### Title

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### Journal

Canadian Journal of Veterinary Research, 77(4)

### ISSN

0830-9000



### Authors

Sampieri, Francesca
Vannucci, Fabio A
Allen, Andrew L
et al.



### Publication Date

2013-10-01

Peer reviewed

# Species-specificity of equine and porcine *Lawsonia intracellularis* isolates in laboratory animals

Francesca Sampieri, Fabio A. Vannucci, Andrew L. Allen, Nicola Pusterla, Aphroditi J. Antonopoulos, Katherine R. Ball, Julie Thompson, Patricia M. Dowling, Don L. Hamilton, Connie J. Gebhart

## Abstract

*Lawsonia intracellularis* infection causes proliferative enteropathy (PE) in many mammalian species, with porcine and equine proliferative enteropathy (PPE and EPE) known worldwide. Hamsters are a well-published animal model for PPE infection studies in pigs. There is no laboratory animal model for EPE infection studies and it is not known whether there is species-specificity for equine or porcine isolates of *L. intracellularis* in animal models. The objective of this study was to determine whether it is possible to generate typical EPE lesions in hamsters after inoculation with an equine strain of *L. intracellularis* (EPE strain) and whether it is comparatively possible to generate PPE lesions in rabbits after inoculation with a porcine strain of *L. intracellularis* (PPE strain). In 2 separate trials, 4-week-old and 3-week-old weanling golden Syrian hamsters were challenged with EPE strains and compared to uninfected (both trials) and PPE-infected controls (Trial 2 only). Concurrently, 6 female New Zealand white juvenile rabbits were infected with PPE strain and observed concomitantly to 8 similar rabbits infected with EPE strain for a different experiment. Hamsters and rabbits were observed for 21 to 24 days post-infection (DPI), depending on the experiment. Neither infected species developed clinical signs. The presence of disease was assessed with diagnostic techniques classically used for pigs and horses: immune-peroxidase monolayer assay on sera; quantitative polymerase chain reaction (qPCR) detection of molecular DNA in feces; and hematoxylin and eosin (H&E) stain and immunohistochemistry (IHC) on intestinal tissues. Our results showed that EPE-challenged hamsters do not develop infection when compared with PPE controls (IHC, $P = 0.009$; qPCR, $P = 0.0003$). Conversely, PPE-challenged rabbits do not develop typical intestinal lesions in comparison to EPE-challenged rabbits, with serological response at 14 DPI being significantly lower ($P = 0.0023$). In conclusion, PPE and EPE strains appear to have different host-specificities for hamsters and rabbits, respectively.

## Résumé

*L'infection par* Lawsonia *intracellularis provoque une entéropathie prolifération chez de nombreuses espèces de mammifères; celle des porcins (EPP) et des équidés (EEP) sont connues mondialement. Les hamsters sont un modèle animal bien connu pour l'étude de l'EPP. Il n'existe pas de modèle animal de laboratoire pour étudier l'EEP, et on ne sait pas s'il y a spécificité d'espèce pour les isolats équins ou porcins de* L. intracellularis *dans des modèles animaux. L'objectif de la présente étude était de déterminer s'il est possible de générer des lésions typiques d'EEP chez les hamsters après inoculation d'une souche équine de L. intracellularis (souche EEP) et s'il est également possible de générer des lésions d'EPP chez des lapins après inoculation d'une souche porcine de L. intracellularis (souche EPP). Dans 2 essais séparés, des hamsters dorés syriens sevrés âgés de 4 semaines et de 3 semaines ont été inoculés avec des souches EEP, et ont été comparés à des témoins non infectés (les deux essais) et à des témoins infectés avec EPP (essai 2 seulement). Parallèlement, 6 jeunes lapines Nouvelle-Zélande ont été infectées par la souche EEP et observées de façon concomitante à 8 lapins similaires infectés par la souche EPP pour une expérience différente. Les hamsters et les lapins ont été observés pendant 21 à 24 jours après l'infection (JAI), en fonction de l'expérience. Aucune des espèces infectées n'a développé de signes cliniques. La présence de maladie a été évaluée par des techniques classiques de diagnostic utilisées pour les porcs et les chevaux : l'essai par immuno-peroxydase sur monocouche pour les sérums; la détection par réaction d'amplification en chaîne par la polymérase quantitative (qPCR) de l'ADN moléculaire dans les selles; la coloration hématoxyline-éosine et l'immunohistochimie (IHC) sur des tissus intestinaux. Nos résultats ont montré que les hamsters inoculés avec EEP ne développent pas d'infection comparativement aux EPP témoins (IHC* P *= 0,009; qPCR* P *= 0,0003). À l'inverse, les lapins inoculés avec EPP ne développent pas des lésions intestinales typiques comparativement aux lapins inoculés avec EEP, avec une réponse sérologique à 14 JAI significativement plus faible (*P *= 0,0023). En conclusion, les souches d'EPP et d'EEP semblent avoir des spécificités d'hôte différentes chez les hamsters et les lapins, respectivement.*

*(Traduit par Dr. J.M. Dhillon)*

## Introduction

*Lawsonia intracellularis* is a Gram-negative, obligate intracellular bacterium infecting the enterocytes of the large or small intestines of a wide range of domestic, wildlife, avian, and laboratory animal species (1–5). An intense proliferation is induced by intra-cytoplasmic replication of *L. intracellularis* in the host's enterocytes, principally in the jejunum and ileum, wherein the hyperplasic activity corresponds

Department of Veterinary Biomedical Sciences (Sampieri, Antonopoulos, Ball, Dowling, Hamilton) and Department of Veterinary Pathology (Allen), Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B4; Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota, Saint Paul, Minnesota 55108, USA (Vannucci, Gebhart); Department of Veterinary Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, California 95616, USA (Pusterla); Canadian Light Source, Saskatoon, Saskatchewan (Thompson).

Address all correspondence to Francesca Sampieri; telephone: (502) 727-3460; fax: (270) 767-7540; e-mail: francesca.sampieri@usask.ca or fsampierivet@hotmail.com

Received July 19, 2012. Accepted November 1, 2012.

to loss of function. Such lesions cause a variety of acute and chronic clinical signs in affected individuals, ranging from malabsorption, diarrhea, depression, weight loss, abdominal pain, and even death (6–8). It is known that *L. intracellularis* causes porcine and equine proliferative enteropathies (PPE and EPE, respectively): the former represented a challenge for the swine industry for more than 50 y; the latter emerged more recently and, after sporadic and isolated beginnings, is now diffused worldwide (1,8–10). Proliferative enteropathies adversely affect both the porcine and equine industries and their impact goes beyond the presence of challenging clinical signs, as slow and long recoveries or severely reduced growth performance are common consequences (5,8,10).

Proliferative enteropathy has been studied experimentally in pigs for years, using either porcine *L. intracellularis* isolates grown *in vitro* or porcine tissues (11–12). Similarly, among laboratory animals, hamsters have been known to be naturally and experimentally affected by the disease since long before *L. intracellularis* was defined and named in 1995 (13). Naturally affected hamsters undergo diarrhea (also known as "wet-tail") and weight loss, with severe hyperplasia of the ileal segment in the small intestine, which is similar to lesions observed in pigs (14). Hamsters have therefore been used as an animal model for PPE studies, whether infection is produced through ileal homogenates harvested from naturally diseased pigs or bacteria grown in pure cell culture (7,14–19). A few researchers have attempted to infect other rodents (mice and rats) and chickens, with variable success (20–22). Unlike PPE studies, the research on EPE animal models is limited to a recently established foal model and to the preliminary results of a rabbit model, both obtained from the same virulent EPE strain (23–25).

