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The in vitro diagnosis of anthelmintic resistance in cyathostomins

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ABSTRACT

Cyathostomins are the primary parasitic pathogens of equids. For over 40 years, these nematodes have been controlled using broad spectrum anthelmintics. Three classes of anthelmintic are currently available for this use but, unfortunately, resistance to each of these has now been recorded in cyathostomin populations. As part of an optimal strategy to control cyathostomin infections in the field, it will be important to identify drug-resistant worms at as early a stage as possible. This objective needs to be supported by methodologies that will allow the accurate comparison of anthelmintic resistance in different nematode populations. At present, the faecal egg count reduction test is considered the most suitable method for initial screening for anthelmintic resistance in equine nematode populations. However, in its current state, this test lacks sensitivity. It is also costly and time-consuming to perform. Laboratory-based techniques, such as the egg hatch assay, larval development assay, larval migration inhibition assay and the larval feeding inhibition assay offer alternative options for assessing anthelmintic resistance in nematode populations. All of these tests have been investigated for their utility in measuring drug resistance in sheep nematode populations and some have proven useful. The egg hatch assay, larval development assay and larval migration inhibition assay have been investigated for use in measuring levels of drug resistance in equine nematode populations. However, at best, the results obtained thus far indicate that these tests require further refinement.

1. Introduction

The Cyathostominae are the most important metazoan parasites of equids. These nematodes of the large intestine comprise a group of over 50 species, the majority of which can infect horses (Lichtenfels et al., 2008). Cyathostomins are currently controlled using anthelmintic drugs, which belong to three distinct classes: the benzimidazoles (BZ), the tetrahydropyrimidines (THP) and the avermectins/milbemycins (AM). However, drug resistance in cyathostomins is now a major issue, with resistance to BZ anthelmintics being geographically widespread (Kaplan, 2002) and, in some regions, resistance to the THP drugs is common (Kaplan et al., 2004). Furthermore, cyathostomin resistance to members of the AM class of anthelmintics has recently been reported (Molento et al., 2008). No new class of anthelmintics has reached the equine market in more than 25 years; however, two new classes, the amino-acetonitrile derivatives (Kaminsky et al., 2008), and the spiroindoles (Little et al., 2010) have recently been launched for use in sheep. It would seem that release of an amino-acetonitrile derivative product for use in horses is some way off, and due to toxicity in horses, a spiroindole product is unlikely. For these reasons, the efficacy of the existing anthelmintic classes against equine nematodes needs to be maintained for as long as possible.

To facilitate the investigation of clinical problems and for the purpose of designing optimal nematode control

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strategies, anthelmintic resistance needs to be detected at as early a stage as possible. The currently used 'gold standard' test, the faecal egg count reduction test (FECRT), has been quoted as being reliable only when >25% of the nematodes present in a given population are resistant (Martin et al., 1989). Thus, in using this test, when the proportion of genotypically resistant individuals is relatively low in a population, these are likely to be missed. Since the last set of recommendations for the detection of anthelmintic resistance in veterinary nematodes was published (Coles et al., 1992), the problem of cyathostomin drug resistance has worsened. There is a now a requirement for improved methods for drug resistance detection. As outlined above, the FECRT lacks sensitivity, whilst in vivo procedures such as critical and controlled anthelmintic efficacy tests are only appropriate for particular research purposes because of ethical issues and the costs that are involved. In this review, we detail the progress made using in vitro assays to detect drug resistance in parasitic nematodes, with emphasis on the cyathostomins.

2. Laboratory-based assays for detecting anthelmintic resistance in nematode species of veterinary importance

A number of in vitro assays are available and these have been primarily developed for investigations into drug resistance in sheep nematode species (Coles et al., 2006). The techniques that are currently available include the egg hatch assay (EHA), the larval development assay (LDA), the larval feeding inhibition assay (LFIA) and the larval migration inhibition assay (LMIA). Molecular genetic tests have also been investigated for their potential to detect anthelmintic resistance. In particular, polymerase chain reaction based tests have been designed to identify single nucleotide polymorphisms associated with resistance to BZs in cyathostomins (Drogemuller et al., 2004a; Hodgkinson et al., 2008). Also available, are preliminary sequence data on possible molecular targets for drug resistance assays for AM drugs (Drogemuller et al., 2004b). However, this work is in its infancy and the data obtained thus far has been well reviewed recently (Hodgkinson, 2006; Von Samson-Himmelstjerna et al., 2007) and so will not be included here.

