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Variability of the egg hatch assay to survey benzimidazole resistance in nematodes of small ruminants under field conditions

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ABSTRACT

The egg hatch assay (EHA) is one of the main *in vitro* methods for detection of benzimidazole resistance in nematode parasites of small ruminants. However, although the EHA has been standardised at the laboratory level, the diagnostic performance of this method has not been fully characterised for field screenings. In the present work, monthly variation of benzimidazole resistance estimated by EHA was surveyed over two years in three sheep flocks and in one goat and an additional sheep flock sharing the same pastures. Resistance was measured by calculating both the effective dose of thiabendazole (TBZ) that inhibited hatching of ≥50% of parasite eggs (ED_{50}) and the proportion (P_{dd}) of eggs hatching at a discriminating dose of 0.1 μg/ml TBZ. P_{dd} exhibited higher variability than ED_{50}, in agreement with the higher sensitivity of P_{dd} to changes in resistance levels. Both resistance parameters, however, were highly correlated, and their variation was similarly related to the same factors. Resistance levels differed among sheep flocks, and the resistance level of the goat flock was higher than that measured for the sheep flock sharing the same pasture. Moreover, monthly variation of resistance in goats did not mirror that recorded in sheep. Resistance levels varied seasonally, with the highest values recorded in the spring, and they were inversely related to the number of days that samples were stored under anaerobic conditions. In addition, they were directly associated with the relative abundance of *Teladorsagia* spp. but inversely related to the relative abundance of *Haemonchus* spp. After controlling for the effects of these identified factors for variation, inter-monthly sampling variation due to unknown factors was the main source of variability, accounting for more than 60–70% of variance for both resistance parameters and yielding absolute estimation errors higher than 0.06 for ED_{50} or 0.2 for P_{dd} when resistance was estimated from a single sampling. Optimum sample size, estimated from variance components, suggested that at least 4–5 samplings would be needed to halve this absolute error, whereas additional samplings would slightly increase precision but at the cost of substantially increasing sampling effort. More research is needed to identify the main factors involved in this inter-sampling variation to standardise the implementation of EHA under field conditions.

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1. Introduction

Infections with gastro-intestinal nematodes remain the most prevalent parasitic diseases affecting small ruminants worldwide. For many years, the management of gastro-intestinal nematode parasites has relied heavily on the use of anthelmintic drugs, causing parasites to develop resistance (Gilleard, 2006).

Anthelmintic resistance among parasites of small ruminants has occurred for at least four decades, and a varying degree of resistance against broad-spectrum anthelmintic drug groups has been widely reported throughout the world, although in most countries where anthelmintic resistance occurs, widespread benzimidazole (BZ) resistance characterises the situation (Jabbar et al., 2006).

In the face of increasing anthelmintic resistance, the ability to reliably detect it is a crucial part of resistance management, as accurate diagnosis of resistance would assist in preventing it from becoming widespread. In this way, a variety of *in vitro* tests are available to monitor BZ resistance, but each suffers to some degree in terms of reliability, reproducibility, sensitivity and ease of interpretation (Taylor et al., 2002).

The egg hatch assay (EHA) is recommended by the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) as the most reliable *in vitro* test for detection of BZ resistance in nematode parasites (Coles et al., 1992). This test, first described by Le Jambre (1976) and Coles and Simpkin (1977), is based on the ovicidal properties of the benzimidazoles and the ability of eggs from resistant strains to embryonate and hatch in the presence of higher drug concentrations than susceptible strains. Usually, the BZ resistance level is measured by estimating the effective dose of thiabendazole that inhibits the hatching of ≥50% of parasite eggs (ED_{50}), although in a modified form, the same test can be used with a choice of a single discriminating concentration (Whitlock et al., 1980; Kemp and Smith, 1982; Coles et al., 2006; Cudeková et al., 2010).

Given that EHA can be performed in one farm visitation, it is fast (results can be obtained in 2 days) and simple to perform, and it has been used on many occasions to characterise the resistance of nematode parasites of sheep, goats, horses or even cattle in the field (*e.g.*, Craven et al., 1999; Várady et al., 2006; Saeed et al., 2010; Calvete et al., 2012; Demeler et al., 2012). Despite its great potential, however, there are some difficulties in performing this test, as its sensitivity is affected negatively if less than 25% of the resistant parasite population is involved (Martin et al., 1989), and there is considerable day-to-day variation in ED_{50} values during the patent period (Le Jambre, 1976; Borgsteede and Couwenberg, 1987; Kerboeuf and Hubert, 1987; Várady et al., 1995).

Moreover, due to the lack of standardisation and the high variability of EHA estimations, there are difficulties in comparing results between and within laboratories (Johansen, 1989; Von Samson-Himmelstjerna et al., 2009). Accordingly, Von Samson-Himmelstjerna et al. (2009) have performed, to date, the most complete study aimed to evaluate the repeatability of data generated within laboratories and reproducibility of results between laboratories. From this survey, several proposals to reduce EHA inconsistencies have been derived to reduce protocol variations at the laboratory level, mainly (among others) by using deionised water and making dilutions of thiabendazole in dimethyl sulphoxide (DMSO). However, even after standardisation, results from this survey highlighted the difficulties in dealing with the variability of this test.

As the EHA for BZ resistance is also used for field screening surveys, standardisation of the method is also required at the field implementation level, and therefore characterisation of the variation of EHA results under field conditions is needed. For this reason, in the present survey, BZ resistance was estimated monthly by EHA in one goat and four sheep flocks over two years. The main goals of this work were to obtain a first approximation of the variation in BZ resistance levels measured by EHA under field conditions, to identify putative factors associated with this variation and to estimate the optimum sample size required for a determined precision. The three goals were implemented for resistance levels estimated both by DE_{50} and by discriminating dose.

2. Materials and methods

2.1. Flocks and flock sampling

From April 2008 to March 2010, four sheep flocks and one goat flock were sampled once monthly to estimate BZ resistance levels. All flocks were located across the Middle Ebro Valley in northeast Spain in a climatic area characterised by annual mean precipitation ranging from 250 to 350 mm and an annual mean temperature ranging from 13.5 to 15 °C. Although all sheep flocks included were raised for meat production under an extensive management system representative of ovine breeding activity in the region, they differed in census, breed or type of pasture. They are briefly described below.

- Flock IR1: Located on irrigated agricultural lands neighbouring the Ebro River, it had a census of 925 Rasa Aragonesa breed sheep grazing in public and communal pastures.
- Flock IR2: Similar to the previous flock, it was located on irrigated lands, but pastures were private and therefore no other flocks grazed in the same area. It comprised approximately 600 sheep of the Rasa Aragonesa breed.
- Flock NIR1: Sheep flock located on non-irrigated lands and grazing in public and communal pastures. The breed was also Rasa Aragonesa, and it comprised approximately 1700 sheep.
- Flock NIR2: Sheep flock also located on non-irrigated lands but grazing in private pastures shared with goat flock NIR2g. The census was approximately 160 sheep of the Ansotana (60) and Rasa Aragonesa (100) breeds.
- Flock NIR2g: Goat flock comprised of 40 Angora breed individuals not used for production. This flock grazed simultaneously with sheep flock NIR2 in the same pastures during the entire survey.

Each flock was sampled monthly to estimate BZ resistance levels. In each flock, faecal samples were taken from the rectum of 40–50 randomly selected adults, whereas