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Research paper

Species composition of larvae cultured after anthelmintic treatment indicates reduced moxidectin susceptibility of immature *Cylicocyclus* species in horses

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ABSTRACT

For the control of cyathostomins in horses, the macrocyclic lactones (MLs), moxidectin (MOX) and ivermectin (IVM) are the most commonly used anthelmintics. However, reduced activity, observed as shortening of the egg reappearance period (ERP) has been described. Shortening of the ERP may be caused by a decreased susceptibility of immature worms for MLs. Alternatively, immature worms may develop faster into egg producing adults as a result of repeated ML treatments. The species composition of the larval cultures obtained shortly after ML and pyrantel (PYR) treatment can confirm the hypothesis of decreased ML susceptibility, as this is often class-specific, whereas faster development would also occur after treatment with anthelmintics with a different mode of action. From 3 farms with a known history of shortened ERP, 8 horses per farm were selected and divided into 2 groups. The MOX-PYR-MOX group was treated twice with MOX (day 0 and 126) and once with PYR (day 84) and the IVM-PYR-IVM group was treated twice with IVM (day 0 and 98) and once with PYR (day 56). Cultured infective larvae (L3s) were counted and differentiated with the reverse line blot on pooled samples. Per cyathostomin species, the number of larvae per gram was calculated. The efficacy of all ML treatments was 100% and a shortened ERP was found on all 3 farms. The species composition of the larval cultures after ML treatment did not differ significantly from that after PYR treatment in the IVM-PYR-IVM group, but it did differ in the MOX-PYR-MOX group. The larval cultures obtained after MOX treatment consisted mostly of *Cylicocyclus nassatus*, while after PYR treatment *Cylicostephanus longibursatus* was the most abundant species. In the cultures from 42 days after MOX treatment 6 cyathostomin species from 3 genera were found on the farm with the lowest activity (farm 1), while on the farm with the highest activity (farm 3) only 3 species from one genus were found in the same number of examined L3s. The high numbers of L3s of *Cylicocyclus* species 42 days after MOX treatment and the low numbers 42 days after PYR treatment can be explained by reduced susceptibility of the immature worms to MOX, but not by a faster development. In conclusion, shortening of the ERP following MOX treatment is most likely a process in which an increasing number of immature worms from an increasing number of species is becoming less susceptible to the active compound.

1. Introduction

Cyathostomins are the most prevalent nematodes in equids worldwide. Macrocyclic lactones (MLs) are currently the most used anthelmintics, of which ivermectin (IVM) and moxidectin (MOX) are registered for the control of cyathostomins in horses (Reinemeyer and Rohrbach, 1990; Nielsen et al., 2014).

The egg reappearance period (ERP) is the time between treatment and the reappearance of eggs in the feces. The ERP for IVM and MOX at the time of introduction has been reported to be 8–9 weeks (Borgsteede et al., 1993; Parry et al., 1993; Boersema et al., 1996, 1998) and 12–25 weeks (Demeulenaere et al., 1997; DiPietro et al., 1997; Boersema et al., 1998), respectively. However, worldwide reduced ERP has been reported both for IVM and MOX (von

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Samson-Himmelstjerna et al., 2007; Lyons et al., 2011b; Molento et al., 2012; Geurden et al., 2014).

Several mechanisms can explain the shortening of the ERP. After ML treatment, the ERP is often shorter than the shortest pre-patent period (PPP) of 57 days for cyathostomins (Lyons et al., 2011a) and shortening of the ERP has also been observed in housed horses (van Doorn et al., 2014). This indicates that larvae already present in the host at the time of treatment must be responsible for the shortening of the ERP. Therefore, a reduced ERP is regarded as an early sign of development of resistance (Lyons et al., 2009). A shift within the cyathostomin population towards species with a shorter development time is not a likely cause, as horses with and without a shortened ERP showed a similar species composition before treatment (van Doorn et al., 2014). Furthermore, 20 years of ML treatment did not result in a change in the cyathostomin species composition within the same herds (Chapman et al., 2002). Therefore, shortening of the ERP is most likely caused by decreased susceptibility of immature stages to MLs or by a faster pace of development of immature stages into egg producing adults. In a critical test described by Lyons et al. (2010), the efficacy of MOX against luminal larval stages in one horse was only 82%, while the efficacy against adult cyathostomins was still 100% in the same horse. Critical tests performed by the same group demonstrated a effectivity of IVM against L4s of 36–80% (Lyons et al., 2009) and 3 years later the effectivity against L4s has dropped to only 0–16% (Lyons and Tolliver, 2013). The effectivity against adults was in both cases higher than 99%. The authors suggested that this was caused by reduced efficacy of MLs against the immature luminal stages, but they could not exclude the possibility of a fast development of mucosal stages into luminal stages in the 6 days between ML treatment and necropsy.

Experimentally, it may be possible to distinguish faster development from decreased susceptibility for MLs because a faster pace of development from L4 into egg laying adult should also occur after treatment with an anthelmintic outside the ML group e.g. pyrantel (PYR). A faster development can be advantageous for the parasite. It increases the chance to reproduce in between 2 subsequent treatments. Plasticity of the development time in nematodes has been demonstrated before, for example in *Anguillicola crassus*, a parasitic nematode of the Japanese and European eel (Weclawski et al., 2013).