Due to the increasing costs of research for horses and pigs, which are the main species affected, using laboratory animal models instead of large animals could reduce expenses. Standardized welfare conditions could still be maintained and environmental and infection control could be maximized over larger populations at one time, which could potentiate the statistical value of results. Although the "3Rs" tenet (replacement, reduction, and refinement) of the Canadian Council for Animal Care (CCAC) is meant to responsibly control the use of animals in research rather than reduce experimental costs, animal models are advocated when *in-vitro* models have great limitations, as in the case of *L. intracellularis* (26,27). The studies described here were part of a larger project investigating EPE in relation to a novel pharmaceutical compound, which contains the post-transition metal gallium, whose environmental impact after excretion has not been clarified (28). To that end, modeling EPE on smaller surrogate animals was considered to be liberally in agreement with the "3Rs" tenet.

Our interest was to verify whether *L. intracellularis* exhibits a species-restricted host-susceptibility or if one given strain could generate infection in multiple species (4,5). Understanding the role of cross-infection in the pathogenesis of proliferative enteropathy would also be epidemiologically useful in order to define which species may amplify the bacterial shedding in the environment. Such an understanding could help to determine to what extent and for how long these species would represent a challenge for epidemiology boundaries in horse and pig farms. Even more importantly, determining the ability of *L. intracellularis* to cross-infect from one host-species to another will help researchers to choose appropriate animal models, thereby reducing costs, refining experimental methods and, ultimately, sparing animals. The specific objective of the present study was to determine whether it is possible to generate typical EPE lesions in hamsters after inoculation with an equine strain of *L. intracellularis* (EPE strain) and whether it is comparatively possible to generate PPE lesions in rabbits after inoculation with a porcine strain of *L. intracellularis* (PPE strain).

# Materials and methods

All the studies described were approved by the Animal Research Ethics Board of the University of Saskatchewan and conducted according to CCAC guidelines.

## Hamsters

In Trial 1, 29 pathogen-free golden Syrian hamster *(Mesocricetus auratus)* weanlings (Strain 049, VAF Hamsters; Charles River Canada, Pointe Claire, Quebec) were born at the Animal Care Unit, Western College of Veterinary Medicine, University of Saskatchewan. The hamsters originated from immuno-competent animal colonies monitored by serology, polymerase chain reaction (PCR), bacteriology, parasitology, or gross pathology tests for absence (pathogen-free and virus antibody-free, or VAF/Plus hamsters) of Reovirus, Sendai virus, Lymphocytic choriomeningitis virus, *Bordetella bronchiseptica, Streptococcus pneumonia, Campylobacter jejuni, Clostridium piliforme, Corynebacterium kutscheri, Encephalitozoon cuniculi, Helicobacter* spp., *Mycoplasma pulmonis, Salmonella* spp., *beta-hemolytic Streptococcus*), and *Streptococcus* spp. (serogroups A, B, and G); all intestinal helmints (*Syphacia* spp., *Hymenolepis* spp., etc.) and protozoa species (*Coccidia* spp., *Trichomonadaceae, Entamoeba* spp., etc); dermathophytosis and all arthropods ectoparasites (Strain 049, VAF Hamsters; Charles River Canada).

The hamsters were housed with their dams in rodent cages in a Containment Level (CL)-1 Room until weaning (21 d from birth), bedded on dust-free shavings, fed rodent chow pellets (5P00, Prolab RMH 3000, LabDiet; PMI Nutritional International, Brentwood, Missouri, USA) fresh water *ad libitum*, and maintained in standard husbandry conditions (12/12 h light/dark cycle and 20°C ± 2°C room temperature). Newborn hamsters were visually monitored once daily for the first 5 days of life and then twice daily until weaning. At weaning, they were weighed, ear-punched, and randomly assigned to 2 groups: uninfected controls (9 hamsters) and EPE-strain-infected (20 hamsters). Hamsters were then separated into pairs or triplets, according to group (controls *versus* EPE-infected) and gender. Hamsters were inoculated with *L. intracellularis* 1 wk after weaning. After inoculation, hamsters were housed in rodent cages in a CL-2 Room, bedded on dust-free shavings in identical husbandry conditions, and provided with the same feed and water sources, as previously described.

In Trial 2, an additional 24 pathogen-free and VAF golden Syrian hamsters (Strain HsdHan AURA; Harlan Laboratories, Indianapolis, Indiana, USA) were born at the same Animal Care Unit facility described for Trial 1. For both experimental subjects and their dams, housing, husbandry, and daily monitoring conditions were identical to those in Trial 1. The health monitoring of the immuno-competent

originating hamster colonies included serology, gross pathology, and microscopic analysis tests to verify the absence of pathogens and related antibodies for Reovirus, Sendai virus, Lymphocytic choriomeningitis virus, Simian Virus 5, *Bordetella bronchiseptica, Clostridium piliforme, Corynebacterium kutscheri, Salmonella* spp., *Campylobacter jejuni, L. intracellularis, Helicobacter* spp., *Klebsiella* spp., *Mycoplasma pulmonis, Proteus mirabilis, Pseudomonas aeruginosa, Pneumocystis* spp., *Pasteurella pneumotropica,* beta-hemolytic *Streptococcus* spp., *Streptococcus pneumoniae, Staphyloccus aureus;* ectoparasites, and endoparasites, *Demodex* spp., and *Encephalitozoon cuniculi* (Strain HsdHan AURA; Harlan Laboratories).

On day 21, hamsters were weaned, ear-punched, weighed, and randomly assigned to 3 groups: controls — uninfected (6 hamsters); PPE-strain group — inoculated with a porcine strain of *L. intracellularis* (9 hamsters); and EPE-strain group — inoculated with an equine strain of *L. intracellularis* (9 hamsters). Hamsters in Trial 2 were inoculated at weaning to increase the impact of stress on disease reproduction.

## Rabbits

Six 4- to 5-week-old female New Zealand white, pathogen-free rabbits *(Oryctolagus cuniculus)* (Strain 052 VAF Rabbits; Charles River Canada) were used. Animals originated from colonies tested regularly by serology, gross and histo-pathology, bacteriology, and parasitology tests for absence of pathogen agents and related antibodies (VAF) [Lymphocytic choriomeningitis virus, Parainfluenza virus 1 and 2, Rabbit hemorrhagic disease virus, Reovirus, Rotavirus, *Bordetella bronchiseptica, CAR-Bacillus, Clostridium piliforme, Campylobacter jejuni, Corynebacterium kutscheri, Helicobacter* spp., *Klebsiella* spp., *L. intracellularis, Mycoplasma pulmonis, Proteus mirabilis, Pseudomonas aeruginosa, Pneumocystis* spp., *Pasteurella pneumotropica, Salmonella* spp., beta-hemolytic *Streptococcus* spp., *Streptococcus pneumonia, Staphylococcus aureus, Encephalitozoon cuniculi, Giardia* spp., *Toxoplasma gondii, Treponema cuniculi,* pinworms, and several tests for typical rodent diseases, but used annually in rabbits by the provider (Charles River Canada) for internal control purposes], as described for the previous study on the *L. intracellularis* rabbit model (25). Also, 8 additional does of the same age, weight, and origin (Charles River Canada) were used as a comparative control to the PPE-strain study, as they had been EPE-infected for a different experiment.

The 2 studies were purposefully conducted at the same time in order to compare the lesions at the peak of infection (14 DPI). No sham-challenged rabbits were used in this experiment, as it has previously been shown that unexposed rabbits do not develop infection (25). On arrival, all rabbits underwent a health check and were identified with a permanent marker felt-pen on the right ear. Rabbits were group-housed in 2 separate and isolated pens in different areas within the same CL-2 Room. Pens were contained by stainless steel bars and polycarbonate panels. Both EPE- and PPE-infected rabbits were inoculated concurrently and managed identically, with strict reverse isolation procedures maintained for the PPE-strain group. All rabbits were fed rabbit pellets (Co-op Whole Earth Rabbit Ration; Federated Co-op, Saskatoon, Saskatchewan) and water *ad libitum* and maintained in standard husbandry conditions, as previously described.

