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Efficacy of gallium maltolate against *Lawsonia intracellularis* infection in a rabbit model

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Antimicrobial efficacy against *Lawsonia intracellularis* is difficult to evaluate *in vitro*, thus, the effects of gallium maltolate's (GaM) were investigated in a rabbit model for equine proliferative enteropathy (EPE). Juvenile (5–6-week-old) does were infected with 3.0×10^8 *L. intracellularis*/rabbit and allocated into three groups ($n = 8$). One week postinfection, one group was treated with GaM, 50 mg/kg; one, with doxycycline, 5 mg/kg; and one with a sham-treatment (control). Feces and blood were collected daily and weekly, respectively, to verify presence of *L. intracellularis* fecal shedding using qPCR, and seroconversion using immunoperoxidase monolayer assay. Rabbits were sacrificed after 1 week of treatment to collect intestinal tissues focusing on EPE-affected sections. Intestinal lesions were confirmed via immunohistochemistry. No difference was noted between treatments regarding EPE-lesions in jejunum ($P = 0.51$), ileum ($P = 0.74$), and cecum ($P = 0.35$), or in *L. intracellularis* fecal shedding ($P = 0.64$). GaM and doxycycline appear to have similar efficacy against EPE in infected rabbits.

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INTRODUCTION

The obligate intracellular gram-negative bacterium *Lawsonia intracellularis* is the etiological agent of proliferative enteropathy in a number of wild and domestic animal species, most notably pigs and horses (Lawson & Gebhart, 2000). *L. intracellularis* causes different degrees of enterocytic hyperplasia in the distal jejunum and ileum (occasionally cecum and colon), and affects intestinal absorption (Herbst *et al.*, 2003; Horiuchi *et al.*, 2008; Pusterla & Gebhart, 2009; Wong *et al.*, 2009). Equine proliferative enteropathy (EPE) is an emerging disease in foals and weanling horses (McOrist *et al.*, 1995a; Pusterla & Gebhart, 2009). Clinical signs range in severity and chronicity, from generalized chronic wasting and pronounced ventral edema (associated with altered colloid-osmotic pressure and hypoalbuminemia), to profuse diarrhea, fever, colic, acute shock, and death (Pusterla & Gebhart, 2009; Page *et al.*, 2012). Thus, *L. intracellularis* infection is a significant burden for both the equine and swine industries, with economic losses due to animal mortality, poor growth rates, and costly treatment, as well as lost revenue from recovered animals (Frazer, 2008).

Currently, antimicrobial treatment of EPE relies either on oral macrolides or azalides, often combined with rifampin (Lavoie *et al.*, 2000; Schumacher *et al.*, 2000; Feary *et al.*, 2007), or oral or parenteral tetracyclines, with no further alternative proposed since 2006 (Sampieri *et al.*, 2006; Frazer, 2008). These antimicrobials are lipophilic, capable of permeating cell membranes to reach intracellular micro-organisms that are otherwise inaccessible to the water-soluble agents (*i.e.* beta-lactams and gentamicin) utilized commonly in equine therapy. *Lawsonia intracellularis* presents a therapeutic challenge, as these bacteria use the 'sanctuary' location of enterocytes to avoid antimicrobial activity and local and cellular host immune defenses. Although not specifically evaluated, treatment failures (Frazer, 2008; Page *et al.*, 2012), indicate the potential for *L. intracellularis* to develop a specific antimicrobial susceptibility pattern, or even resistance (Frazer, 2008). To date, antimicrobial susceptibility studies for EPE have not been conducted *in vitro*, but several studies were designed to

evaluate porcine proliferative enteropathy (PPE) strain susceptibility to a variety of antimicrobials. Although those studies attempted to mimic clinical conditions, there were clear technical limitations, possible geographical strain differences between the PPE strains currently known and difficulty in maintaining *L. intracellularis* in pure cell-culture (Lawson *et al.*, 1993; McOrist *et al.*, 1995b; Wattanaphansak *et al.*, 2009). In the early years of PPE research, two clinical studies showed the effectiveness of tetracyclines in affected pigs and a hamster PPE model (La Regina *et al.*, 1980), and a few others confirmed it against several other intracellular bacteria (Moulder, 1985; McOrist *et al.*, 1995b). However, for EPE, most therapeutic knowledge has been achieved through clinical experience.

It is postulated that a novel gallium-based compound, gallium maltolate (GaM), might be capable of reaching intracellular bacteria through the oligoelement absorption mechanism (specifically iron, zinc and aluminum) in the small intestine, without generating specific grounds for antimicrobial resistance (Gunther & Wright, 1983; Berry *et al.*, 1984; Caspary, 1992). Elemental gallium (GaIII), a post-transition metal belonging to group IIIA of the periodic table, seems ideal for the so-called 'trojan horse' mechanism, due to an electric charge, ionic radius, valence and electronic footprint similar to ferric iron (FeIII), which is essential to pathogen survival (Bernstein, 1998; Collery *et al.*, 2002). Gallium is preferentially absorbed by phagocytic cells during the inflammatory process and appears to copiously converge at sites of infection, entering macrophages through mechanisms dependent and independent of the iron-transporter transferrin (Tsan, 1986; Chitambar & Zivkovic, 1987). Bacteria take up FeIII, whereas mammalian cells and structures (*e.g.,* heme) use ferrous iron (FeII) and, subsequently, process it through various redox reactions (Logan *et al.*, 1981; Bernstein, 1998). Thus, at sufficiently high concentrations, GaIII can be absorbed by bacteria, altering the first step of the bacterial ferric metabolism (Olakanmi *et al.*, 2000). Unlike iron, gallium is unable to complete a redox-cycling reaction in several iron metabolic pathways and enzymes (*e.g.,* ribonucleotide reductase) necessary for DNA replication and cell functions (Bernstein, 1998). The blockage of these iron-dependent metabolic steps has been clearly ascribed to elemental gallium in *Mycobacteria*, and it was hypothesized in *Rhodococcus equi* (Olakanmi *et al.*, 2000; Harrington *et al.*, 2006). The activity of GaIII appears sufficient to reduce bacterial survival and replication (Bernstein, 1998). In the United States, gallium is approved for treatment of human cancer-related hypercalcemia (citrate-chelated gallium nitrate) and for diagnostic imaging (*i.e.*, radioisotope Ga^{67}) (Chitambar, 2010).