A shortened ERP does not necessarily involve all present cyathostomin species. One species may be more prone to develop resistance or may have a higher level of plasticity than another. Although differentiation of the eggs or cultured L3s by morphological means is not possible, they can be differentiated by molecular methods (Traversa et al., 2007; van Doorn et al., 2010, 2014; Cwiklinski et al., 2012). These studies used the reverse line blot (RLB), a method based on the hybridization of species specific probes with an amplified fragment of the intergenic spacer (IGS) region. Individual L3s can be differentiated into 21 of the most common cyathostomin species (Cwiklinski et al., 2012). Recently, a RLB on pooled L3s was described for the differentiation of larval cultures allowing an estimation of the larvae per gram (LPG) per cyathostomin species (Kooyman et al., 2016).

The aims of this study were to determine which species are involved in the shortening of the ERP and to determine the cause of shortening of the ERP: reduced susceptibility to MLs or a faster development of the species involved.

2. Material and methods

2.1. Farms and horses

This study was designed as a field study in the Netherlands with horses naturally infected with cyathostomins and lasted from February to September 2013. Three farms were selected based on the presence of a shortened ERP in 2012 after ML treatment (Geurden et al., 2014). Farms NEO1, NEO2 and NEO3 from that study correspond to farm 1–3, respectively in the present study. The 3 farms practiced regular deworming (at least twice a year). On each farm, 8 horses (2–3 years old) with ≥150 eggs per gram (EPG) were randomly allocated to one of the two treatment groups. The IVM-PYR-IVM group was treated with IVM (Eqvalan® oral paste, Merial at 0.2 mg/kg bodyweight) at day 0 and day 98 and in between (day 56) with PYR (Strongid-P® paste, Pfizer at 19 mg/kg bodyweight). The MOX-PYR-MOX group was treated with MOX (Equest® oral gel, Pfizer at 0.4 mg/kg bodyweight) at day 0 and day 126 and in between (day 84) with PYR. The PYR treatments in between the ML treatments were timed according to the regular ERP of the respective ML products. Before each treatment the bodyweight was estimated with a girth tape. In order to minimize under-dosing, the dose was based on 110% of the estimated bodyweight. Fecal samples were collected at day −5 to day −2 from both groups and at day 14, 42, 56, 70, 98, 112, 140, 154 from the IVM-PYR-IVM group and at day 14, 42, 56, 84, 98, 126, 140, 168, 182 and 210 from the MOX-PYR-MOX group. All fecal samples were used for egg counts and larval cultures and all larval cultures were counted and differentiated.

2.2. Egg counts and larval cultures

Egg counts and culturing of L3s were performed as described (van Doorn et al., 2014) with a detection limit of 25 EPG and 0.4 larvae per g (LPG). The recovery of the L3s (100% × LPG/EPG) was calculated for all samples with positive EPG and positive LPG. The mean recovery of a group is the mean of the recoveries of all individual samples from that group.

2.3. Drugs activity and egg reappearance period (ERP)

There are no formal guidelines for obtaining one clear estimate for the activity of anthelmintics by fecal egg count reduction test (FECRT) in horses (Vidyashankar et al., 2012). For the present study the activity of a drug for the herd was determined by taking the mean of the arcsine transformed activity of the individual horses (Pook et al., 2002; Relf et al., 2014). For small groups of horses, it is an advantage that no control group is needed when using the individual activity. The activity at day 14 after treatment is defined as the efficacy (EFF) and determines whether there is resistance or not. A mean EFF–2 SE ≤95% at day 14 after ML treatment was defined as resistance. ERP is defined here as the period between treatment and the moment that the EPG increased to ≥10% of the pre-treatment EPG (Borgsteede et al., 1993; von Samson-Himmelstjerna et al., 2007; van Doorn et al., 2014).

2.4. Polymerase chain reaction (PCR) and reverse line blot (RLB)

PCR and RLB on pooled L3s as well as the transformation of RLB score into a frequency and a LPG per cyathostomin species was performed as described (Kooyman et al., 2016). In brief, for the differentiation of one culture, 4 pools with 10 L3s each were subjected to PCR and RLB with 19 species specific probes allowing the differentiation into 17 cyathostomin species belonging to 7 genera. For convenience, the genera were abbreviated to 3 or 4 letters, instead of the official 1 letter. Differentiated species were: *Cylicocyclus (Cyc. ashworthi, Cyc. insigne, Cyc. nassatus, Cyc. leptostomum), Cylicostephanus (Cys. calicatus, Cys. goldi, Cys. longibursatus, Cys. minutus var. A, Cys. minutus var. B), Cyathostomum (Cya. catinatum, Cya. pateratum, Cya. tetracanthum), Coronocyclus (Cor. coronatus, Cor. labiatus, Cor. labratus), Cylicodonthophorus (Cyd. bicoronatus) Parapoteriostomum (Para. mettami)* and *Poteriostomum (Pot. imparidentatum)*. The presence of a species within a pool was determined