## Inoculum preparation

Control inoculum (medium only) consisted of buffered sucrose/phosphate/glutamate (SPG) solution and was administered to the uninfected hamster groups in both trials (controls), but not to the rabbits.

**EPE strain —** Hamsters in Trials 1 and 2 and the EPE-infected rabbits were challenged with the equine strain of *L. intracellularis* E40504, prepared as described in previous studies (23,29). The *L. intracellularis* infectious material originated from ileal mucosal scrapings of a foal that succumbed to EPE. It was subsequently cultured on McCoy cells (ATCC CRL 1696) in a defined environment (27,30). Infectious virulent isolate from low passage pure cell-cultures was prepared and suspended in buffered SPG medium for all the EPE-infected animals, as described in previous studies (23,29). In the hamster trials, inoculum concentration was between $4.5 \times 10^7$ bacteria/mL and $7.5 \times 10^7$ bacteria/mL (31). For the positive control, EPE-infected rabbits were inoculated at a dose of 5.5 mL/rabbit to give a final inoculum of between 1.3 and $2.5 \times 10^8$ bacteria per rabbit (25).

**PPE strain —** For the Trial 2 hamsters and PPE-infected rabbits, inocula were prepared from PPE lesions harvested from an infected pig's ileal mucosa (PHE/MN1-00) and cultured on McCoy cells (ATCC CRL 1696) in a defined environment (27,29,30). Infectious inocula were prepared as described previously from low passage pure cell-culture isolate. The main difference between inocula used for hamsters and rabbits was the final concentration and the volume. For hamsters, the final concentration of the inocula was $3.2 \times 10^7$ bacteria/mL and the volume was as close as possible to 1 mL, whereas the volume was 8.5 mL per rabbit (target dose of inoculum was $4.25 \times 10^8$ bacteria per animal).

For hamsters in Trial 1, a total dose of 0.45 mL/hamster (approx. $3.375 \times 10^7$ bacteria/hamster), suspended in commercial fruit-flavored pudding (Jell-O; Kraft Foods, Canada), was administered to each animal once on day 0. In Trial 2, an equal inoculum dose was given, but no palatable medium was added. Subjects were 1 wk younger and lighter in body weight (BW), thus the total target volume (1 mL) was divided into 3 administrations over 2 d (once on day 0, twice on day 1). The dosing volume calculations were based on a maximum volume of 20 mL/kg BW and repeated inoculation was used to maximize exposure (32). All challenge material was stored in a −20°C freezer and thawed overnight at 4°C, 12 h before the experiments.

## Inoculation procedures

**Hamsters —** The hamsters were inoculated using 2 different techniques. In Trial 1, hamsters were fed the inocula through a syringe because of the palatable medium added. In Trial 2, hamsters were inoculated by oral gavage to ensure that the entire intended dose was administered to the smaller animals.

**Rabbits —** Rabbits were inoculated after a 1-week mandatory acclimation period (University of Saskatchewan AUC internal guidelines). Before infection, pen-pooled fecal samples were collected from each separate pen (PPE-infected and EPE-infected rabbits, respectively). Each rabbit was weighed, its general health was assessed, a blood sample was collected for serology analysis, and intragastric

administration of infective challenge was done through a nasogastric tube, as described in a previous study (25).

## Collection of samples

**Hamsters —** Changes in body weight (BW) were monitored daily in hamsters for 21 DPI in Trial 1 and for 24 DPI in Trial 2. In both trials, demeanor, gross appearance, fecal consistency and quality, as well as self- and mutual grooming were monitored twice daily with a 4-step grading system, with 0 corresponding to normal and 3 to severely abnormal findings (Table 1a). Treats of apples and small amounts of fruit-flavored pudding (Jell-O; Kraft Foods) were given twice daily to check the appetite and awareness of each animal. Lack of response to treats and to researchers' voices was considered a sign of decreased well-being (33).

In both trials, pooled fecal samples were collected from the cages once weekly and on the predetermined days of euthanasia, when individual samples were collected from the sacrificed animals.

Blood samples for serologic evaluation were collected at the time of euthanasia by intracardiac puncture. In Trial 1, 4 EPE-infected hamsters were euthanized at 7, 11, 14, 17, and 21 DPI; 1 control was euthanized at 7, 11, and 21 DPI; and 3 controls were sacrificed at 14 and 17 DPI. In Trial 2, 8 hamsters (3 from EPE group, 3 from PPE group, and 2 controls) were euthanized at 17, 21, and 24 DPI. In both trials, animals were euthanized with an inhalatory anesthetic overdose of isofluorane (Isoflurane, 99.9%; Halocarbon Products, River Edge, New Jersey, USA) in a glass chamber. Selection for euthanasia was randomized within each group. To evaluate the presence of proliferative enteropathy lesions, sections of the ileum, jejunum, cecum, and colon were harvested at necropsy, prepared within histology cassettes, and immediately placed in 10% phosphate-buffered formalin solution for histopathologic (hematoxylin and eosin — H&E) and immunohistochemical (IHC) examination (7).

**Rabbits —** Changes in BW were monitored daily. Demeanor, gross appearance, fecal consistency and quality, appetite, and self- and mutual grooming were monitored twice daily with a 4-step grading system, with 0 corresponding to normal and 3 to severely abnormal findings, as previously described (Table 1b) (25). Lack of interest in treats (apples and carrots, in this case) was again considered a sign of decreased well-being (33).

Once weekly and at the time of euthanasia, blood samples (1 mL each) for serology were collected with a sterile needle and syringe from the ear's central artery, after a local block and disinfection were applied, as described in a previous study (25). Pooled fecal samples were also collected from the pen once weekly and individual fecal samples were collected at the time of euthanasia. Two PPE-infected rabbits per week were humanely euthanized with an intravenous overdose (720 mg/rabbit) of pentobarbital (Euthanyl; Bimeda-MTC Animal Health, Cambridge, Ontario). To observe for evidence of lesions and to collect multiple samples (1.25 to 2.5 cm long) from the gastrointestinal tract (GIT) for histopathology and IHC examination, selection for euthanasia was randomized (25). Samples were immediately placed in phosphate-buffered 10% formalin solution and prepared routinely for staining. At 14 DPI, EPE-infected rabbits were euthanized by the same means as the PPE-infected rabbits and samples were collected following the protocols reported in a previous study (25).

## Sample analysis

**Macroscopic examination —** Regardless of group and trial, shortly after euthanasia the alimentary tract of both hamsters and rabbits was examined visually from stomach to rectum. The sampling protocol for hamsters consisted of collecting from the terminal jejunum, ileum, ileo-cecal valve, cecal *ampulla,* and colon, as reported in a previous study by Vannucci and colleagues (7). Fecal pellets were collected from the terminal colon and rectum. The sampling protocol for rabbits was identical to that described in a previous study of our research group (25).

**Immunohistochemistry —** Formalin-fixed sections were paraffin-embedded, cut, and stained by the streptavidin method, including anti-*L. intracellularis*-specific mouse monoclonal antibody (34). The positive labeled *L. intracellularis*-specific antigen in the crypts was evaluated with a previously published 5-grade IHC scoring system (7,34). For each animal, the negative control for each tissue section consisted of a corresponding tissue section IHC-labeled, but omitting the primary antibody. Furthermore, pig ileal tissues that were 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 analysis —** Anti-*L. intracellularis*-specific immunoglobulin G (IgG) in serum was measured by an immuno-peroxidase monolayer assay (IPMA), as reported in a previous study by Guedes and colleagues (35). Positive serum samples were end-point titrated from 1:30 up to 1:1920. Control samples consisted of serum from a rabbit before (negative control) and after (positive control) hyperimmunization with *L. intracellularis,* purified from cell culture. Also, serum total protein concentration was measured with the refractometer method to investigate changes over time.