Recently, the antimicrobial activity of several gallium-based compounds was explored for several intracellular bacteria of veterinary interest (Harrington *et al.*, 2006; Fecteau *et al.*, 2011; Arnold *et al.*, 2012). Gallium salts have poor solubility in aqueous solution and are often unsuitable for oral or parenteral administration, due to the formation of insoluble by-products (*e.g.,* gallium hydroxide, gallate), which limits enteral absorption and bioavailability, or causes nephrotoxicity (Bernstein, 1998). GaM is safe following oral administration to humans, mice, rats, dogs, and foals (Bernstein *et al.*, 2000; Martens *et al.*, 2007, 2010) and it is not known to cause nephrotoxicity (Bernstein *et al.*, 2000). Although knowledge of the replication processes of *L. intracellularis* are still incomplete, it is assumed that GaM should deliver GaIII in the vicinity of the bacteria, particularly those in the intestinal brush border (Bernstein, 1998).

This study compared the efficacy of repeated oral dosing with doxycycline (5 mg/kg), the antimicrobial used most commonly to treat EPE or GaM (50 mg/kg), in comparison with untreated controls, in a rabbit EPE infection model (Sampieri *et al.*, 2013a). The dosage regimens were based on the pharmacokinetics of doxycycline in horses (Davis *et al.*, 2006) and GaM in healthy and EPE-infected rabbits (Sampieri *et al.*, 2014), and published evidence for the efficacy of doxycycline against a number of intracellular bacteria (*e.g., N. risticii* and *R. equi*) (Womble *et al.*, 2007).

MATERIAL AND METHODS

This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Animals

A published protocol for inducing lesions with the equine strain *L. intracellularis* in juvenile female rabbits was followed to achieve infection (subjects = 24 New Zealand white rabbits, (*Oryctolagus cuniculi*, Strain – 052 VAF Rabbits; Charles River Canada, Pointe Claire, QC, Canada), 5 to 6-week-old, bodyweight = 1.2–1.5 kg). Rabbits were group-housed in separate pens and monitored for attitude, bodyweight changes, ear hematomas (subsequently to sampling), food and water intake, and fecal consistency (Sampieri *et al.*, 2013a).

Each rabbit was inoculated via nasogastric intubation with a dose of 3.0×10^8 *L. intracellularis* (low-passage cell-culture inoculum of strain E04504, harvested from an EPE-affected foal's ileal mucosa) suspended in 5.5 mL of buffered sucrose-phosphate-glutamate medium, followed by a 3 mL of distilled water. Onset of infection was detected *ante mortem* using current diagnostics for EPE, such as immunoperoxidase monolayer assay (IPMA) serology and TaqMan qPCR on fecal material (Guedes *et al.*, 2002a; Pusterla *et al.*, 2009; Sampieri *et al.*, 2013a). The *postmortem* assessment was based on gross pathology examination, hematoxylin-eosin staining, and immunohistochemistry labeling of *L. intracellularis* with murine anti-*L. intracellularis*-antibody, on samples previously fixed in 10% buffered formalin (Guedes *et al.*, 2002b).

Study design

Immediately prior to administration, gallium maltolate, GaM (provided by L.R.B., Terrametrix, Menlo Park, CA, USA), was dissolved in sterile double-distilled water until a clear solution

was achieved (final concentration: 8 mg/mL, final pH = 5.8–6) (Harrington *et al.*, 2006).

Rabbits were allocated to three treatment groups of eight animals each: doxycycline group (Group D), GaM group (Group G), and control group (Group C). Treatments were started on day 7 postinfection (PI), a time when EPE lesions begin to appear, with a so-called 'infection peak' around 12–14 days PI in the rabbit model (Sampieri *et al.*, 2013a). Group D received doxycycline in an oral apple-flavored suspension, at a 5 mg/kg *q* 24 h dose. Oral administration was chosen because the volume of the suspension was too low (<1 mL) for administration by nasogastric tubing. Group G was administered the GaM solution via nasogastric tube [5 Fr., 40 cm long feeding tube (Kendall Sovereign, Tyco Health Care Group LP, Mansfield, MA, USA)] (Sampieri *et al.*, 2013a) at a 50 mg/kg *q* 48 h dose. Group C received a 6 mL suspension of water and Jell-O (Kraft Foods, Calgary, AB, Canada), administered *q* 48 h by nasogastric intubation, or, after three unsuccessful attempts at nasogastric intubation, orally.

Nasogastric intubation was performed after a local mucosal block (Xylocaine Gel 2%; Astra Zeneca Inc., Missisauga, ON, Canada) was applied to the medial aspect of the nostril of interest, in both Groups C and G. Blood samples were collected using a needle inserted into the ear's central artery, after a local skin block (EMLA Cream; Astra Zeneca Canada Inc., Missisauga, ON, Canada).

Sample collection

Pooled fecal samples were collected daily from each group and frozen at −20 °C until analysis. Arterial blood samples (1 mL) were collected at the time of infection, and then once weekly until the day of euthanasia (day 14 PI) from each doe. Due to a need to examine *L. intracellularis* lesions at 'infection peak', euthanasia was on day 8 of treatment. Necropsy, limited to the gastrointestinal tract, was conducted in all rabbits by examiners blinded to Group C. The gastrointestinal tract, from duodenum to rectum, underwent a visual examination for thickness, discoloration, and type of content, as *L. intracellularis* lesions are not located in any other organ (Sampieri *et al.*, 2013a). Samples of duodenum, midjejunum, and terminal jejunum, ileum, ileocecal valve (including *ampulla coli* and *sacculus rotundus*), cecum (transitional area between cecum and cecal appendix), terminal portion of the cecal *appendix*, large colon (proximal to the *fusum coli*), terminal colon and rectum were collected (Sampieri *et al.*, 2013a). One rabbit in group G died on the last morning of the experiment; its necropsy indicated aspiration pneumonia as cause of death, likely due to an error in treatment administration.

