise it was always <0.1 μg/ml. The corresponding EC_{95} value for the MHco8 isolate found in Sk was with 1.82 μg TBZ per ml extremely high but also all other laboratories also found EC_{95} values >0.1 μg TBZ per ml. For the other four susceptible isolates tested between laboratories, a considerable degree of variability concerning the EC_{95} values was recorded. These ranged between 0.047 and 0.353 μg TBZ per ml with seven out of a total of 37 individual experiments showing EC_{95} values >0.1 μg TBZ per ml.

Numbers of eggs in the resistant samples were rather low limiting the numbers of tests or repeats, but in all analyzed experiments resistance was correctly identified (Fig. 6). The mean EC_{50} values for the resistant isolates corresponded with the known phenotype (Table 4). The testing of three
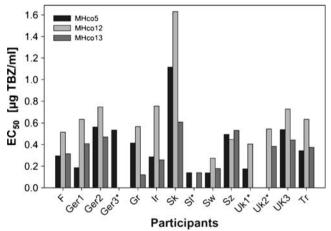

Fig. 5 Calculated EC_{50} values of five BZ-susceptible *H. contortus* isolates tested in July 2005 by ten different laboratories. The MHco1, MHco3, MHco6, MHco8, and MHco9 isolates originated from Slovakia, Netherlands, Switzerland, Kenya, and Germany, respectively. Laboratories marked with *asterisk* had cases where the data were not analyzed due to insufficient hatch rates in the controls or R^2 values >0.8

Fig. 6 Calculated EC_{50} values of three BZ-resistant *H. contortus* isolates tested in March 2006 by 14 different laboratories. The MHco5 was previously named *H. contortus* IRE and originated from The Netherlands. The MHco12 and MHco13 are Swiss isolates coming from places named Cournillens and Courtion, respectively. Laboratories marked with *asterisk* had cases where the data were not analyzed due to insufficient hatch rates in the controls or R^2 values >0.8

Table 4 Mean effective concentration given in micrograms TBZ per milliliter required for 50% (EC$_{50}$) and 95% (EC$_{95}$) hatch inhibition, corresponding standard deviation (SD), mean control hatch rates, corresponding SD for ring test used in July 2005 benzimidazole-susceptible (MHco1, 3, 6, 8, and 9) and in March 2006 resistant (MHco5, 12, and 13) isolates of *H. contortus*

Isolate	Mean EC$_{50}$/SD	Mean EC$_{95}$/SD	Mean control hatch %/SD
MHco1	0.039/0.022	0.111/0.100	69.7/29.8
MHco3	0.033/0.001	0.071/0.029	84.0/12.6
MHco6	0.038/0.017	0.084/0.042	84.2/12.9
MHco8	0.059/0.026	0.241/0.102	89.6/6.7
MHco9	0.031/0.022	0.092/0.053	87.1/4.9
MHco5	0.401/0.265	1.532/0.896	89.4/14.5
MHco12	0.657/0.339	1.864/0.842	94.4/3.3
MHco13	0.352/0.155	1.355/0.956	87.8/14.4

BZ-resistant *H. contortus* isolates was characterized by overall high R^2 values of >0.9, except for one experiment (i.e., MHco13 where UK2 data showed an R^2 value of 0.81). The calculated EC$_{50}$ values of all three isolates were always >0.1 μg TBZ per ml in all laboratories. Also, the lower limit of the EC$_{50}$ value 95% confidence interval was always >0.1 except for one experiment with the MHco5 isolate (0.056 μg/ml in Ger1). The EC$_{95}$ values for the three BZ-resistant isolates were always >0.35 μg TBZ per ml and the highest value was 9.81 μg/ml (Sk for MHco12). The corresponding EC$_{95}$ value 95% confidence intervals often were very wide, sometimes over three orders of magnitude and also differed between labs in the same range (data not shown).

Between 2006 and 2008, five and four rounds of ring tests each using eggs from susceptible *O. ostertagi* and *C. oncophora* isolates, respectively, were performed, following the protocol established previously using *H. contortus* isolates. The following laboratories participated at this ring testing initiative within the framework of the EU PARASOL-project: B, Ger2, Sk, Sw, and UK2 (for abbreviations see Table 1). A total of 43 individual EHT were performed during this ring testing, of which 34 EHT were included into the analysis while other tests were excluded due to technical problems (e.g., errors during setup of TBZ concentration) or insufficient hatch rates (<70%) in the control samples. Throughout all testing, both isolates were shown to be very susceptible to TBZ with a dose of 0.1 μg TBZ per ml inhibiting hatching in virtually all eggs and in all experiments with EC$_{95}$ values always below this concentration (Table 5). The EHT provided highly reproducible results and

the R^2 value for in individual experiments was always >0.88 in all laboratories. The upper limit of the EC$_{50}$ value 95% confidence interval was always below 0.1 μg TBZ per ml (data not shown).

