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Rapid selection for β-tubulin alleles in codon 200 conferring benzimidazole resistance in an *Ostertagia ostertagi* isolate on pasture

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ABSTRACT

Resistance to benzimidazoles (BZs) is widespread in sheep nematodes and increasing in those of cattle. Several reasons including the predominant use of pour-on anthelmintics and lack of scales in field conditions lead to under-dosing of cattle and therefore to increased selection pressure. In an field experiment the frequency of BZ-resistance associated allele (TAC) in codon 200 in the β-tubulin isotype 1 gene of *Ostertagia ostertagi* was monitored over one grazing season (approximately 30 weeks). Group 1, consisting of four calves, was experimentally infected with a pure *O. ostertagi* population displaying ~50% of the TAC allele. The subsequently following groups of calves (four groups of two calves each) acquired natural infections by grazing contaminated pastures. Each group was treated with increasing percentages of sub-therapeutic dosages of albendazole (35–65%). Larvae obtained from faecal cultures pre and post treatment were subjected to species/genus-specific PCR as well as pyrosequencing to determine allele frequencies. PCR revealed the presence of *Ostertagia*, *Trichostrongylus*, *Haemonchus* and *Cooperia* in pre-treatment samples and predominantly *Ostertagia* as well as some *Trichostrongylus* in post treatment samples. Faecal egg count reduction was always less than 90% 7–10 days post treatment. In naturally infected calves TAC allele frequencies were significantly increased ($p < 0.05$) after treatment and they also rapidly increased during the grazing season (pre: 15–63%; post: 55–89%). The more than 4-fold increase in resistant genotypes before treatment indicates how fast selection for BZ resistance can occur when sub-therapeutic dosages are combined with a high treatment frequency, even under moderated climatic conditions and in the presence of a refugium.

1. Introduction

Ostertagia ostertagi and other gastrointestinal (GI) parasites impose a potential health risk for grazing animals and are among the major economic constraints for farmed livestock (Coles, 2005). Though clinical parasitism such as ostertagiosis occurs relatively rarely and mainly in first season grazing calves, subclinical parasite infections are found more or less ubiquitous (Corwin, 1997; Fox et al., 2007; Hawkins, 1993; Szyszka and Kyriazakis, 2013). Due to the acquired but usually not fully protective immunity against GI parasites subclinical infections also affect dairy cows which grazed for several years. Studies have shown that subclinical infections, which may also be caused by the use

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of only partially effective anthelmintic treatments, are considered to cause production losses. In particular *O. ostertagi* has frequently been correlated with decreased productivity (Sanchez et al., 2004; Gibb et al., 2005). The inflammation of the abomasum leads to pain, decreased food up-take and a lower feed conversion ratio which results in decreased weight gain (Charlier et al., 2009; Fox, 1993) and lower milk production (Gross et al., 1999; Kloosterman et al., 1985; Sanchez et al., 2004). Sanchez et al. (2004) reviewed 27 studies and concluded that anthelmintic treatment of adult dairy cattle infected mainly with *O. ostertagi*, leads to an increase of milk production of ~0.35 kg/cow per day. Furthermore, cattle treated with eprinomectin were found to have a significantly higher daily milk yield (+1.68 kg/cow) compared to untreated controls (Gibb et al., 2005).

To prevent animal suffering and production losses caused by GI parasites it is important that affected animals are treated with efficient anthelmintics. Often animals receive prophylactic or strategic treatments and since weighing of calves is often not feasible on pasture, incorrect dosing including under-dosing regularly occurs. The frequent use combined with under-dosing and lack of appropriate grazing management systems may lead to the increased development of anthelmintic resistant cattle nematode (Sutherland and Leathwick, 2011) populations as already observed for sheep nematodes worldwide (Wolstenholme et al., 2004). Another important reason for accelerated development of anthelmintic resistance (AR) is a decreased refugium (Coles, 2005), either in terms of susceptible larvae on pasture, in untreated animals or even in treated animals, e.g. in the form of inhibited third stage larvae (L3). A reduced refugium results in higher selection pressure (van Wyk, 2001) while it was shown by Martin et al., 1981 that a substantial refugium delays AR development against benzimidazoles (BZs) in *Haemonchus contortus.* Under temperate climate conditions the main reasons for a small-sized refugium are the treatment of all animals because targeted treatment or targeted selective treatment approaches have not frequently been implemented in most grazing management systems and a high stocking rate. Particularly in areas with summer drought, hostile environmental conditions, reducing the number of free living stages of GI nematodes on pasture, can contribute to decreasing refugia.