Verification of infection

Histology and immunohistochemistry. Two adjacent, formalin-fixed and paraffin-embedded sections per sample were cut and stained by hematoxylin and eosin and immunohistochemistry with streptavidin method, using murine anti-*L. intracellularis*-specific monoclonal antibody, to detect proliferative lesions of the intestinal epithelium and the presence of the antigen within the cells, respectively. The *L. intracellularis*-specific antigen in the enterocytes was evaluated blindly with a five-grade immunohistochemistry scoring system (with grade 0 equal to no lesions; and grade 4, equal to all enteric crypts affected), as previously reported (Guedes *et al.*, 2002b). For each rabbit, a negative control for each intestinal section consisted of a corresponding immunohistochemistry-labeled tissue section, with the exclusion of the primary antibody. Furthermore, pig ileal tissues known to be negative and positive for *L. intracellularis* infection were labeled with the murine anti-*L. intracellularis* monoclonal antibody to confirm the antibody's specificity and sensitivity, respectively.

Serology. Anti–*L. intracellularis*–specific immunoglobulin (Ig) G titer was measured in serum using IPMA (Guedes *et al.*, 2002a). Positive serum samples were end-point titrated from a dilution of 1:30 up to 1:1920. Control samples consisted of serum from a rabbit prior to (negative control) and after (positive control) hyperimmunization with purified *L. intracellularis*.

Quantitative PCR. qPCR was conducted on fecal samples, as previously reported (Pusterla *et al.*, 2010; Sampieri *et al.*, 2013a). The purified DNA was analyzed by qPCR for presence of *L. intracellularis aspA* gene copies (Pusterla *et al.*, 2009).

Statistics

Statistical analysis was conducted using a commercially available software program (GraphPad Prism 5.4; GraphPad Software, Inc., Software, La Jolla, CA, USA). Contingency tables with Chi-square tests were used for comparison of the immunohistochemistry for results in intestinal tissue sections. One-way analysis of variance (ANOVA) with Bonferroni *posthoc* test was used for [Ga] and [Fe] in intestinal tissues, bodyweight gains, qPCR analysis and serology. Alpha was set at 5%.

RESULTS

Clinical appearance

All rabbits tolerated well the infection, handling, and multiple treatments. Bodyweight gain decreased around 11–12 days PI, although this was not significant ($P = 0.98$). There were no significant changes in food intake, fecal consistency, grooming, and behavior in all groups. A marginal decrease in bodyweight gain was observed in group G on day 8, 10, 12, 14 PI. This was smaller than half the average daily bodyweight gain, observed throughout the experiment, and was attributed to the dosing volume (often over 10 mL), as observed previously (Sampieri *et al.*, 2014) (Fig. 1a, b).

Gross pathology

Lesions consistent with *L. intracellularis* infection were visible in jejunum and ileum, but they appeared less thickened and edematous in group D. Such changes were consistent with

