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Oral tylosin administration is associated with an increase of faecal enterococci and lactic acid bacteria in dogs with tylosin-responsive diarrhoea

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ABSTRACT

The term tylosin-responsive diarrhoea (TRD) is used for canine recurrent diarrhoea cases for which no underlying cause can be found after extensive diagnostic investigations, but which show a response to the antibiotic tylosin in a few days. The objective of this prospective, one-arm longitudinal trial was to assess the effects of oral tylosin administration on the faecal levels of potentially probiotic bacteria, such as *Enterococcus* spp. and lactic acid bacteria (LAB), in dogs with TRD.

This trial included 14 client-owned suspected TRD dogs that were on tylosin treatment and had firm faeces. Treatment was then terminated and dogs were followed up for up to 2 months to determine the recurrence of diarrhoea. Once diarrhoea started, dogs received tylosin (orally, 25 mg/kg, once daily for 7 days). At the end of the treatment period, stools were firm again in 11 dogs (TRD dogs); three dogs continued having diarrhoea and were excluded from the study. Faecal samples were collected at all three time-points for culture of LAB and enterococci. In TRD dogs, the colony counts of *Enterococcus* spp. ($P = 0.003$), LAB ($P = 0.037$), tylosin-resistant *Enterococcus* spp. ($P < 0.001$) and LAB ($P < 0.001$) were significantly higher when the dogs were on tylosin treatment and had normal faecal consistency compared to when they had diarrhoea following discontinuation of tylosin. In conclusion, cessation of diarrhoea in TRD dogs with tylosin treatment could be mediated by selection of a specific lactic acid population, the *Enterococcus* spp., due to their potential probiotic properties.

Introduction

The macrolide antibiotic tylosin is widely used in the treatment of canine antibiotic-responsive diarrhoea (Hall, 2011) and as an adjunctive treatment in inflammatory bowel disease (IBD) (Van Kruiningen, 1976; Simpson and Jergens, 2011) and exocrine pancreatic insufficiency (Westermarck et al., 1993; Westermarck and Wiberg, 2012). All of these conditions are common in dogs and typically manifest as chronic diarrhoea. In recent years, tylosin has been shown to be effective in treating idiopathic recurrent diarrhoea, leading to the term tylosin-responsive diarrhoea (TRD) (Westermarck et al., 2005; Kilpinen et al., 2011, 2014). A specific feature of TRD is that recurrent diarrhoea ceases within a few days of initiating tylosin treatment and that stools remain normal for as long as treatment continues. After discontinuation of tylosin, diarrhoea reappears in many dogs within a few weeks.

The exact mode of action of tylosin on TRD remains obscure. Known pathogenic bacteria have been excluded as the underlying cause of TRD (Westermarck et al., 2005; Kilpinen et al., 2011). The antibacterial effect of tylosin may play a minor role in terminating recurrent diarrhoea and the immunomodulatory properties of tylosin could explain its favourable effect. Cao et al. (2006) proposed an anti-inflammatory effect of tylosin in vitro due to the modulation of cyclo-oxygenase-2 (COX-2), nitric oxide synthase (NOS) and cytokines in macrophages and monocytes. An involvement of cytokines in the mechanism of action of tylosin has also been suggested by Er et al. (2010) in a study performed with lipopolysaccharide (LPS)-treated mice. A protective effect of tylosin against intestinal inflammation was suggested by Menozzi et al. (2005) and Blackwood et al. (2008) who showed that tylosin reduced the macroscopic lesion scores and the severity of histological lesions in the colon of rats with chemically induced colitis and rhesus macaques with naturally occurring colitis, respectively.

Suchodolski et al. (2009) investigated the effect of tylosin on the composition and diversity of the jejunal microbiota of healthy dogs and showed that during tylosin administration, the proportion of *Enterococcus*-like organisms increased significantly. They

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speculated that tylosin may promote the growth of potentially beneficial commensal bacteria, such as *Enterococcus* spp., which may have probiotic characteristics. Further, they suggested that tylosin might have suppressed the commensal microbiota and allowed for the proliferation of this bacterial group due to its resistance to tylosin (De Graef et al., 2004; De Leener et al., 2005; Jackson et al., 2009).