BZs have been used since the 1960 as broad-spectrum anthelmintics against GI parasites. BZs bind to the β-tubulin isotype 1 of the parasites with high affinity and only with considerably lower affinity to that of mammalian β-tubulin. Binding causes the inhibition of microtubule polymerisation (Lacey, 1988) which in combination with ongoing dissociation leads to the disappearance of the microtubules over time. Resistance to BZs is mainly mediated by specific changes in the β-tubulin gene. One single nucleotide polymorphism (SNP) has been found sufficient to cause resistance against BZs (von Samson-Himmelstjerna et al., 2007). For example SNPs in the codons 167 or 200 of the β-tubulin isotype 1 gene causing changes from phenylalanine (TTC) to tyrosine (TAC) or in codon 198 from glutamate (GAA) to alanine (GCA) correlate with resistant phenotypes (Kotze et al., 2014). AR may develop slowly in the beginning and so remain mostly undetected, but then increases rapidly to high levels (Jackson and Coop, 2000).

It has been shown previously that pyrosequencing is a highly sensitive method to detect early development of resistance in terms of increased SNP frequency. In comparison, detection of BZ resistance using in vitro (egg hatch assay) or in vivo (faecal egg count reduction test, FECRT) methods did reveal correlation of data but appeared to be less sensitive (Demeler et al., 2013a; von Samson-Himmelstjerna et al., 2009). However, most investigations have been performed using GI nematodes of sheep and only limited work is available regarding GI nematodes of cattle. Most publications reporting resistance to BZs in cattle GI nematodes are based on data obtained from FECRTs (Rendell, 2010; Soutello et al., 2007; Walker et al., 2013; Yazwinski et al., 2009) and/or controlled tests (Gasbarre et al., 2009). Only one study has so far been reported in which FECRT data and in vitro assay data are available for the same GI nematode population, but in the absence of BZ-resistant populations no correlation could be obtained (Demeler et al., 2012a). In addition only very limited publications describe parallel investigations using the FECRT and/or β-tubulin genotypes (Chaudhry et al., 2014; Demeler et al., 2013a).

The intention of the current study was to investigate, how rapid (i) sub-therapeutic and (ii) frequent anthelmintic treatment will lead to an increase of the BZ-associated SNP frequency in the β-tubulin isotype 1 gene in a partially resistant *O. ostertagi* population. For that purpose an *O. ostertagi* population, in which a SNP in codon 200 was already present with a resistant allele frequency of approx. 54%, was chosen to infect calves grazing on pasture in the presence of a BZ susceptible refugium.

2. Materials and methods

2.1. Parasites

Infective L3 of an *O. ostertagi* isolate were used for the initial experimental infection (infection dose 40,000 L3) of the calves. The *O. ostertagi* isolate used in this experiment was a mixture consisting approximately 50% of a population from the field (found to be resistant to BZs) and 50% of a laboratory *O. ostertagia* isolate susceptible to anthelmintics (as previously described in Demeler et al. (2010)). Prior to infection the population was examined for the presence of BZ resistance associated SNPs in the β-tubulin isotype 1 gene.

2.2. Calves and pasture

Four months old nematode-naïve weaned calves were purchased from an all-year indoor dairy farm with no reports of GI nematode infections in the past 3 years. Prior to the experiment the faeces were examined for the presence of strongyle eggs using the Mini-FLOTAC® technique (Barda et al., 2013) with a sensitivity of 5 eggs per gram (epg). The pasture consisted of 1065 m^2 and was not grazed by cattle during the past 5 years. However, sheep and goats were grazed on this pasture and potentially able to contaminate the pasture in the previous grazing periods with