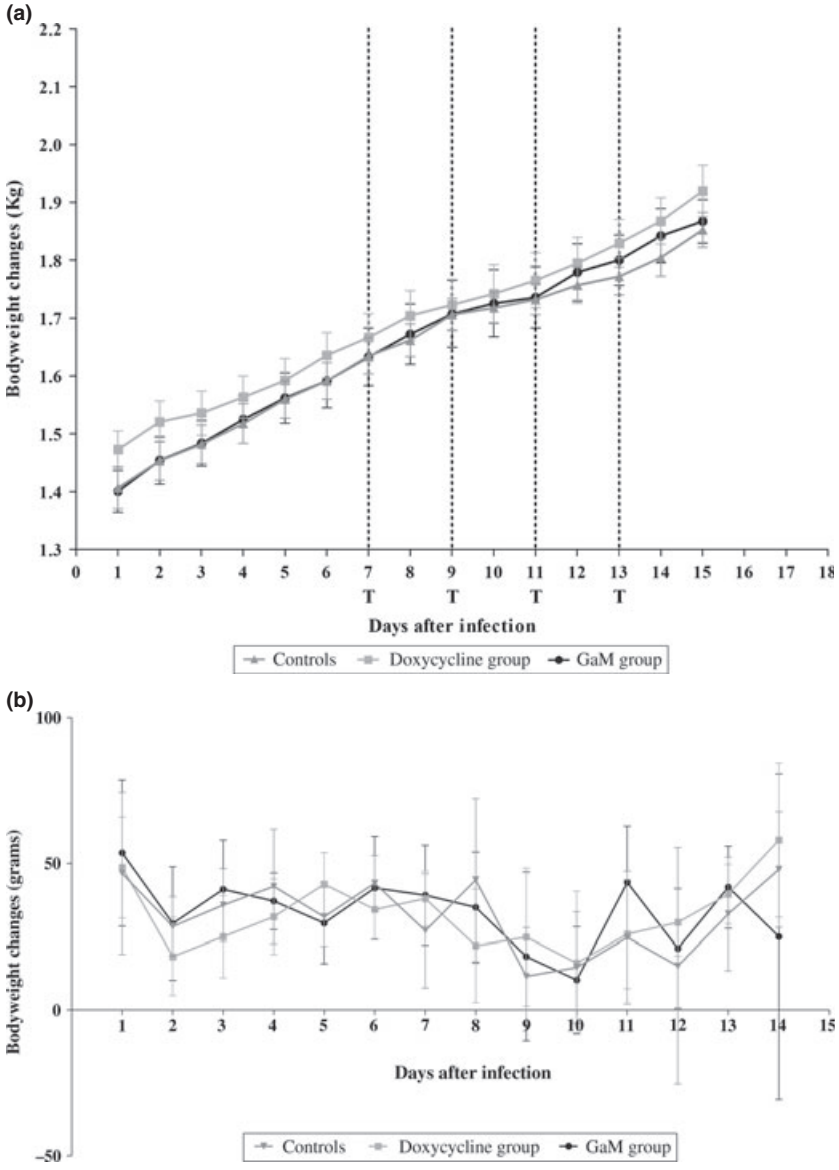

Fig. 1. (a) Mean ± SD bodyweight gain (kg) of rabbits (n = 8/group) inoculated orally with *Lawsonia intracellularis* and treated with vehicle-only (Group C or controls) (6 mL every second day), doxycycline (Group D) (5 mg/kg once daily) or gallium maltolate (Group G) (50 mg/kg every second day) by nasogastric tube, beginning at 7 days PI. No significant difference was noted between the three groups. In all groups, a reduction of bodyweight gain was noted between 8 and 12 days PI. The dashed vertical lines and 'T' indicate gallium maltolate or vehicle-only treatment. (b) Mean ± SD daily bodyweight changes (in grams) in rabbits (n = 8/group) inoculated orally with *L. intracellularis* and treated with vehicle-only (Group C or controls) (6 mL every second day), doxycycline (Group D) (5 mg/kg once daily) or gallium maltolate (Group G) (50 mg/kg every second day) by nasogastric tube beginning 7 days PI. Note the trend in suppression of bodyweight gain between 8 and 12 days PI.

partial recovery of the lesions on the serosa, particularly on the antimesenteric aspect, of the jejunum. This was an unexpected finding and it was not quantified.

Serology

All antibody titers were negative at 7 days PI (low titer: 60), except for 1 group G rabbit. Seroconversion was demonstrated in all rabbits by 14 days PI, with titers ranging between, 120 to >1920 in group D, 480 to >1920 in group G, and 480 to >1920 in group C, $P = 0.56$, confirming the presence of infection and an immune response, as previously published (Sampieri *et al.*, 2013a).

Histology and immunohistochemistry

Typical lesions were confirmed in jejunum (Group D: three rabbits; Group G: one rabbit; Group C: two rabbits, respectively), ileum (Group D: two rabbits; Group G: one rabbit; Group C: one rabbit, respectively), and cecum (Group D: no lesions; Group G: one rabbit, Group C: no lesions, respectively), and were not significantly different between treatments (for jejunum, $P = 0.51$; for ileum, $P = 0.74$; for cecum, $P = 0.35$). In summary, 62.5% of group D, 37.5% of group G and 37.5% of group C rabbits demonstrated lesions when all three sections with positive immunohistochemistry-labeled lesions were counted.

PCR

Fecal shedding of *L. intracellularis* was noted inconsistently starting at 2 days PI in two groups and consistently from 5 days PI in all groups, and persisted for the rest of the study (Sampieri *et al.*, 2013a). There were no statistically significant differences in daily assessments between groups ($P = 0.61$) (Fig. 2) (Sampieri *et al.*, 2013a), among all groups, considering

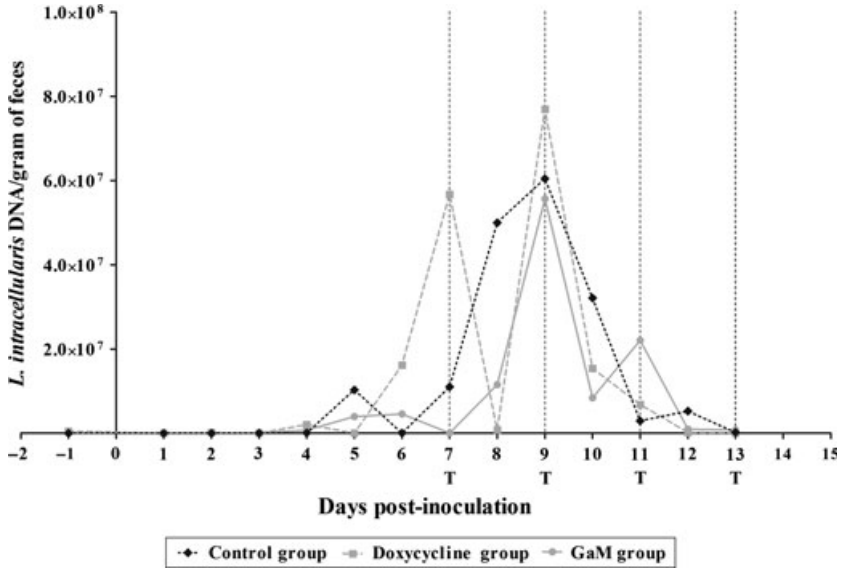

Fig. 2. qPCR results reporting daily fecal *Lawsonia intracellularis* DNA levels, in rabbits inoculated orally with *L. intracellularis* and treated with vehicle-only (Group C or controls) (6 mL every second day), doxycycline (Group D) (5 mg/kg once daily) or gallium maltolate (Group G) (50 mg/kg every second day) by nasogastric tube, beginning at 7 days PI. Fresh feces were collected as pooled samples for each group. The dashed vertical lines and 'T' indicate gallium maltolate or vehicle-only treatment.

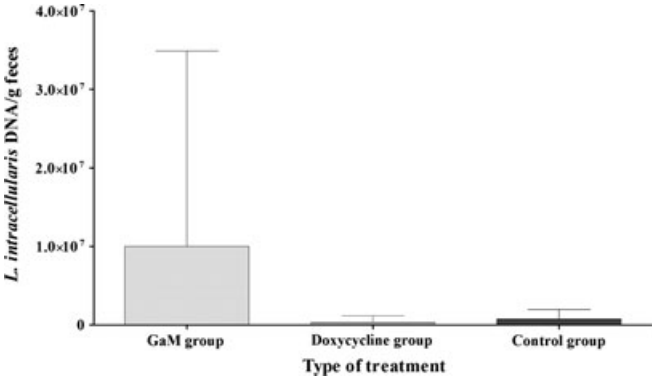

Fig. 3. Mean ± SD of qPCR results of fecal *Lawsonia intracellularis* DNA concentrations collected at necropsy in three groups of rabbits (n = 8/group), after inoculation with *L. intracellularis* and 7 days treatment with vehicle-only (Group C or controls) (6 mL every second day), doxycycline (Group D) (5 mg/kg once daily) or gallium maltolate (Group G or GaM) (50 mg/kg every second day) by nasogastric tube. Although not statistically different, fecal *L. intracellularis* DNA concentration levels for both Groups C and D were lower than Group G, but still in the range 10^6 to $>10^7$.

