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Rapid Communication

RAPID COMMUNICATION

Evaluation of swine enteroids as in vitro models for *Lawsonia intracellularis* infection[1,2]

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Abstract

The enteric pathogen *Lawsonia intracellularis* is one of the main causes of diarrhea and compromised weight gain in pigs worldwide. Traditional cell-line cultures have been used to study *L. intracellularis* pathogenesis. However, these systems fail to reproduce the epithelial changes observed in the intestines of *L. intracellularis*-infected pigs, specifically, the changes in intestinal cell constitution and gene expression. A more physiologically accurate and state-of-the-art model is provided by swine enteroids derived from stem cell-containing crypts from healthy pigs. The objective of this study was to verify the feasibility of two-dimensional swine enteroids as in vitro models for *L. intracellularis* infection. We established both three- and two-dimensional swine enteroid cultures derived from intestinal crypts. The two-dimensional swine enteroids were infected by *L. intracellularis* in four independent experiments. Enteroid-infected samples were collected 3 and 7 d postinfection for analysis using real-time quantitative PCR and *L. intracellularis* immunohistochemistry. In this study, we show that *L. intracellularis* is capable of infecting and replicating intracellularly in two-dimensional swine enteroids derived from ileum.

Key words: in vitro model, intracellular bacteria, *Lawsonia intracellularis*, organoids, pathogenesis, proliferative enteropathy

Introduction

Proliferative enteropathy (PE) is globally identified in pig herds and is considered one of the main enteric diseases in pigs (Vannucci et al., 2019). *Lawsonia intracellularis*, the etiologic agent of PE, is an obligate intracellular pathogen that replicates in the cytoplasm of intestinal epithelial cells. This infection results in crypt hyperplasia, decreased numbers of goblet cells, and compromised feed digestion that leads to poor weight gain in affected pigs (Vannucci et al., 2019). The mechanisms involved in the intestinal

epithelium changes caused by *L. intracellularis* infection are still unclear.

Proliferative enteropathy can be prevented in pig herds by vaccination (McOrist and Smits, 2007; Roerink et al., 2018). Despite the efficacy of the commercial vaccines, some producers are still resistant to the incorporation of this strategy in their herds, and rely on antimicrobial therapy, in feed, water, or individually administrated, to prevent and control PE (Holyoake et al., 2009; Resende et al., 2015). Most of the current investigations of alternatives to antimicrobial treatment of PE are not strategically designed toward specific targets due to the lack of understating of PE pathogenesis. *Lawsonia intracellularis* is the only species in the genus and the lack of information regarding its virulence factors has limited the development of alternative prevention and control methods (Vannucci and Gebhart, 2014).

For determining the events related to intestinal epithelial changes observed during *L. intracellularis* infection, in vitro models would be the method of choice because of the ability to control variables. Although single cell cultures are routinely used for laboratory propagation of *L. intracellularis*, they fail in reproducing the cellular changes observed in the intestinal epithelium of PE-affected pigs (Resende et al., 2019b). Although mice are not a natural host for *L. intracellularis* infection, we recently evaluated three-dimensional mouse enteroids as an in vitro model for *L. intracellularis* pathogenesis. However, despite the organotypic similarity offered by the model, mouse enteroids were an inadequate model since *L. intracellularis* did not significantly propagate or produce detectable changes in the host cells (Resende et al., 2019a). Therefore, an adequate in vitro model for the investigation of *L. intracellularis* pathogenesis is still needed.

Swine enteroids obtained from intestinal stem cells have been described recently (Gonzalez et al., 2013; Khalil et al., 2016) and there are only a few reports of their application as in vitro systems for studying intestinal physiology, nutrition, and pathogenesis of infectious diseases (Koltes and Gabler, 2016; Ferrandis Vila et al., 2018; Li et al., 2019). Enteroids can be cultured as three-dimensional structures composed of intestinal epithelial cells which can be polarized with the apical membrane forming a sphere-like system with the center acting as intestinal lumen where cell debris are shed, and metabolites are secreted (Sato and Clevers, 2013; Yin et al., 2019). This three-dimensional structure makes it necessary to use microinjection systems to add the treatments into the enteroid lumen, which is time consuming, laborious and makes control of dosing and exposure variable between enteroids (Wilson et al., 2015; Resende et al., 2019a), Alternatively, enteroids can be cultured as two-dimensional enteroids by using transwells. Enteroids cultured this way still contain all types of intestinal epithelial cells present in the intestine, with the advantage of having an easy-access cell apical membrane, which simplifies the infection process and analyses (Braverman and Yilmaz, 2018; Thorne et al., 2018). The objective of this study was to evaluate two-dimensional swine enteroids as in vitro models for *L. intracellularis* infection.