2.1. The egg hatch assay (EHA)

The EHA (Le Jambre, 1976) is only of use for detecting BZ resistance. This assay has the advantages of being inexpensive and relatively quick to perform (Craven et al., 1999). Thiabendazole is the anthelmintic most often used in this test because it has comparatively high water solubility. In the EHA, clean nematode eggs are isolated from faeces, incubated in serial concentrations of anthelmintic and the percentage of eggs that hatch (or die) at each concentration is determined. The data is then corrected for the levels of egg mortality that are observed in control (i.e. no drug) wells and a dose-response line is plotted against drug concentration. The data is transformed to generate a linear regression from which an EC_{50} value is obtained. The EC_{50} value is the concentration of anthelmintic at which 50% of eggs are killed. Eggs that have embryonated are less sensitive to BZ, and this can be a confounding factor to the assay (Le Jambre, 1976). To reduce the impact of embryonation of eggs on the results of the assay, ovine faecal samples have been stored anaerobically (Hunt and Taylor, 1989) or at 4 °C (Smith-Buijs and Borgsteede, 1986) prior to the test. Despite an extensive number of publications detailing the use of EHA with sheep nematodes, recent experiments with *Haemonchus contortus* have indicated that the EHA still requires a good deal of inter-laboratory validation and optimization (Coles et al., 2006). The EHA was recently 'ring-tested' for nematode species by a number of EU research groups and, following optimization and standardization of the procedure at various points, the assay was shown to provide consistent data amongst the laboratories (Von Samson-Himmelstjerna et al., 2009).

A number of studies have analyzed cyathostomins in the EHA. Some of these have compared the EC_{50} values with data derived from matching BZ FECRTs. In some cases, significant correlations between the EHA and the FECRT data were observed (Craven et al., 1999; Königová et al., 2003). Nevertheless, the correlations obtained were not particularly 'exact' and a discriminating concentration for BZ resistance in the EHA has not been established. Of note, poor correlations between EHA data and values derived from an LDA using cyathostomin populations have been observed (Craven et al., 1999: see Section 2.2 for more details on the LDA). In the latter work, the authors assumed that the discrepancies arose because the EHA and LDA measure different attributes of the nematode response to anthelmintic. In addition to these issues, the main disadvantage of the EHA for use with cyathostomin populations is that most anthelmintics used in horses now belong to the AM and THP classes and BZ drugs are used at low levels (see Lind et al., 2007). Furthermore, as BZ resistance is widespread in cyathostomins in many geographical locations, a test that is confined only to detecting resistance to this class of anthelmintic is of limited value.

2.2. The larval development assay (LDA)

The LDA measures the development of eggs through to third stage larvae (L3). This test has advantages over the EHA in that anthelmintics of different classes can be examined, embryonation of eggs is not a concern, and where multiple-species populations are an issue, in some cases, L3 can be characterized at the end of the test. Two versions of this test have been developed; one, a liquid-based test (Hubert and Kerboeuf, 1992) and one, which is agar-based (Gill et al., 1995). An agar-based LDA kit, developed at the Commonwealth Scientific and Industrial Research Organization in Australia in the 1990s (DrenchRite®, Horizon Technology, Australia) is commercially available. This assay has been used to detect anthelmintic resistance with success in both goat (Howell et al., 2008; Kaplan et al., 2007; Terrill et al., 2001) and sheep (Ancheta et al., 2004; Howell et al., 2008) nematodes. Recently, a liquid-based LDA was adapted for use with cattle nematodes with relatively good success for ivermectin (IVM) (Demeler et al., 2010b). Comparison of a susceptible and resistant isolate of *Cooperia oncophora* yielded a resistance ratio of 5.3 for IVM.