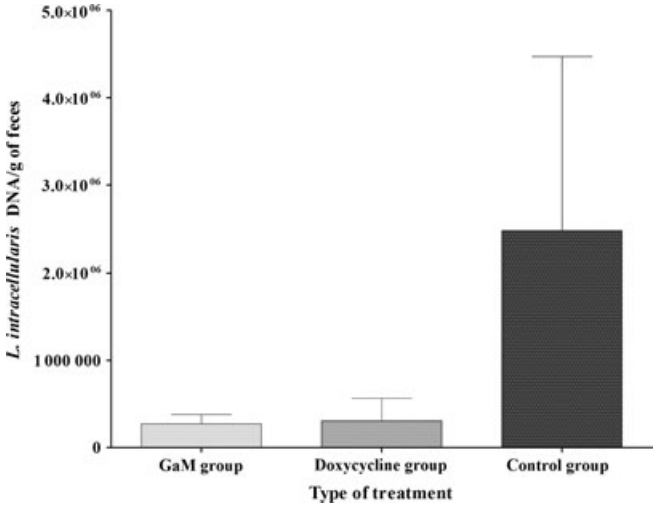

Fig. 4. Mean ± SD of qPCR results of cecal content *Lawsonia intracellularis* DNA concentrations collected at necropsy in three groups of rabbits (n = 8/group), after inoculation with *L. intracellularis* and 7 days treatment with vehicle-only (Group C or controls) (6 mL every second day), doxycycline (Group D) (5 mg/kg once daily) or gallium maltolate (Group G or GaM) (50 mg/kg every second day) by nasogastric tube. Although not statistically different, cecal *L. intracellularis* DNA concentration for both Groups D and G were lower than Group C, but still in the range 10^5 to $>10^6$, showing that untreated animals may carry (and shed) live bacteria in their intestines, contaminating the environment with low to undetectable amounts of bacteria for a long time.

the amount of *L. intracellularis* DNA detected in the feces (P = 0.64) (Fig. 3), and in the cecal content (P = 0.32) at the time of euthanasia (Fig. 4).

DISCUSSION

This study is the first to compare the efficacy of antimicrobial treatments for EPE in an infection model. Repeated oral bolus doses of doxycycline, GaM, or vehicle-only were clinically well tolerated by EPE-infected rabbits. However, no differences were detected through several diagnostic methodologies between treatment outcomes. Our data suggests that GaM and doxycycline have similar efficacy in a rabbit model of EPE.

While developing this rabbit model, we have repeatedly observed a depression in bodyweight gain, on or around 12 days PI, corresponding to infection peak (Sampieri *et al.*, 2013a). In the present study, we expected the added stress associated with the repeated dosage regimen (*i.e.,* repeated nasogastric intubation and dosing) to affect bodyweight gain more significantly (*i.e.,* remaining well below ≈28.5 g/day in 5 to 6-week-old does [Charles River Canada]) (Sampieri *et al.*,

2013a), but this was not observed. Furthermore, treatment with GaM or doxycycline did not alter this depression in body-weight gain, demonstrating that, in spite of treatment, EPE-infected animals still lose body condition (Frazer, 2008; Wong *et al.*, 2009). It appears that the changes in weight gain were more pronounced in Group G during treatment days, presumably due to the large volumes administered. However, the overall weight gain was similar in all groups.

We used a rabbit model to compare antimicrobial efficacy against EPE infection because the rabbit represents a 'time-compressed' model, as specific IgG antibodies against *L. intracellularis* are typically detectable in rabbits within 14 days PI, and occasionally from 7 days PI (Sampieri *et al.*, 2013a,b), unlike the foal model of EPE (Pusterla *et al.*, 2010).

The present study used rabbits that were 3 weeks younger than in our previous experiments (Sampieri *et al.*, 2013a), as younger animals were hypothesized to develop more severe lesions, due to their immature gastrointestinal tract and lower immuno-competence (Pakandl *et al.*, 2008). Weaned rabbits are known to mount a sufficient immune response when challenged with infectious agents, although this depends on infection type and load (Guedes & Gebhart, 2003; Pakandl *et al.*, 2008). It is not currently known if there are age-related changes in immune-competence related to *L. intracellularis* in suckling rabbits. Rabbits in all groups developed a pronounced immune response to *L. intracellularis* infection, with IgG titers comparable, or higher, to those observed previously in 8–9 weeks old rabbits. This may explain the low incidence of severe lesions on pathological and immunohistochemical analyses.

An additional consideration is age. The variation between the present study and the previous research on the same model suggests a potential difference between the maturation of systemic (IgG) and local (IgA and IgE) immune systems and how it affects the response to infection in recently weaned rabbits. Undoubtedly, such a consideration warrants further research (not only in rabbits), as similar findings were obtained in porcine models of PPE (personal communication of Dr. C.J. Gebhart – University of Minnesota). Interestingly, gross pathological examination of the intestines suggested that doxycycline-treated rabbits showed an apparently faster return to normal of the affected digestive tract, but this was not supported by immunohistochemistry. Reductions in serosal edema and wall thickness were observed following doxycycline therapy moving progressively from proximal to distal small intestine, and this should be investigated further.

