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Standardization of the larval migration inhibition test for the detection of resistance to ivermectin in gastro intestinal nematodes of ruminants

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ABSTRACT

Resistance to anthelmintics is an increasing problem in sheep, goat and cattle industries worldwide. For parasite management on farms reliable methods for the detection of resistance are required and it is important that tests give comparable, reproducible and reliable results in different laboratories. The ability of the larval migration inhibition test (LMIT), to detect ivermectin resistance in cattle and sheep nematodes has been evaluated through ring testing in up to six different laboratories in Europe, supported by an EU sixth Framework Project (PARASOL). Third stage larvae of *Ostertagia ostertagi*, *Cooperia oncophora*, and *Haemonchus contortus* with a known resistance status were obtained from faecal cultures of experimentally infected calves and sheep. Following a series of ring tests using identical protocols, reproducible results were obtained within and between participating laboratories. In all tests dose–response curves with R^2 values >0.90 were obtained by all laboratories. Resistance ratios of 8.3 and 8.4 were found when susceptible and IVM-resistant isolates of *C. oncophora* and *H. contortus* were compared and differences in the EC_{50} values were highly significant ($p < 0.0001$). Protocols for the LMIT and the preparation of ivermectin solutions are described in a supplementary file.

1. Introduction

Monitoring anthelmintic resistance is a key requirement for the sustainable control of nematodes in livestock industries. For many years the faecal egg count reduction test (FECRT) has been used as the golden standard. A disadvantage of this procedure is that tested animals must be sampled on two different occasions. The detection of resistance in the FECRT is further hindered by (i) the fact that the output of eggs by female worms does not sufficiently correlate with actual worm burden (Eysker and Ploeger, 2000), (ii) non-uniform distribution of eggs in the faecal samples and (iii) variable distribution of worms in host populations (Vidyashankar et al., 2007). In addition, it has been shown that the FECRT is only capable of detecting resistance after >25% of the nematode population carries the resistance allele (Martin et al., 1989). Therefore it is of great importance to detect resistance at an early stage in

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order to change parasite management and to determine which drug classes still remain active against particular nematode populations.

As more cost effective alternatives, various *in vitro* tests or bioassays have been developed for the detection of resistance (Taylor et al., 2002; von Samson-Himmelstjerna et al., 2009). Several modifications of larval development tests (LDT) have been published for the detection of resistance to benzimidazoles, levamisole and macrocyclic lactones (Coles et al., 1988; Gill and Lacey, 1998; Gill et al., 1995; Giordano et al., 1988; Taylor, 1990). The LDT is thought to detect effects of substances on the pharyngeal muscles of larvae (Gill et al., 1995) and affected larvae fail to develop to infective third stage larvae presumably through starvation. Anthelmintics causing paralysis in somatic muscles can be evaluated using motility or migration tests which principally involve incubation of third stage larvae in the presence of drugs and the measurement of motility by observation (Martin and Le Jambre, 1979), electronic detectors (Folz et al., 1987) or by migration through a sieve (Sangster et al., 1988). A larval paralysis test for the detection of resistance to thiabendazole was developed by Sutherland and Lee (1990) and Gill et al. (1991) reported a larval motility test for the detection of resistance to macrocyclic lactones (MLs). A number of these tests have been adapted for use with different parasite species and substances and therefore a wide range of similar tests have since been published (Douch and Morum, 1994; Gamble and Zajac, 1992; Gatongi et al., 2003; Rabel et al., 1994; Wagland et al., 1992).

However, to date no data on the repeatability of results generated within the same laboratory and the reproducibility of results between different laboratories has been published for any migration test. A protocol for a larval migration inhibition test (LMIT), consisting of the use of a migration system which enables the physical separation of motile from non-motile larvae through the use of sieves, has been recently published (Demeler et al., 2010). In the studies described here, stock solutions of drug were provided by one laboratory to minimize variations. Susceptible and, if available, resistant isolates of *Cooperia oncophora*, *Ostertagia ostertagi* and *Haemonchus contortus* were ring tested in six different laboratories in five different countries. The final objective of the ring test was to prepare a detailed standard operating procedure (SOP) and a protocol for the preparation of test solutions for the LMIT.

2. Materials and methods

2.1. Parasites

The drug susceptible *C. oncophora* isolate (*C. oncophora* sus.) used in this ring test was a Weybridge-isolate originally obtained from BAYER Animal Health GmbH in 2002. The IVM-resistant *C. oncophora* isolate (*C. oncophora* res.) was originally obtained within field studies from a UK farm, where the therapeutic dose of IVM failed to eliminate the parasites *in vivo* (Coles et al., 1998). The isolate used in this study was obtained from BAYER Animal Health GmbH in 2002 and further maintained in Hannover including challenge with therapeutic doses of IVM until the use in this study.

The susceptible *O. ostertagi* (*O. ostertagi* sus.) used was also a Weybridge-isolate obtained from BAYER Animal Health AG in 2002 and had no history of exposure to MLs. In all passages it remained susceptible to therapeutic doses of MLs and benzimidazoles, resulting in a 100% reduction of the egg count in infected calves up to 28 days after treatment.

The susceptible *H. contortus* isolate (*H. contortus*. sus.) used for the ring tests was the *H. contortus* McMaster isolate. The second isolate was the IVM-resistant *H. contortus* CAVR isolate. The susceptible *H. contortus* McMaster isolate had no history of exposure to MLs. *H. contortus* CAVR was resistant to IVM and other MLs and showed decreased sensitivity to moxidectin *in vivo* (Le Jambre et al., 1995).

All isolates were individually and regularly passaged in 4–5-month-old male calves or 3–6 months old sheep, respectively. All susceptible isolates remained susceptible to the therapeutic doses of MLs and/or benzimidazoles. The *C. oncophora* res. isolate and the *H. contortus* CAVR isolate were additionally challenged with the recommended therapeutic dose of IVM (0.2 mg/kg body weight). While the *H. contortus* CAVR isolate showed high resistance against IVM (FEC reduction between 0 and 25%), the FEC of the *C. oncophora* res. isolate dropped to 0 for the first 4 days after treatment. On day 5, FECs reappeared but generally they did not reach the number of eggs per gram observed before treatment. Mean FEC reduction on days 5–12 after treatment varied from 35 to 78%.

2.2. Drug solutions

Ivermectin was purchased from Sigma (I8898). Stock dilutions of ten different concentrations of IVM were made in dimethyl sulphoxide (DMSO, 100%) and sent out to participating laboratories prior to every ring test. For each of the tested isolates, eight drug concentrations were tested. The tubes were numbered (1–8) and colour-coded (blue for the test with the susceptible isolate and red for the resistant isolate) but the actual concentration of the IVM in the different tubes remained unknown to the examiners in the participating laboratories (exception Hannover, where the tubes were prepared). Following provided instructions (see detailed standard operating procedure [SOP] in supplementary file 1), further dilutions were made with deionised water by the personnel of the participating laboratories, maintaining a stable concentration of 0.5% DMSO in the wells for final use in the test.

The final concentration of IVM used ranged between 10^{-5} and 5×10^{-10} M. Appropriate concentration ranges were determined in evaluation studies (Demeler et al., 2010).

2.3. Larval migration inhibition test (LMIT)

From October 2007 until July 2008, four ring tests per species (total number 12) were performed in four and/or five different laboratories. This action was part of the PARASOL project, funded by the European Union (FOOD-CT-2005-022851) and all participating laboratories