**Quantitative PCR analysis —** Quantitative polymerase chain reaction (qPCR) analysis was conducted on fecal samples as reported in a previous study by Pusterla and colleagues on the EPE foal model (23). The purified DNA was analyzed by qPCR for the presence of *L. intracellularis aspA* gene copies (4,36). For each target gene, 2 primers and an internal, fluorescent-labeled *Taq*Man probe [5′end, reporter dye FAM (6-carboxyflourescein), 3′end, quencher dye TAMRA (6-carboxytetramethylrhodamine)] was designed using Primer Express software (Applied Biosystems, Foster City, California, USA) The "*L. intra* system" forward primer and reverse primer were "bcL. intra-114f" (CACTTGCAAACAATAAACTTGGTCTTC) and "bcL. intra-263r" (CATTCATATTTGTACTTGTCCCTGCA), respectively, with the aspartate ammonia lyase *(aspA)* gene as target and "bc*L. intra*-201p" (TCCTTGATCAATTTGTTGTGGATTGTATTCAAGG) as probe. The "Pan-Bacteria system" forward primer and reverse primer were "PB.283f" (GGATGATCAGCCACACTGGA) and "PB.352r" (CCAATATTCCTCACTGCTGCC), respectively, with 16S ribosomal ribonucleic acid (rRNA) as a target and "PB.305" (CCCGTAGGAGTCTGGACCGTGTCTCA) as a probe. *Taq*Man PCR systems were validated using 2-fold dilutions of genomic deoxyribonucleic acid (gDNA) testing positive for the target genes. Dilutions were analyzed in triplicate and a standard curve was plotted against the dilutions. The slope of the standard curve was used to calculate amplification efficiencies using the formula $E = 10^{1/-s}-1$. Each system

**Table 1a. "Humane Intervention Monitoring Parameters" format used as a monitoring spreadsheet for hamsters enrolled in the infection animal model for porcine and equine proliferative enteropathy during the validation phase. This table was used to record clinical observations for each hamster twice daily, except for body weight, which was assessed only once daily**

| Experimental day: | Date: | Hamster code: | |
|---|---|---|---|
| | Variable | | Individual score |
| | **Daily Feed Intake** | | AM |
| 0 | Same or increased from previous day | | |
| 1 | Slight decrease from previous day | | |
| 2 | Marked decrease from previous day | | PM |
| 3 | Complete anorexia | | |
| | **Daily Water Intake** | | AM |
| 0 | Same or increased from previous day | | |
| 1 | Slight decrease from previous day | | |
| 2 | Marked decrease from previous day | | PM |
| 3 | No water taken in | | |
| | **Body Weight (BW)** (average weight gain for young hamsters is 1 to 2 g/d or ≈ 10 g/wk) | | AM |
| 0 | Increasing weight ~ 20%/wk | | |
| 1 | Increasing weight ~ 10%/wk | | (Calculated gain |
| 2 | Maintaining weight, no weight gain | | from last BW |
| 3 | Rapid weight loss (20% to 25%/wk) or losing weight for more than 2 d | | measurement) |
| | **Stool** | | AM |
| 0 | Normal feces | | |
| 1 | Feces formed but softer than usual | | |
| 2 | Feces not formed but not liquid | | PM |
| 3 | Liquid feces (for > 2 d), leading to emaciation | | |
| | **Physical Appearance** | | AM |
| 0 | Normal | | |
| 1 | Lack of grooming | | |
| 2 | Rough coat, nasal/ocular discharge | | PM |
| 3 | Very rough coat, abnormally hunched posture, distended abdomen (> 3 d) | | |
| | **Behavior** | | AM |
| 0 | Normal, alert, playful, comes to front of cage | | |
| 1 | Hyperactive, agitated | | |
| 2 | Decreased activity, minor depression, unwilling to move | | PM |
| 3 | Depressed, head turned to corner of cage, still moves when stimulated | | |

needed to be greater than 95% efficiency to be considered. The detection limit for "*L. intra* system" is between 5 to 10 copies/uL of DNA (determined with plasmid DNA). The "*L. intra* system" detects all *L. intracellularis,* regardless of the host species. Known positive controls and no template controls were run on every plate and met previously established standardization values. The quality of DNA was determined by the "PanBacteria system," with a control value under 30, to pass quality control (37). A real-time PCR assay targeting a universal sequence of the bacterial 16S rRNA gene was used as quality control, i.e., efficiency of DNA purification and amplification, and as an indicator of fecal inhibition (38).

**RT-reaction and real-time *Taq*Man PCR —** Each PCR reaction contained 20x primer and probes for the respective *Taq*Man system with a final concentration of 400 nM for each primer and 80 nM for the *Taq*Man probe and commercially available PCR Mastermix *(Taq*Man Universal PCR Mastermix; Applied Biosystems), containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, and 0.625 U Ampli*Taq* Gold DNA polymerase per reaction; 0.25 U AmpErase UNG per reaction; and 1 μL of the DNA sample in a final volume of 12 μL. The samples were placed in a 384 well plate and amplified in an automated fluorometer (ABI Prism7900 HTA Fast; Applied Biosystems). Applied Biosystem's standard amplification conditions were used: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 60 s at 60°C. Fluorescent signals were collected during the annealing temperature and cycle threshold (CT) values extracted with a threshold of 0.1 and baseline values of 3 to 12 for all samples using SDS Software, version 2.2.1 (Applied Biosystems). Absolute quantitation was calculated by a standard curve and expressed as copy numbers of the *L. intracellularis aspA* gene/g of feces.

## Statistics

For the hamster trials, BW findings were analyzed through a Mann-Whitney test, as Trial 1 had 2 uneven groups, and Kruskal-Wallis test with Dunn's *post hoc* testing, as Trial 2 had 3 uneven groups. Immunohistochemistry (IHC) results were analyzed through contingency tables with the Fisher Exact Test to compare the 2 categorical outcomes (lesions *versus* no lesions) and to estimate odds

**Table 1b. "Humane Intervention Monitoring Parameters" format used as a monitoring spreadsheet for the rabbits enrolled in the rabbit animal model for porcine and equine proliferative enteropathy during the validation phase. This table was used to record clinical observations for each rabbit twice daily, except for body weight, which was assessed only once daily**

| Experimental day: | Date: | Rabbit code: | |
|---|---|---|---|
| | Variable | | Individual score |
| | **Daily Feed Intake** | | AM |
| 0 | Same or increased from previous day | | |
| 1 | Slight decrease from previous day | | |
| 2 | Marked decrease from previous day | | PM |
| 3 | Complete anorexia | | |
| | **Daily Water Intake** | | AM |
| 0 | Same or increased from previous day | | |
| 1 | Slight decrease from previous day | | |
| 2 | Marked decrease from previous day | | PM |
| 3 | No water taken in | | |
| | **Body Weight (BW)** (average weight gain for young rabbits is 200 g/wk) | | AM |
| 0 | Increasing weight ~ 200 g/wk | | |
| 1 | Increasing weight ~ 100 g/wk | | (Calculated gain |
| 2 | Maintaining weight, no weight gain | | from last BW |
| 3 | Losing weight for more than 2 d | | measurement) |
| | **Stool** | | AM |
| 0 | Normal feces | | |
| 1 | Feces formed but softer than usual | | |
| 2 | Feces not formed but not liquid | | PM |
| 3 | Liquid feces | | |
| | **Physical Appearance** | | AM |
| 0 | Normal | | |
| 1 | Lack of grooming | | |
| 2 | Rough coat, nasal/ocular discharge | | PM |
| 3 | Very rough coat, abnormal posture, ears low | | |
| | **Behavior** | | AM |
| 0 | Normal, alert, comes to front of cage | | |
| 1 | Minor depression but still moves when stimulated | | |
| 2 | Grinding teeth, depressed, head turned to corner of cage | | PM |
| 3 | Very depressed, almost unresponsive | | |

ratio and relative risks. For analyses of qPCR results, a 1-way analysis of variance (ANOVA) was used, with Bonferroni's *post hoc* testing.