Multiple GaM doses failed to decrease fecal shedding of *L. intracellularis* DNA (Fig. 3) and fecal shedding rates were similar to those identified in our original studies (Sampieri *et al.*, 2013a). Interestingly, the concentrations of *L. intracellularis* DNA in feces were not significantly different in the three groups, although Group G showed somewhat increased values. Quantitative PCR detects DNA material (from live and dead bacteria alike), confirming the occurrence of bacterial replication within the intestine (concentrations range: 10^5–10^7) during antimicrobial therapy with varying concentrations in different sections of the intestine. Higher fecal shedding of DNA in antimicrobial treated rabbits may be related to faster inactivation and subsequent expulsion of bacteria. However, this finding contradicts the reported association of antimicrobial treatment and decreased bacterial fecal shedding, which prompts clinicians to diligently collect fecal samples before therapy initiation, to increase diagnostic accuracy (Dauvillier *et al.*, 2006). In rabbits, the higher cecal concentrations of *L. intracellularis* of Group C could be related to *L. intracellularis* colonization in the cecum (Duhamel *et al.*, 1998; Horiuchi *et al.*, 2008), examined even more closely in the context of the EPE model studies. A better detailing of such differences between the rabbit model and horses would be useful to understand the true implications of therapeutic responses, or lesions (and clinical signs) development. At present, a detailed understanding of differences in bacterial concentrations of the foal's gastrointestinal tract sections is not available (Pusterla *et al.*, 2010).

The results obtained through immunohistochemistry (Guedes *et al.*, 2002b) showed no significant differences between the count of gut lesion or the percentage of rabbits affected between groups. It is important to consider the location of the immunohistochemistry labeling within the gut mucosa, in view of the effects of age (in terms of further time-compression) in this infection model. Lesion location allows us to approximate the time of lesion appearance closer to either the time of onset or the time of recovery. This type of differentiation can highlight underlying differences between the affected groups (Sampieri *et al.*, 2013a). In Group D (62.5%) and Group G (37.5%), immunohistochemistry-labeled lesions clusters were principally located in the mucosa and still progressing toward the *lamina propria.* These findings suggest an important delay in lesion development and progression, ascribable to antimicrobial therapy, likely explaining the enhanced *L. intracellularis* fecal shedding in Group G at time of euthanasia, as active lesions remained present in the gut. On the contrary in Group C, lesions were found only in 37.5% rabbits, but immunohistochemistry labeling was detected at the level of the *lamina propria*, where bacteria are processed and destroyed. This suggests that these rabbits were in the recovery phase, typically observed around 21 days PI, in 9–10 weeks old rabbits (Sampieri *et al.*, 2013a). In our model, timing of euthanasia with duration of treatment was crucial, for both the study design and the understanding of lesion progression and fecal shedding of *L. intracellularis,* after the initiation of antimicrobial therapy.

In this study, lesion remission occurred more rapidly than our previous study (14 vs. 21 days PI). This observation may relate to the age difference of the rabbits used in each study. Possibly, younger rabbits exposed to EPE proceed more quickly from 'onset' to 'remission' than older does (9–10 weeks), showing fecal shedding and seroconversion as expected, but 'compressing' even further the timeframe of lesion appearance (Sampieri *et al.*, 2013a). This concept may have epidemiological relevance for the contamination of horse premises (Pusterla *et al.*, 2012), but it was an unwanted complication for our study.

This comparative study calls into question the use of GaM as a potential treatment for EPE. Recent studies showed that prophylactic treatment with GaM prior to experimental infection, at a dose capable of achieving the minimum inhibitory concentration (MIC), was unable to prevent *R. equi* associated disease (Harrington *et al.*, 2006; Coleman *et al.*, 2010; Chaffin *et al.*, 2011). For *L. intracellularis*, where *in vitro* susceptibility studies are very difficult (Lawson *et al.*, 1993), our use of a rabbit model of EPE clearly indicated a delay in lesion development following GaM therapy, suggesting that it is unlikely that GaM prophylactic treatment could have better therapeutic results, unlike what could have been expected with the *R. equi*-infected foals (Chaffin *et al.*, 2011). Until *in vitro* studies clearly demonstrate the sensitivity of *L. intracellularis* to GaM, we suggest that further studies *in vivo* are not warranted and would conflict with replacement, reduction and refinement of animal use in scientific studies (Fenwick *et al.*, 2009).

Although our rabbit model represents a mild form of EPE infection, GaM offered no treatment advantage over doxycycline. There was no evidence to support the use of repeated doses of GaM, at 50 mg/kg (2.5 times the dose used in foals). Further investigation with GaM in EPE-infected foals is not warranted at this time. Until *in vitro* MIC studies are available and suggest that GaM should be re-evaluated in foals, we recommend that EPE-infected foals be treated with the current standard of care.

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REFERENCES

Arnold, C.E., Bordin, A., Lawhon, S.D., Libal, M.C., Bernstein, L.R. & Cohen, N.D. (2012) Antimicrobial activity of gallium maltolate against *Staphylococcus aureus* and methicillin-resistant *S. aureus* and *Staphylococcus pseudintermedius*: an *in vitro* study. *Veterinary Microbiology*, **155**, 389–394.

Bernstein, L.R. (1998) Mechanisms of therapeutic activity for gallium. *Pharmacology Reviews*, **50**, 665–682.

Bernstein, L.R., Tanner, T., Godfrey, C. & Noll, B. (2000) Chemistry and pharmacokinetics of gallium maltolate, a compound with high oral gallium bioavailability. *Metal Based Drugs*, **7**, 33–47.

Berry, J.P., Poupon, M.F., Galle, S. & Escaig, F. (1984) Role of lysosomes in gallium concentration by mammalian tissues. *Biologie of the Cellulaire*, **51**, 43–51.

Caspary, W.F. (1992) Physiology and pathophysiology of intestinal absorption. *American Journal of Clinical Nutrition*, **55**, 299S–308S.

Chaffin, M.K., Cohen, N.D., Martens, R.J., O'Conor, M. & Bernstein, L.R. (2011) Evaluation of the efficacy of gallium maltolate for chemoprophylaxis against pneumonia caused by *Rhodococcus equi* infection in foals. *American Journal of Veterinary Research*, **72**, 945–957.

Chitambar, C.R. (2010) Medical applications and toxicities of gallium compounds. *International Journal of Environmental Research and Public Health*, **7**, 2337–2361.