However, it is unclear whether the proposed immunomodulatory effect is induced by tylosin directly or is mediated by a shift of intestinal microbiota towards potentially beneficial commensal bacteria. As some enterococci strains are used as probiotics (Franz et al., 2011), proliferation of probiotic enterococci in the intestine might contribute to the cessation of diarrhoea in dogs responding to tylosin.

The primary objective of this study was to assess the effects of oral tylosin administration on the levels of potentially probiotic bacteria, such as lactic acid bacteria (LAB) and *Enterococcus* spp., a genus of LAB, in dogs with clinical signs. We wished to test the hypothesis that colony counts of *Enterococcus* spp. and LAB increase during oral tylosin administration in dogs diagnosed with TRD. A second aim was to investigate whether tylosin had a stronger effect on the increase in the colony counts of *Enterococcus* spp. in relation to the total LAB community. The third objective aimed at characterising the reasons underlying the assumed increase of these bacteria. We hypothesised that tylosin administration causes a selection pressure towards tylosin-resistant bacteria, thus increasing the levels of tylosin-resistant *Enterococcus* spp. and LAB.

Materials and methods

Study design, study population and eligibility criteria

The study population of this prospective, one-arm longitudinal trial consisted of client-owned dogs that were initially involved in a placebo-controlled, double-blinded, randomised controlled trial evaluating the efficacy of tylosin treatment in dogs with chronic recurrent diarrhoea (Kilpinen et al., 2011). Dogs over 6-months old were eligible to participate if they had been treated for recurrent diarrhoea at least once successfully with tylosin prior to enrolment in the trial. Further, no oral antimicrobials besides tylosin, non-steroidal anti-inflammatory drugs (NSAIDS) or corticosteroids were allowed in the 30 days preceding or during the trial. Exclusion criteria were pregnancy, lactation and concurrent systemic diseases causing secondary diarrhoea.

An initial examination was conducted to assess eligibility for the trial, including clinical history, physical examination and analysis of blood samples for complete blood count (CBC) and serum biochemistry profile, including concentrations of cobalamin, folate and canine trypsin-like immunoreactivity (TLI). Urinalysis, faecal examination for parasites and pathogenic bacteria, and a gastroduodenoscopy (including small intestinal mucosal biopsies) were also undertaken as reported previously (Kilpinen et al., 2011).

Study protocol

The study was conducted at the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, University of Helsinki. The National Animal Experiment Board in Finland approved the study protocol, and informed consent was obtained from all owners (protocol number: Vetcare 1698).

When starting the current trial (time-point A), all dogs were on oral tylosin treatment with individual courses and had normal faecal consistency. Tylosin treatment was then discontinued and a follow-up period of a maximum of 2 months commenced to determine the recurrence of diarrhoea. Owners evaluated and recorded the faecal consistency of their dogs according to a standardised faecal scoring system described earlier (Kilpinen et al., 2011). In brief, the faecal scoring system was based on a nine-point scale, consisting of scores from 1 to 5, with half-point intervals. The recurrence of diarrhoea was defined as the dog's faeces having a consistency score of ≥ 4 for at least two consecutive defaecations.

After onset of diarrhoea (time-point B), the dogs received tylosin (tylosin tartrate tablets 120 mg and 240 mg containing 100 mg and 200 mg of tylosin, respectively, University Pharmacy) orally at 25 mg/kg once daily for 7 days. On day 7 of the treatment period (time-point C), faecal consistency of the dogs was confirmed at a clinic visit. Dogs with firm faeces (faecal score ≤ 3) were considered tylosin-responsive diarrhoea dogs (TRD dogs); dogs with faecal scores of > 3 were considered tylosin-non-responsive diarrhoea dogs and were excluded from the study. All faecal samples were collected from the rectum at all three time-points and immediately after collection stored at –20 °C until bacteriological culturing.