Only descriptive statistics were used for PPE-infected rabbits, as the sample number was insufficient for statistical analysis. When the comparison was applicable to the EPE-infected animals, Mann-Whitney, Kruskal-Wallis, and unpaired *t*-tests were used (BW and serum total protein concentration). For all statistical analyses in hamsters and rabbits, commercial software (GraphPad Prism 5.4; Applied Biosystems) was used and alpha was set at 5%.

# Results

## Clinical appearance

No hamsters showed signs of diarrhea throughout either trial and their enthusiastic interest in treats was never diminished (33). The only behavioral change was a mild to moderate hyperactivity that was noted after 14 DPI, as some PPE-infected animals rearranged the entire cage's content within a few hours in the daylight, which suggests abnormal behavior. There was no difference in daily weight gain between infected and uninfected hamsters in either trial ($P = 0.12$ for Trial 1; 0.88 for Trial 2). There was no difference between 3- and 4-week-old hamsters ($P = 0.14$), either due to age (3- *versus* 4-week-old) or breeding colony (hamsters from Charles River Canada versus those from Harlan Laboratories), or in infected and uninfected hamsters (for EPE group: $P = 0.77$ and for EPE and PPE combined group: $P = 0.6$).

No clinical abnormalities were noted in the PPE-infected rabbits during the study. Two does showed signs of aggression toward other pen-mates; 1 improved over time and 1 developed this behavior toward the end of the trial. No significant depression or suppression in BW gain was noted in any PPE-infected rabbit, not even at around 14 DPI (peak of disease), as was observed in EPE-infected rabbits (25). Three PPE-infected rabbits had negative BW gains at different stages, which were unrelated to each other and quickly compensated for (within 1 d) as the total BW gain of PPE-infected rabbits (7 DPI: 171 to 309 g; 14 DPI: 506 to 667 g; 21 DPI: 781 to 871 g) was above the average of 200 g/wk that was recommended by the provider

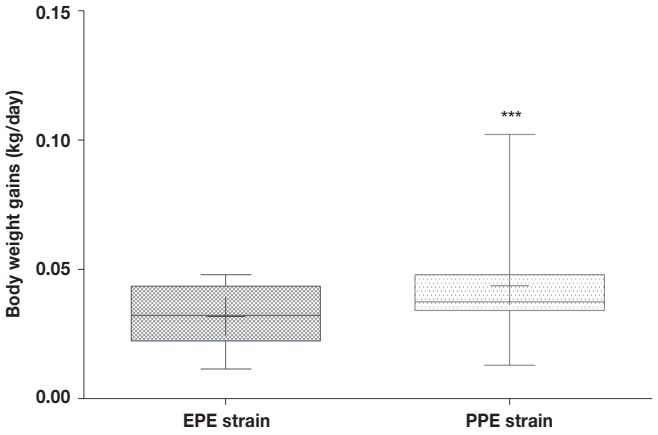


**Figure 1. Comparison of body weight gain in 5-week-old rabbits infected with the EPE strain and those infected with the PPE strain of *L. intracellularis*. By 14 DPI, the PPE-infected rabbits gained more weight overall than their EPE-infected counterparts ($P = 0.04$). The mean in the box plot is represented by a cross hair, whereas the whiskers represent the 5 to 95 percentile interval. Note that the preliminary phases of the EPE infection model in rabbits show a trend of reduced growth performance around the infection peak at approximately 14 DPI, which is not matched by the PPE-infected rabbits.**

(Charles River Canada). A comparison between daily average BW gain in the EPE and PPE groups at 14 DPI (Figure 1) showed that EPE-does had a suppression in BW gain ($P = 0.036$).

## Gross pathology

There were no gross lesions in the intestines of any hamsters in either trial. In Trial 1, more abdominal fat was noted in older animals. Intestinal content appeared adequate for the tracts examined and the diameter of the intestinal sections (ilea) at the level of the ileo-cecal valve in Trial 1 controls was smaller ($P = 0.0011$) than the ilea in EPE-infected hamsters (1.5 to 2.5 mm *versus* 2 to 3 mm). In Trial 2, however, the diameter of the jejunum and ileum in hamsters was the same in EPE-infected, PPE-infected, and control groups ($P = 0.9$ and 0.26, respectively).

In PPE-infected rabbits, mild edematous changes of the serosal layer of the terminal portion of the jejunum and ileum were noted and were comparable to the serosal findings for EPE-infected rabbits (Figure 2). No *rugae* were observed in the mucosa, however, and by 14 DPI, only the serosal and mucosal edema were apparently increased.

## Immunohistochemistry

In Trial 1, only 1 out of the 20 EPE-infected hamsters showed mild (grade 1) IHC labeling, which statistically was not different ($P = 1.0$) than controls. The odds ratio showed 2.586 higher chances of infecting a hamster when it was challenged with the inoculum. In Trial 2, a statistical difference ($P = 0.013$) was noted between the infected and uninfected hamsters, with greatly reduced (0.04) odds ratios of having PE lesions in uninfected animals. Comparing the presence of IHC-labeled lesions between PPE-infected hamsters and uninfected controls, however, increased the statistical significance ($P = 0.0003$), whereas comparing PPE-strain and EPE-strain groups showed that the PPE-strain had higher ability than the EPE-strain to infect hamsters ($P = 0.009$) (Figures 3a and 3b). The relative risk

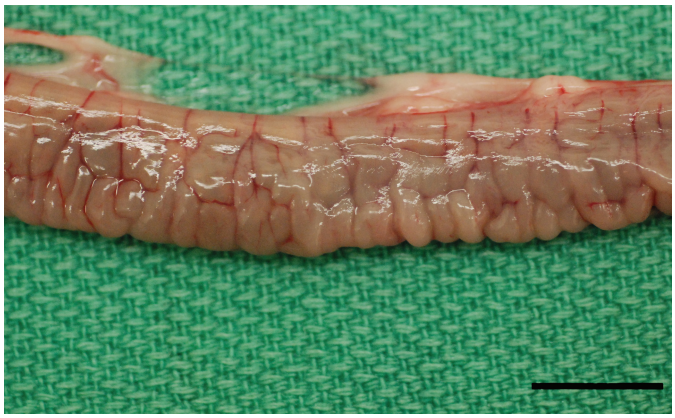

**Figure 2. Example of the macroscopic appearance of a tract of jejunum, approximately 3.5 cm long (bar scale = 1 cm) in rabbits infected with the PPE strain of *L. intracellularis*. Note that the serosal edema and "cobblestone" appearance on the anti-mesenteric aspect of the sample are similar to the macroscopic lesions of the EPE infection model in rabbits, although such serosal appearance was not related to mucosal lesions in the PPE-infected rabbits.**

of inducing IHC detectable lesions in hamsters after PPE-strain inoculation was 3 times higher than after inoculating with EPE-strain, with an odds ratio of 35.29 in favor of PPE-strain infection. The results of the challenge in Trial 2 are represented in Figure 4. One last observation about EPE-strain is that there was no difference ($P = 0.28$) found in either trial related to age (3-week-old *versus* 4-week-old), level of stress (inoculated a week after weaning *versus* at weaning), or method of inoculation (oral feeding *versus* oral gavage), even though the relative risk appears 3.3 times higher in younger, stressed hamsters.

In the PPE-infected rabbit experiment, no characteristic lesions were detected through IHC labeling at any stage of the study. Only fragments of reactive DNA were noted at the level of the *lamina propria* (Figures 5a and 5b). In the EPE-infected rabbits that were euthanized at the same time, IHC detectable lesions were found in several rabbits in at least 2 GIT sections (jejunum and ileum) (unpublished data), which mirrored the results of the initial model development (25).