Chitambar, C.R. & Zivkovic, Z. (1987) Uptake of gallium-67 by human leukemic cells: demonstration of transferrin receptor-dependent and transferrin-independent mechanisms. *Cancer Research*, **47**, 3929–3934.

Coleman, M., Kuskie, K., Liu, M., Chaffin, K., Libal, M., Giguere, S., Bernstein, L. & Cohen, N. (2010) *In vitro* antimicrobial activity of gallium maltolate against virulent *Rhodococcus equi*. *Veterinary Microbiology*, **146**, 175–178.

Collery, P., Keppler, B., Madoulet, C. & Desoize, B. (2002) Gallium in cancer treatment. *Critical Reviews in Oncology/Hematology*, **42**, 283–296.

Dauvillier, J., Picandet, V., Harel, J., Gottschalk, M., Desrosiers, R., Jean, D. & Lavoie, J.P. (2006) Diagnostic and epidemiological features of *Lawsonia intracellularis* enteropathy in 2 foals. *Canadian Veterinary Journal*, **47**, 689–691.

Davis, J.L., Salmon, J.H. & Papich, M.G. (2006) Pharmacokinetics and tissue distribution of doxycycline after oral administration of single and multiple doses in horses. *American Journal of Veterinary Research*, **67**, 310–316.

Duhamel, G.E., Klein, E.C., Elder, R.O. & Gebhart, C.J. (1998) Subclinical proliferative enteropathy in sentinel rabbits associated with *Lawsonia intracellularis*. *Veterinary Pathology*, **35**, 300–303.

Feary, D.J., Gebhart, C.J. & Pusterla, N. (2007) *Lawsonia intracellularis* proliferative enteropathy in a foal. *Schweizer Archiv für Tierheilkunde*, **149**, 129–133.

Fecteau, M.E., Whitlock, R.H., Fyok, T.L., McAdams, S.C., Boston, R.C. & Sweeney, R.W. (2011) Antimicrobial activity of gallium nitrate against *Mycobacterium avium subsp. paratuberculosis* in neonatal calves. *Journal of Veterinary Internal Medicine*, **25**, 1152–1155.

Fenwick, N., Griffin, G. & Gauthier, C. (2009) The welfare of animals used in science: how the "Three Rs" ethic guides improvements. *Canadian Veterinary Journal*, **50**, 523–530.

Frazer, M.L. (2008) *Lawsonia intracellularis* infection in horses: 2005–2007. *Journal of Veterinary Internal Medicine*, **22**, 1243–1248.

Guedes, R.M. & Gebhart, C.J. (2003) Preparation and characterization of polyclonal and monoclonal antibodies against *Lawsonia intracellularis*. *Journal of Veterinary Diagnostic and Investigation*, **15**, 438–446.

Guedes, R.M., Gebhart, C.J., Deen, J. & Winkelman, N.L. (2002a) Validation of an immunoperoxidase monolayer assay as a serologic test for porcine proliferative enteropathy. *Journal of Veterinary Diagnostic and Investigation*, **14**, 528–530.

Guedes, R.M., Gebhart, C.J., Winkelman, N.L., Mackie-Nuss, R.A., Marsteller, T.A. & Deen, J. (2002b) Comparison of different methods for diagnosis of porcine proliferative enteropathy. *Canadian Journal of Veterinary Research*, **66**, 99–107.

Gunther, R.D. & Wright, E.M. (1983) Na^+, Li^+, and Cl^- transport by brush border membranes from rabbit jejunum. *Journal of Membrane Biology*, **74**, 85–94.

Harrington, J.R., Martens, R.J., Cohen, N.D. & Bernstein, L.R. (2006) Antimicrobial activity of gallium against virulent *Rhodococcus equi in vitro* and *in vivo*. *Journal of Veterinary Pharmacology and Therapeutics*, **29**, 121–127.

Herbst, W., Hertrampf, B., Schmitt, T., Weiss, R. & Baljer, G. (2003) Diagnosis of *Lawsonia intracellularis* using the polymerase chain reaction (PCR) in pigs with and without diarrhea and other animal species. *Deutsche Tierarztliche Wochenschrift*, **110**, 361–364.

Horiuchi, N., Watarai, M., Kobayashi, Y., Omata, Y. & Furuoka, H. (2008) Proliferative enteropathy involving *Lawsonia intracellularis* infection in rabbits (*Oryctlagus cuniculus*). *Journal of Veterinary Medical Science*, **70**, 389–392.

La Regina, M., Fales, W.H. & Wagner, J.E. (1980) Effects of antibiotic treatment on the occurrance of experimentally induced proliferative ileitis of hamsters. *Laboratory Animal Science*, **30**, 38–41.

Lavoie, J.P., Drolet, R., Parsons, D., Leguillette, R., Sauvageau, R., Shapiro, J., Houle, L., Halle, G. & Gebhart, C.J. (2000) Equine proliferative enteropathy: a cause of weight loss, colic, diarrhoea and hypoproteinaemia in foals on three breeding farms in Canada. *Equine Veterinary Journal*, **32**, 418–425.

Lawson, G.H. & Gebhart, C.J. (2000) Proliferative enteropathy. *Journal of Comparative Pathology*, **122**, 77–100.

Lawson, G.H., McOrist, S., Jasni, S. & Mackie, R.A. (1993) Intracellular bacteria of porcine proliferative enteropathy: cultivation and maintenance *in vitro*. *Journal of Clinical Microbiology*, **31**, 1136–1142.

Logan, K.J., Ng, P.K., Turner, C.J., Schmidt, P.R., Terner, U.K., Scott, J.R., Lentle, B.C. & Noujaim, A.A. (1981) Comparative pharmacokinetics of 67 Ga and 59Fe in humans. *International Journal of Nuclear Medicine and Biology*, **8**, 271–276.

