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

F. SAMPIERI*
A. L. ALLEN*
J. ALCORN†
C. R. CLARK*
F. A. VANNUCCI‡
N. PUSTERLA§
S. M. MAPES§
K. R. BALL*
P. M. DOWLING*
J. THOMPSON¶
L. R. BERNSTEIN**
C. J. GEBHART‡ &
D. L. HAMILTON*

*R: Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada; †: College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK, Canada; ‡: College of Veterinary Medicine, University of Minnesota, Saint Paul, MN, USA; §: School of Veterinary Medicine, University of California, Davis, CA, USA; *: Canadian Light Source, Saskatoon, SK, Canada; **: Terrametrix, Menlo Park, CA, USA

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 enterocyte 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 (Tian, 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 Ga67) (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, body-weight = 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., Terramatrix, 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, orally, 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, mid-jejunum, and terminal jejenum, 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 post hoc 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
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...
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 $\approx 28.5$ g/day in 5 to 6-week-old does [Charles River Canada]) (Sampieri et al.,...
Undoubtedly, such a consideration warrants further research 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 serum 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; Horiiuchi 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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