## Serology

Control samples for the IPMA procedure consisted of serum from a rabbit before (negative control) and after (positive control) hyperimmunization with *L. intracellularis*, purified from cell culture. No IPMA serology results consistent with immune response were noted in hamsters in either trial, whether they were terminated at 21 or 24 DPI.

No antibody titers were evident in the PPE-infected rabbits until 14 DPI. The IgG titers at that time (Figure 6) appeared significantly lower ($P = 0.0023$) in PPE-infected rabbits (range: 60 to 960) than in the EPE-infected group of rabbits (range: 480 to 1920) of the same age, gender, litter, and housing conditions. Serum total protein concentration was measured to investigate changes, as decreased values are reported in foals naturally and experimentally affected by EPE (5,23). No statistical difference was noted over time ($P = 0.88$, as it ranged from 49 to 60 g/L on day 0; 45 to 53 g/L at 7 DPI; 40.5 to 49 g/L at 14 DPI; and 47.5 to 53.2 g/L at 21 DPI) in PPE-infected

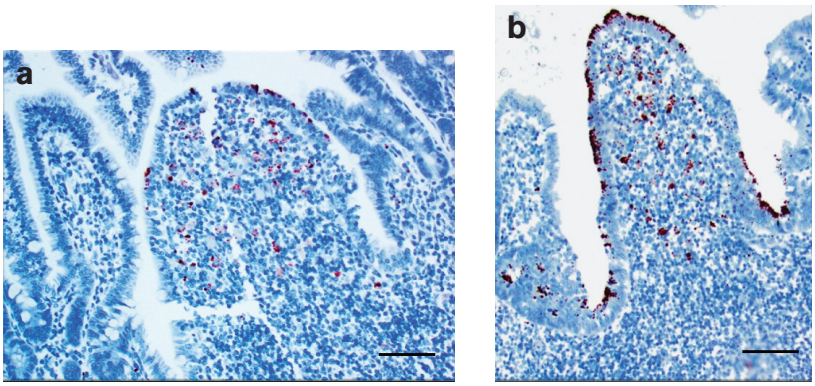


**Figure 3. Comparison of IHC labeling in the EPE-infected and PPE-infected hamsters. Staining: streptavidin method, including anti-*L. intracellularis*-specific mouse monoclonal antibody, with 3-amino-9-ethylcarbazol (AEC) substrate-chromogen and counterstained with Mayer's hematoxylin to observe for the presence of the antigen within the cells. Scale bar = 100 μm. a — Ileum of an EPE-infected hamster. Note that distribution of antigen (red dots) in the mucosal enterocytes of ileal *villi* is very limited and no antigen is detectable within the enteric crypts. b — Ileum of PPE-infected hamster. Note the much larger distribution of detectable antigen in the mucosal enterocytes.**

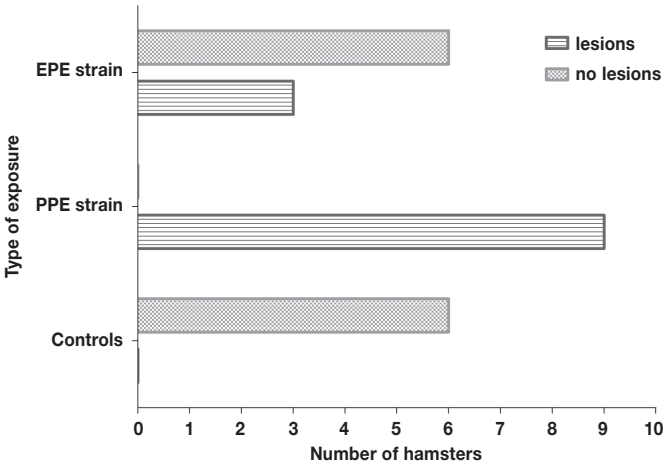


**Figure 4. In Trial 2, the predominance of infection obtained in hamsters inoculated with the PPE-strain of *L. intracellularis* versus the EPE-strain and uninfected controls is shown. Note that all the PPE-challenged hamsters were showing IHC-detectable antigen in typical lesion sites (enteric crypts and mucosal enterocytes), whereas only 3 out of 9 hamsters showed mild EPE lesions (not in the intestinal crypts) after the EPE-strain challenge.**

rabbits, although a decreased concentration was noted in at least 4 rabbits at around 14 DPI, which was soon recovered by the 2 survivor rabbits by 21 DPI.

## Quantitative PCR

On day 0 of Trial 1, 5 hamsters from the EPE-infected group showed positive fecal qPCR, 1 being quite high (693609.1 *aspA* gene copies/g feces — see Figure 7a). For Trial 1, qPCR results showed no difference ($P = 0.051$) over time, when compared to the "day 0" group, although at the limits of significance (Figure 7a). By 21 DPI, however, 3 out of 4 infected hamsters were still shedding *L. intracellularis*. After infection with EPE-strain *L. intracellularis*, a comparison between 3- and 4-week-old hamsters showed that younger hamsters have a higher likelihood of shedding *L. intracellularis* in feces ($P < 0.0001$).

Conversely, in Trial 2 (Figure 7b), despite an initial higher fecal shedding of *L. intracellularis* for EPE-infected hamsters by 7 DPI, the overall comparison of the EPE-strain and PPE-strain over time, from day 0 to 24 DPI, showed higher shedding in the PPE-strain group ($P = 0.0003$) for both time and infection factors (Figure 7c).

A comparison between the detection of PPE-strain versus EPE-strain *L. intracellularis* in rabbits is shown in Figure 8, which indicates higher fecal shedding in the EPE-infected rabbits ($P = 0.0089$).

# Discussion

These studies show that *L. intracellularis* cross-infection can occur in laboratory animals, but not with ease. The goal was to reproduce PE disease, or lesions, from equine and porcine strain isolates in order to individuate a surrogate species small enough to be cost-effective, yet large enough to endure humane research procedures, such as repeated blood samples.

The PPE infection model in hamsters was considered the term of comparison for our hamster studies. Replication of infection in hamster weanlings from a pure cell culture of PPE-strain of *L. intracellularis* that was isolated from naturally diseased pigs was in agreement with previous publications, which showed that inoculation of hamsters yielded a typical PE infection (7,14,16,17). In theses studies, no clinical signs were noted in the infected hamsters, as is commonly reported with pure cell culture inoculation, both in hamsters and pigs (7,17,30). The only exception was some degree of hyperactivity during daylight hours, which is atypical for hamsters as they are nocturnal animals. In addition, intestinal lesions were detected by H&E and confirmed with IHC labeling. Quantitative PCR showed that *L. intracellularis* DNA was shed in feces in exponentially increasing concentrations throughout the study (24 DPI), which demonstrated active replication of the bacteria in the host's intestine (7,14,18,19,39).