Martens, R.J., Mealey, K., Cohen, N.D., Harrington, J.R., Chaffin, M.K., Taylor, R.J. & Bernstein, L.R. (2007) Pharmacokinetics of gallium maltolate after intragastric administration in neonatal foals. *American Journal of Veterinary Research*, **68**, 1041–1044.

Martens, R.J., Cohen, N.D., Fajt, V.R., Nerren, J.R., Chaffin, M.K., Taylor, R.J. & Bernstein, L.R. (2010) Gallium maltolate: safety in neonatal foals following multiple enteral administrations. *Journal of Veterinary Pharmacology and Therapeutics*, **33**, 208–212.

McOrist, S., Gebhart, C.J., Boid, R. & Barns, S.M. (1995a) Characterization of *Lawsonia intracellularis gen*. nov., sp. nov., the obligately intracellular bacterium of porcine proliferative enteropathy. *International Journal of Systematic Bacteriology*, **45**, 820–825.

McOrist, S., Mackie, R.A. & Lawson, G.H. (1995b) Antimicrobial susceptibility of ileal symbiont intracellularis isolated from pigs with proliferative enteropathy. *Journal of Clinical Microbiology*, **33**, 1314–1317.

Moulder, J.W. (1985) Comparative biology of intracellular parasitism. *Microbiology Reviews*, **49**, 298–337.

Olakanmi, O., Britigan, B.E. & Schlesinger, L.S. (2000) Gallium disrupts iron metabolism of mycobacteria residing within human macrophages. *Infection and Immunity*, **68**, 5619–5627.

Page, A.E., Fallon, L.H., Bryant, U.K., Horohov, D.W., Luna, T.W., Marsh, P.S., Slovis, N.M., Sprayberry, K.A. & Loynachan, A.T. (2012) Acute deterioration and death with necrotizing enteritis associated with *Lawsonia intracellularis* in 4 weanling horses. *Journal of Veterinary Internal Medicine*, **26**, 1476–1480.

Pakandl, M., Hlaskova, L., Poplstein, M., Chroma, V., Vodicka, T., Salat, J. & Mucksova, J. (2008) Dependence of the immune response to coccidiosis on the age of rabbit suckling. *Parasitol Research*, **103**, 1265–1271.

Pusterla, N. & Gebhart, C. (2009) Equine proliferative enteropathy caused by *Lawsonia intracellularis*. *Equine Veterinary Education*, **8**, 415–418.

Pusterla, N., Jackson, R., Wilson, R., Collier, J., Mapes, S. & Gebhart, C. (2009) Temporal detection of *Lawsonia intracellularis* using serology and real-time PCR in Thoroughbred horses residing on a farm endemic for equine proliferative enteropathy. *Veterinary Microbiology*, **136**, 173–176.

Pusterla, N., Wattanaphansak, S., Mapes, S., Collier, J., Hill, J., DiFrancesco, M. & Gebhart, C. (2010) Oral infection of weanling foals with an equine isolate of *Lawsonia intracellularis*, agent of equine proliferative enteropathy. *Journal of Veterinary Internal Medicine*, **24**, 622–627.

Pusterla, N., Sanchez-Migallon Guzman, D., Vannucci, F.A., Mapes, S., White, A., DiFrancesco, M. & Gebhart, C. (2012) Transmission of *Lawsonia intracellularis* to weanling foals using feces from experimentally infected rabbits. *Veterinary Journal*, **195**, 241–243.

Sampieri, F., Hinchcliff, K.W. & Toribio, R.E. (2006) Tetracycline therapy of *Lawsonia intracellularis* enteropathy in foals. *Equine Veterinary Journal*, **38**, 89–92.

Sampieri, F., Allen, A.L., Pusterla, N., Vannucci, F.A., Antonopoulos, A.J., Ball, K.R., Thompson, J., Dowling, P.M., Hamilton, D.L. & Gebhart, C.J. (2013a) The rabbit as an infection model for equine proliferative enteropathy. *Canadian Journal of Veterinary Research*, **77**, 110–119.

Sampieri, F., Vannucci, F.A., Allen, A.L., Pusterla, N., Antonopoulos, A.J., Ball, K.R., Thompson, J., Dowling, P.M., Hamilton, D.L. & Gebhart, C.J. (2013b) Species-specificity of equine and porcine *L. intracellularis* isolates in laboratory animals. *Canadian Journal of Veterinary Research*, **77**, 261–272.

Sampieri, F., Alcorn, J., Allen, A.L., Clark, C.R., Vannucci, F.A., Pusterla, N., Mapes, S., Ball, K.R., Dowling, P.M., Thompson, J., Bernstein, L.R., Gebhart, C.J. & Hamilton, D.L. (2014) Pharmacokinetics of gallium maltolate in *Lawsonia intracellularis*-infected and uninfected rabbits. *Journal of Veterinary Pharmacology and Therapeutics*, doi: 10.1111/jvp.12114.

Schumacher, J., Schumacher, J., Rolsma, M., Brock, K.V. & Gebhart, C.J. (2000) Surgical and medical treatment of an Arabian filly with proliferative enteropathy caused by *Lawsonia intracellularis*. *Journal of Veterinary Internal Medicine*, **14**, 630–632.

Tsan, M.F. (1986) Mechanism of gallium-67 accumulation in inflammatory lesions. *Journal of Nuclear Medicine*, **26**, 88–92.

Wattanaphansak, S., Singer, R.S. & Gebhart, C.J. (2009) *In vitro* antimicrobial activity against 10 North American and European *Lawsonia intracellularis* isolates. *Veterinary Microbiology*, **134**, 305–310.

Womble, A., Giguere, S. & Lee, E.A. (2007) Pharmacokinetics of oral doxycycline and concentrations in body fluids and bronchoalveolar cells of foals. *Journal of Veterinary Pharmacology and Therapeutics*, **30**, 187–193.

Wong, D.M., Alcott, C.J., Sponseller, B.A., Young, J.L. & Sponseller, B.T. (2009) Impaired intestinal absorption of glucose in 4 foals with *Lawsonia intracellularis* infection. *Journal of Veterinary Internal Medicine*, **23**, 940–944.