Discussion

The standardization of tests for the diagnosis of resistance against chemotherapeutic agents in biological infectious organisms is a key requirement for the development of an applicable diagnostic tool allowing directly comparable data to be produced in different laboratories. If such standardized protocols are available, they can be employed in routine diagnosis and external quality assurance testing. More than a decade ago, the WHO started a program of quality control and proficiency testing on antimicrobial susceptibility assays, and a series of proficiency testing challenges involving 130 laboratories were performed (Tenover et al. 2001). All laboratories participating in this study used the standard methods developed by the National Committee for Clinical Laboratory Standards. Equivalent methods and standards have so far not been established for in vitro testing of anthelmintic resistance. In the face of increasing anthelmintic resistance, such standardized methods are required, particularly in small ruminant nematodes (Kaplan 2004) but also in bovine nematodes (Waghorn et al. 2006), and to investigate the possible development of resistance in anthelmintic treatment campaigns sponsored by the WHO to control human intestinal worms (Bull World Health Organ vol.82 no.8 Geneva Aug. 2004).

Table 5 Mean effective concentration given in micrograms TBZ per milliliter required for 50% hatch inhibition (EC$_{50}$) for benzimidazole-susceptible *O. ostertagi* and *C. oncophora* isolate tested by five laboratories, overall mean EC$_{50}$ and EC$_{95}$, and corresponding standard deviation (SD)
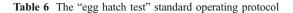
Isolate	Mean EC$_{50}$/SD	Mean EC$_{95}$/SD	Mean control hatch %/SD
C. oncophora	0.046/0.006	0.075/0.005	91.9/1.924
O. ostertagi	0.028/0.006	0.052/0.011	93.6/4.756

The usefulness of the EHT for field testing is supported by data demonstrating agreement between results obtained by this in vitro method with those of the most frequently applied in vivo test. The EC_{50} results obtained with the EHT have been compared with the fecal egg count reduction test (FECRT; Craven et al. 1999; Johansen and Waller 1989; von Samson-Himmelstjerna et al. 2002b), the larval development test (Craven et al. 1999; Várady and Čorba 1999; Königová et al. 2003), and the tubulin-binding assay (Johansen and Waller 1989). In field trials, the majority of FECRTs positive for benzimidazole resistance were also positive using the EHT (Várady et al. 2006; Maingi et al. 1998; Wirtherle et al. 2004); however, the results did not correlate well in all studies published (Craven et al. 1999; Maingi et al. 1998; Königová et al. 2003). Differences between tests may in part be accounted for by the developmental stage of nematodes used in the tests (adult worms in the FECRT and eggs in the EHT) and the host effects in the FECRT, including pharmacokinetics of the anthelmintic used and possibly immunological involvement in worm control.

Failure to correctly identify benzimidazole resistance in essentially all laboratories in the first rounds of tests stresses the need to have a validated protocol which is exactly followed in all laboratories running the EHT. Small inter-laboratory variations in test protocols such as using tap water and dissolving TBZ in DMSO and then diluting in water are not acceptable. The type of water used in the test clearly has the capacity to influence its outcome. Chemical analyses of the different water samples showed considerable differences in calcium and magnesium concentrations as well as in pH values. For example, water with high calcium concentrations abolished the effect of TBZ. Furthermore, tests with water showing the highest magnesium concentration resulted in the second highest EC_{50} values, indicating that the concentration of this other divalent ion might also be of relevance for the TBZ effect in the EHT. Since some of the earliest descriptions of the test (Le Jambre 1976; Coles and Simpkin 1977; Cawthorne and Whitehead 1983) do not specify which water should be used for the test, it is possible that some of the previously reported variance may in fact be attributable to the use of tap water in the assay.

Based on the experience with the present ring testing, a protocol for running the EHT is suggested in Table 6 and a simple protocol for preparing TBZ solutions in Table 2. It is recommended that these protocols must be followed exactly in all laboratories running the EHT. We also recommend the addition of DMSO-dissolved TBZ to water before the addition of eggs to avoid eggs encountering localized high concentration of solvent. In cases where resistance is detected, the genus/species present can be identified by for example performing genus specific real-time PCR (von Samson-Himmelstjerna et al. 2002a) on DNA extracted from the developed larvae.

Table 6 The "egg hatch test" standard operating protocol

Step	Procedure
1	Using a 24-well plate, add 990 µl deionized water to each well
2	Add 10 µl of thiabendazole (TBZ) working solution (see Table 6) to experimental wells (i.e., except control row) and mix the sample by repeated pipetting. Use each concentration at least in duplicate
3	Nematode eggs can be purified by different procedures, e.g., saturated sodium chloride or magnesium sulfate flotation, sugar or glycerol gradient centrifugation. Prior to use in the EHT, the eggs should be washed in deionized water. Add 1 ml of a suspension of freshly isolated (<3 h old) clean eggs to provide between 150 and 200 eggs per well. Alternatively, eggs isolated anaerobically stored samples can be used, but need to be processed immediately after opening. As eggs precipitate quickly, the eggs should be taken from a stirred suspension
4	Seal the plates with tape to prevent evaporation and incubate for 48 h at 25°C.
5	Stop the hatching by the addition of one drop of Gram's iodine. Make sure that iodine is fresh and thus still active to avoid unintended continued development until counting of stages
6	Preferably count total number of eggs and larvae per well. Alternatively, a total of 100 stages per well can be analyzed
7	Calculate the effective concentration required for 50% hatch inhibition (EC_{50}) values and the 95% confidence limits using either the GraphPad Prism software or equivalent procedures. An EC_{50} of >0.1 µg TBZ per ml is considered as indicative of BZ resistance (Coles et al. 1992, 2006). Record percentage of eggs hatching in 0.2 and 0.3 µg TBZ per ml wells. Hatching in these concentrations with an $EC_{50} \leq 0.1$ µg TBZ per ml may indicate resistance in a less frequently represented nematode