The leading hypothesis supporting the idea of the EPE-strain of *L. intracellularis* being capable of infecting hamsters was based not only on the similarities of the bacterial genomic characteristics (98%

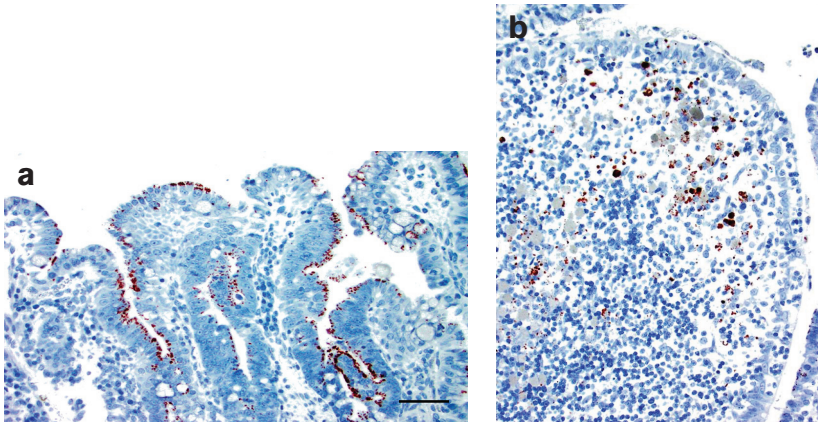


**Figure 5. Comparison of IHC labeling in EPE- and PPE-infected rabbits. Staining: streptavidin method, including anti-*L. intracellularis*-specific mouse monoclonal antibody, with 3-amino-9-ethylcarbazol (AEC) substrate-chromogen and counterstained with Mayer's hematoxylin to observe for typical proliferative lesions of the intestinal epithelium and for the presence of the antigen within the cells. Scale bar = 100 μm. a — Example of ileum of an EPE-infected rabbit at 14 DPI. Note the wide distribution of IHC-detectable antigen in the mucosal enterocytes over ileal *villi* and within enteric crypts. b — Example of cecum of a PPE-infected rabbit at 14 DPI. An ileal tissue counterpart for the PPE strain was unavailable as no antigen was detected anywhere, except in the cecum. Note that not only is the distribution of detectable antigen in the mucosal enterocytes minimal to none, but also the antigenic material appears scattered at the level of the *lamina propria*, where it is destroyed after being engulfed and processed by macrophages.**

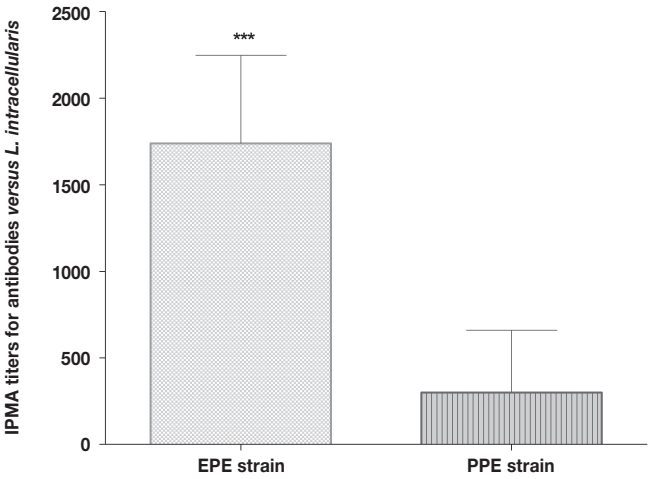


**Figure 6. Immuno-peroxidase monolayer assay (IPMA) findings indicating antibody response at 14 DPI in rabbits infected with the EPE strain and those infected with the PPE strain of *L. intracellularis*. The serological response at 14 DPI in EPE-infected rabbits is visibly different ($P = 0.0023$) than in PPE-infected rabbits. The whiskers on the bar indicate standard deviations.**

between PPE and EPE strains), but also on how easily the PPE-strain can cross-infect from pigs to hamsters (7,17,19,39). Furthermore, similarities in the GIT of hamsters, rabbits, and horses, such as hindgut fermentation and a proportionally large cecum, were considered advantageous, even in the absence of evolutionary correlations (40). In Trial 1, we attempted to cross-infect young, susceptible hamsters (4-week-old) with the EPE-strain, starting from a pure cell culture isolate that is virulent for both horses and rabbits. Since this failed to generate detectable IHC lesions, it was tried again on younger and potentially more vulnerable subjects (3-week-old) (24,25). It should be noted that on day 0 in Trial 1, the fecal material of 5 of the 20 inoculated hamsters became contaminated (see Figure 7a, day 0), despite rigorous experimental procedures being followed in handling each subject. Although such an outcome was unexpected, potential contamination of the feces with fresh inoculum was a plausible explanation. Interestingly, *L. intracellularis* DNA material reached values higher than those detected in the first 11 DPI, with maximum values ranging from $2.0 \times 10^5$ *aspA* gene/g. In the entire experiment, the DNA concentration never rose above the order of magnitude of $10^5$, which is in contrast to the concentrations detected in PPE-infected hamsters (largely over 2 orders of magnitude higher at wk 2 and 3 post-infection).

In Trial 2, the component of stress, which was carefully avoided in Trial 1, as well as young age was added as hamsters were infected immediately after weaning. Despite 2 attempts, cross-infection of hamsters with the EPE strain was unsuccessful, apart from a mild and inconsistent fecal shedding, which even at its maximum value was never as high as the shedding in PPE-infected hamsters (at disease peak, from 17 to 24 DPI). This is particularly striking compared to the textbook-like development of fecal shedding and lesions in PPE-infected hamsters. Although our results originated from a small population, the fact that PPE-challenged hamsters have over 35 times more chance to develop lesions than the EPE-challenged ones is an obvious limitation. This should discourage the further use of hamsters to replace horses in EPE infection models. On a wider perspective, these results constitute evidence to reconsider the ability of the EPE-strain of *L. intracellularis* to cross-infect and cause lesions in other rodents, unlike what was seen for the PPE-strain, whether it depends on their resistance to infection or on specific bacterial tropism for specific hosts (7,21).

As no serologic response was noted in either hamster trial, it is arguable that hamsters may need a longer time to develop a detectable immune response to *L. intracellularis* infection. This