Culturing of faecal samples

Faeces (1 g) were suspended in 9 mL of 0.1% peptone water, after which serial 10-fold dilutions were made from 10^{-1} to 10^{-8}. A volume of 100 µL of each dilution as parallel duplicate cultures was inoculated onto de Man, Rogosa and Sharpe (MRS) agar (Oxoid) and Slanetz and Bartley agar (Scharlab) for enumeration of LAB and enterococci, respectively. The plates were incubated at 37 °C for 48 h under anaerobic conditions using AnaeroGen AN35 sachets (Oxoid). Additionally, a catalase test was performed; catalase-negative bacteria growing on MRS agar and Slanetz and Bartley agar were considered as LAB and enterococci, respectively. Tylosin-resistant *Enterococcus* spp. and LAB were enumerated on corresponding media supplemented with 100 µg/mL of tylosin (Eli Lilly).

Outcome measures

The outcome measures were defined as the mean of the log-transformed colony counts of *Enterococcus* spp. and LAB as well as tylosin-resistant *Enterococcus* spp. and LAB expressed as log CFU/g faeces. The number of colony forming units (CFU) of *Enterococcus* spp. and LAB was enumerated on plates growing a maximum of 150 CFU/plate and was multiplied by the dilution factors to determine the levels of *Enterococcus* spp. and LAB in the faeces. The mean value of the colony counts (CFU/g faeces) in the two duplicates was calculated and used for statistical analysis. When < 1 g of sample was submitted, the result was arithmetically corrected to approximate the count for 1 g.

Statistical analyses

Descriptive statistics were used to summarise the data by time-points. Mean figures with standard error of the mean (SEM) were used to illustrate the data. The difference between time-points in the colony counts of *Enterococcus* spp. and LAB as well as tylosin-resistant *Enterococcus* spp. and LAB was analysed with a one-way repeated measures analysis of variance (ANOVA) model, where the time-point of the sample was used as the only explanatory variable. The estimated proportion of *Enterococcus* spp. of the total amount of LAB was analysed with the same kind of model. Here, the percentage of the *Enterococcus* spp. was used as the response.

All of the analyses were done with logarithmically (to the base 10) transformed data, excluding the proportion analysis, as the normality assumption was not valid with the original scale. In the analysis of the difference between time-points in the colony counts of *Enterococcus* spp. and LAB and the colony counts of tylosin-resistant *Enterococcus* spp. and tylosin-resistant LAB, Tukey's honest significant difference (HSD) test was used in the pair-wise comparisons as a post-hoc test, which takes into account the multiplicity issues in the conducted comparisons. The differences were quantified with pair-wise estimates and 95% confidence intervals (CI) calculated from the fitted models. $P < 0.05$ was considered statistically significant. All statistical analyses were done using SAS System for Windows, v.9.2 (SAS Institute).

Results

Altogether, 14 suspected TRD dogs were enrolled in this trial, assessing the effect of oral tylosin administration on the faecal colony counts of *Enterococcus* spp. and LAB. Eleven dogs responded to the oral tylosin treatment at a dosage of 25 mg/kg once daily for 7 days and had normal faecal consistency at the end of the treatment period. In three dogs, diarrhoea did not resolve with tylosin therapy and they were excluded from the study. The baseline information of the TRD dogs is shown in Table 1. Faecal samples were available for culture from seven dogs at all three time-points (A–C), from three dogs at time-points A and B, and from one dog at time-points B and C.

Mean colony counts of Enterococcus *spp.*

At time-point A, the mean of the log-transformed colony count of *Enterococcus* spp. was 8.016 ± 0.447 CFU/g faeces (Fig. 1). At time-point B, the mean of the log-transformed colony count of *Enterococcus* spp. was significantly lower ($P = 0.003$), 6.352 ± 1.728 CFU/g faeces (Table 2; Fig. 1), than the mean colony count at time-point A. At time-point C, the mean of the log-transformed colony count of *Enterococcus* spp. was significantly higher ($P = 0.003$) than at time-point B, being 8.252 ± 0.923 CFU/g. When comparing the mean colony counts between time-points A and C, no significant difference was noted ($P = 0.967$) (Table 2; Fig. 1).