Problems: Occasionally eggs do not hatch in control wells. The reasons are not certain. It is recommended to accept only those tests where the hatch rate in both control wells was at least 70%

Testing of various susceptible and BZ-resistant *H. contortus* isolates using a standardized EHT protocol demonstrated that by applying the EC_{50} value threshold of 0.1 µg TBZ per ml as recommended by Coles et al. (1992, 2006) all the isolates were correctly identified as susceptible or resistant in all except one out of 77 experiments. In this context, it is important to note that all three resistant isolates tested showed high EC_{50} values relative to the susceptible isolates. Accordingly, a resistance factor of at least five results from the comparison of the mean EC_{50} value of the least susceptible (0.059 for MHco8) with that of the least resistant isolate (0.352 for MHco13).

When the protocols were followed exactly, all five participating laboratories correctly identified susceptibility in *O. ostertagi* and *C. oncophora* isolates. Unfortunately, no BZ-resistant cattle nematode isolates were available, and therefore, it remains to be seen which EC_{50} resistance factor has to be expected for these species. It was found that the results obtained in five different laboratories were highly similar and reproducible. However, it is currently unclear if

the same resistance threshold applied for *H. contortus* isolates can also be applied for eggs from cattle nematodes. Nevertheless, the level of reproducibility found herein indicates that the EHT will be very useful for looking for the development of BZ resistance in bovine nematodes which has already become a widespread problem in New Zealand (Jackson et al. 2006).

It was suggested that EC_{99} values will be more sensitive for the detection of resistance than EC_{50} values and may permit a single discriminating dose to be used unless the degree of resistance is required in which case a range of concentrations will be required (Coles et al. 2006; Várady et al. 2007). Here, the calculation of EC_{95} values was performed from the final two ring tests using *H. contortus*-susceptible and -resistant isolates as well as those from cattle nematodes in order to further characterize the EHT phenotype of these isolates and to test if this value would be suitable as the discriminating dose. Similar mean EC_{95} values as found herein were recently observed by Várady et al. (2006) for more than 15 trichostrongyle field isolates found to be BZ susceptible due to 100% FECR (Várady et al., unpublished data). Since in the present study all isolates considered to be resistant were found to have an EC_{95} value of >0.1 µg TBZ per ml, using this cut off would have resulted in no false negative testing. Interestingly, approximately one third of the experiments using isolates considered to be susceptible also gave EC_{95} value of >0.1 µg TBZ per ml, implying that a considerable proportion of false positive testing would result and using the EC_{99} value as cut off would have increased this even considerably. It is noteworthy that the EC_{95} data from all laboratories would suggest that the MHco8 isolate to be resistant, which would correspond with data from earlier analyses. In these experiments, this isolate was actually considered to be BZ-resistant using a larval development test with an EC_{99} value of 0.16 µg TBZ per ml, which is eight times higher than the discriminating dose for resistance of 0.02 µg TBZ per ml (Várady et al. 2006, 2007). This indicates that the EC_{95} value may be the more sensitive means for the early detection of BZ resistance. However, the considerable variation observed for the mean EC_{95} values expressed in large standard deviations for the isolates examined in this study does not allow an EC_{95} threshold for BZ resistance to be proposed at this stage.

For *H. contortus* ring tests, the decision in which minimum control hatch rate and test R^2 value level should be employed for the inclusion of test data was made empirically. The EC_{50} data from all tests with 60–90% control hatch rate and R^2 between 0.8 and 0.9 did ($n=25$) did not differ significantly (Mann–Whitney rank sum test: $p=0.69$) from those with hatch rates >90% or R^2 values >0.9 ($n=21$). Furthermore, the 95% confidence interval ranges for EC_{50} values of both sets of data completely overlapped. Thus, it was considered suitable to accept these levels as inclusion criteria.

Testing of nematode eggs isolated from horse fecal field samples with EHT is complicated through the high parasite species richness among cyathostomins. This aspect was not addressed in the current study since only one isolate was used. Accordingly, the reproducibility and repeatability of the EHT for cyathostomins needs further investigations.

The EHT protocol resulting from the current ring testing should in future be employed for analyzing the in vitro phenotype of isolates from additional parasitic nematode species for example from canine and human hosts. Similar standardization for other in vitro tests is currently performed in the EU framework program 6 PARASOL project (http://www.parasol-project.org/).

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