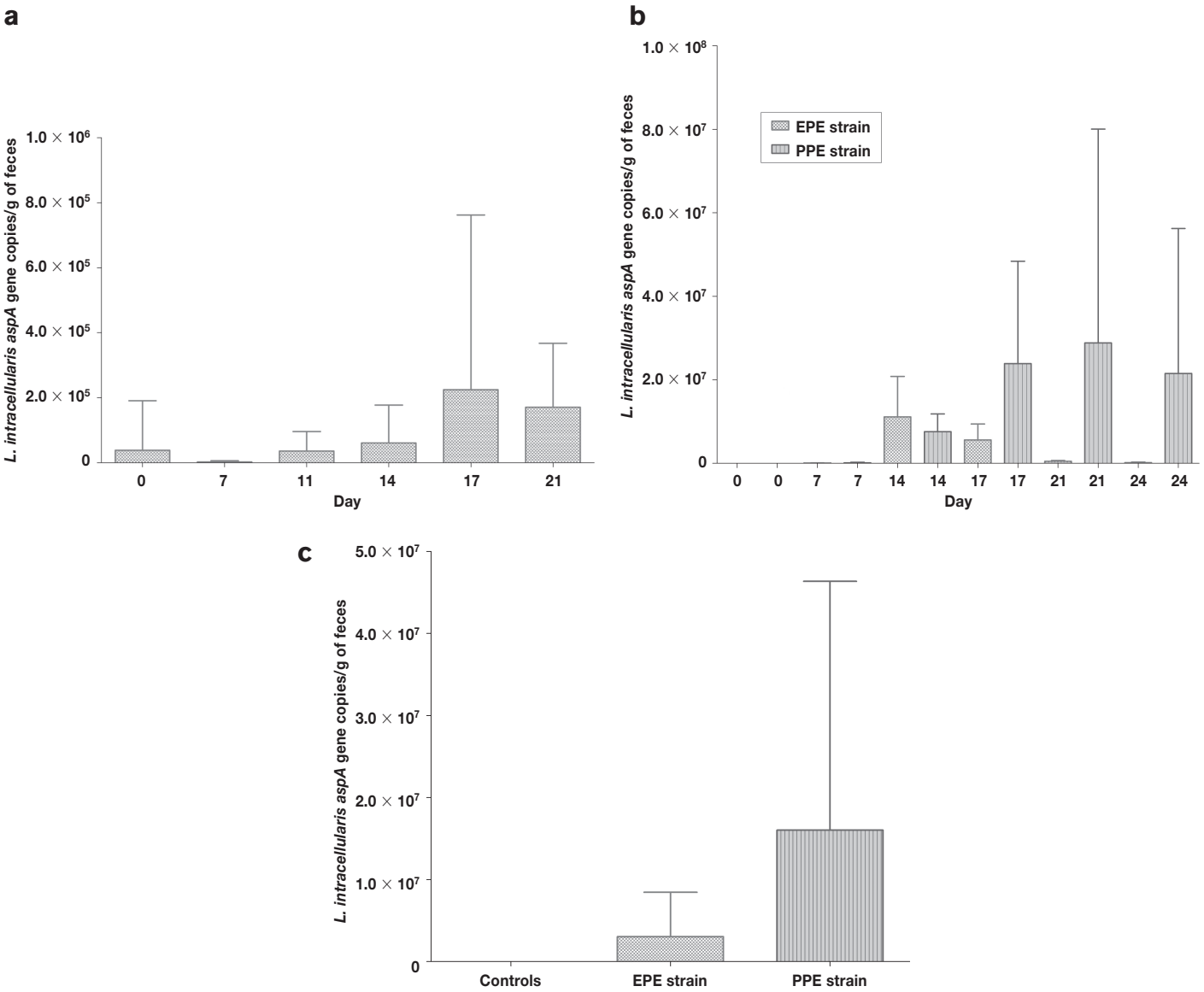


**Figure 7. Detection of *L. intracellularis* DNA gene copies (*aspA* gene) through qPCR analysis in feces (grams) of experimentally infected weanling hamsters. For all 3 graphs, the whiskers on the bar indicate the standard deviations. a — Fecal shedding for EPE-strain *L. intracellularis* during Trial 1, shown from day 0 to 21 DPI. On day 0, some unexplained technical contamination occurred while orally feeding the infectious inocula and this is responsible for the measurable *aspA* gene in the material collected on inoculation day. At 7 DPI, the shedding was minimal and then increased over time. Because of the technical contamination observed, the method of inoculation was changed for Trial 2. Note that the concentration of the *aspA* gene is barely in the range $10^5$ to $10^6$, particularly when compared to the PPE-infected hamsters ($10^7$ to $10^8$ range). b — Fecal shedding for EPE-strain and PPE-strain *L. intracellularis* during Trial 2, shown from day 0 to 24 DPI. While the PPE-strain fecal shedding increases exponentially over time, the EPE-strain fecal shedding is highest at 14 DPI and then decreases significantly over time ($P = 0.003$) to the end of the experiment (24 DPI). c — Overall comparison between strain group (uninfected controls, EPE, and PPE). The different amount of shedding among groups is visible ($P = 0.033$).**

is partially due to the privileged location of the *L. intracellularis*, remote from processing, opsonizing, and exposing units of the reticulo-endothelial system and partly because weanling hamsters may not be as immuno-competent as the adults. In other infection models, older subjects have developed an IgG response in a much more timely fashion (1 wk post-infection) (41). Based on lack of seroconversion and limited fecal shedding, the question now is whether EPE-challenged rodents can become healthy short-term carriers of the disease and in what capacity they contribute to the amplification of infection.

In this context, failing to reproduce a viable PPE-infection in rabbits is significant, as no clinical signs, not even reduced growth performance or IHC lesions, were observed in our PPE-challenged rabbits (25). A transient IHC labeling was noted in the *lamina propria* of only 1 sample out of 6 whole GIT sets of samples (42 samples total), which is consistent with destruction of the bacteria, but not with typical active lesions, which should be located at the apex of enterocytes in the intestinal crypts. This shows that the PPE-strain of *L. intracellularis* is eliminated swiftly by rabbits, despite inoculation with almost twice the dose used in the EPE-challenged rabbits (25)

A recent study showed that the EPE-strain of *L. intracellularis* is unable to cross-infect age-susceptible pigs and the PPE-strain is unable to cross-infect age-susceptible foals, which makes the results of this study on laboratory animals even more relevant (29).

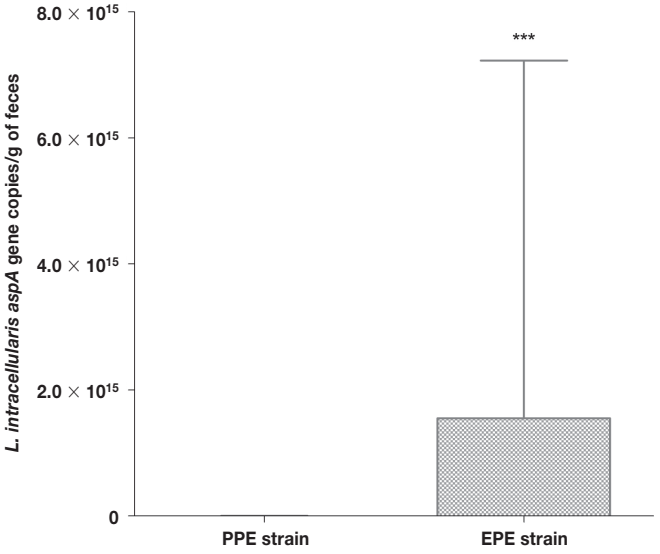


**Figure 8. Comparison of *L. intracellularis aspA* gene copies/g of feces obtained through qPCR between EPE-infected and PPE-infected rabbits ($P = 0.0089$). The whiskers on the bar indicate the standard deviation. Note that the concentration of the *aspA* gene in the feces of EPE-infected rabbits is so high that the values for the PPE-infected group do not appear in the graph, as they remained several orders of magnitude lower ($10^6$ or $10^7$, at the most).**

The inability of the EPE-strain to cause demonstrable infection in hamsters mirrors the results of cross-infection to pigs, although it is clearly capable of infecting and inducing clinical disease and lesions in young horses, as well as cross-infecting rabbits, with mild clinical discomfort, typical intestinal lesions, limited fecal shedding, and high serologic response (25,29). On the contrary, the PPE-strain of *L. intracellularis* is capable of infecting and causing overt (in some cases) disease in pigs and hamsters, but does not cause infection in rabbits or horses, although a limited immune-response and fecal shedding were detected in rabbits as well, similar to some exposure results in sentinel rabbits used in piggeries (7,30,42). Perhaps a secondary host-adaptation, or species-specificity, exists for different *L. intracellularis* strains (25,29,43). In hamsters cross-infected with PPE-strain, the most severe clinical signs were caused by inoculation with homogenates of porcine mucosal ileal scrapings (7,15–17). Future studies may therefore focus on whether the role of passage of the infectious material through cell cultures also hampers the virulence in EPE-strains, as we know that higher passages of PPE-strain in cell cultures definitely immortalize *L. intracellularis*, but reduce its virulence.

In conclusion, although only a small number of animals were used, our investigations help to determine whether different strains of *L. intracellularis* have different secondary host-specificities. Such findings should help to exclude hamsters and rabbits from modeling EPE-strain and PPE-strain infection, respectively. As EPE modeling has been achieved in rabbits, however, 2 important concepts may be extrapolated from our results: studying the infection caused by *L. intracellularis* may necessitate the use of more than 1 animal model and different wildlife species could potentially be a connecting link between differently adapted infectious strains (4).

## Acknowledgments

The authors thank Dr. C. Wheler at the Animal Resources Centre and the support staff of the Animal Care Unit at Western College of Veterinary Medicine, University of Saskatchewan, S. Mapes at University of California at Davis for the PCR explanation, and Dr. J.M. Dhillon for the French translation of the abstract. These studies were funded by a grant from the Equine Health Research Fund of the Western College of Veterinary Medicine. F. Sampieri and KR Ball are fellows of the CIHR-THRUST (Canadian Institutes of Health Research — Training in Health Research Using Synchrotron Techniques Grant). F.A. Vannucci was supported by the Brazilian Government sponsoring agency "Conselho Nacional de Desenvolvimento Cientifico e Tecnologico" (CNPq).

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