Methods

Crypt Isolation

All procedures were conducted in accordance with the guidelines of the Animal Care and Use Manual of the University of Minnesota and were approved by the Institutional Animal Care and Use Committee (1807-36212A). Ileum tissue was collected from a healthy Landrace/Yorkshire cross-bred finisher pig (about 90 kg) euthanized for non-intestinal related research purposes. A 3–4 cm ileum piece was excised and placed in ice-cold Advanced Dulbecco's Modified Eagle Medium (Gibco Advanced DMEM, ThermoFisher) with 1% penicillin/streptomycin (Gibco, ThermoFisher), then transferred to a Petri dish containing ice-cold phosphate-buffered saline solution (PBS) with 1% penicillin/streptomycin and opened longitudinally. After three washes in PBS, the tissue was transferred into calcium and magnesium-free Hanks' Balanced Salt solution (HBSS) containing 30 mM ethylenediaminetetraacetic acid (EDTA, Corning, ThermoFisher), 1 mM dithiothreitol (DTT, Sigma–Aldrich), and 100 µg/mL penicillin/streptomycin, and incubated at 37 °C with shaking for 5–15 min. After incubation, crypts were separated by applying suction using a disposable transfer pipette, which was continued until abundant crypts were observed in the suspension by visual evaluation in an inverted microscope. The crypt suspension was transferred to a 15 mL centrifuge tube and washed with advanced DMEM with 10% fetal bovine serum (FBS, Corning 35011CV, heat inactivated). The tube was centrifuged for 4 min at low speed (300 × *g*) and the supernatant media was aspirated. The pellet was resuspended in DMEM with 10% FBS and centrifuged for 4 min at low speed (300 × *g*). The supernatant media was aspirated, and the pellet resuspended in 1 mL of Intesticult organoid media (StemCell Technology). Crypt density was counted by placing 40 µL of the suspension on a slide and media volume adjusted to have 20–30 crypts / 40 µL. The crypt suspension was suspended in Matrigel (Corning) at a 1:3 ratio, and placed as beads in a 24-well culture plate which was then incubated for 15–20 min at 37 °C for the Matrigel to gelatinize. Once gelatinized, the wells were covered with 400 µL of Intesticult and incubated at 37 °C and 5% CO_2. Intesticult media was replaced every 48 h to grow three-dimensional enteroids.

Three-dimensional Enteroid Passaging and Expansion

Every week, the Matrigel beads containing enteroids (three-dimensional culture) were disrupted using a sterile 1000 µL pipette tip and were transferred along with the media into a 1.5 mL sterile microcentrifuge tube. The enteroids were disrupted by passage through a 1 mL insulin syringe 5–6 times and then pelleted by centrifugation at 600 × *g* for 4 min. After removing the supernatant, the pellet was washed in DMEM and centrifuged again at 600 × *g* for 4 min. The pellet was resuspended in culture media and Matrigel (1:3 ratio) and plated as beads as described above.

Two-dimensional Swine Enteroid Cultures

Twenty-four hours before the passaging of three-dimensional enteroids, 6.5 mm diameter transwell inserts were coated with 150 µL of a 1:100 Matrigel dilution in DMEM and incubated at 37 °C in 5% CO_2 overnight. After this incubation, the excess liquid from the coated transwell membrane was discarded and the membrane was allowed to dry in the incubator for 2 h. Meanwhile, the three-dimensional enteroids were collected and disrupted as described for passage. After the needle-syringe disruption and centrifugation of three-dimensional enteroids, the disrupted enteroid pellet was suspended in Intesticult and 100 µL of the suspension were placed over the Matrigel-coated transwells. Media was replaced every 48 h by aspirating contents of the transwell and well, and adding 100 µL of Intesticult in the insert and 450 µL of Intesticult in the receiving well. Enteroid

growth was monitored using an inverted microscope and when the two-dimensional enteroids achieved confluence of ≥80% (approximately 10 days after plating) they were infected with *L. intracellularis*.

Infection of Two-dimensional Swine Enteroid with *L. intracellularis*

A *L. intracellularis* isolate (PHE/MN1-00, ATCC PTA-3457) at low (≤20) passage (Vannucci et al., 2013) was propagated in McCoy mouse fibroblast cells (ATCC CRL-1696) as described elsewhere (Guedes and Gebhart, 2003a; Resende et al., 2019). On the day of enteroid infection, a *L. intracellularis* suspension obtained from the infected McCoy cultures was filtered through sterile 80 µm filters to clear the suspension of any McCoy cells present, and centrifuged at 8,000 × *g* for 5 min at 4 °C. The bacterial pellet was suspended in Intesticult in a volume sufficient to cover each of the transwells with 100 µL of bacterial suspension. The plate containing negative controls and *L. intracellularis* infected two-dimensional swine enteroids was placed in a microaerophilic atmosphere, as detailed elsewhere (Vannucci et al., 2011; Resende et al., 2019a), at 37 °C.

Lawsonia intracellularis real-time polymerase chain reaction (real-time PCR) was performed as described (Wattanaphansak et al., 2010) using an aliquot of the inoculum from each of the four independent infection experiments (E1 to E4) as PCR template. C_t values were converted to *L. intracellularis* organisms/mL, obtaining 1.99×10^3 *L. intracellularis*/mL for experiment 1 (E1), 9.72×10^2 *L. intracellularis*/mL for E2, 1.04×10^7 *L. intracellularis*/mL for E3, and 5.49×10^4 *L. intracellularis*/mL for E4. In each independent experiment, wells were infected with *L. intracellularis* suspension, or exposed to the medium used to suspend the inoculum only as negative controls. One set of wells was harvested at 3 days postinfection (dpi) and another set was harvested at 7 dpi. The enteroid media collected from the top of the transwell at 3 and 7 dpi was saved for *L. intracellularis* real-time PCR. The set of transwells to be harvested at 7 dpi had its media replaced at 5 dpi, with the used media also being saved for *L. intracellularis* real-time PCR. This procedure resulted in three samples for *L. intracellularis* real-time PCR (3, 5, and 7 dpi) for each independent experiment as described elsewhere (Wattanaphansak et al., 2010).

Immunohistochemistry of Swine Enteroids Infected with *L. intracellularis*

After removing the media from the transwell insert, two-dimensional swine enteroids were fixed for 2 h with 150 and 500 µL of 4% formaldehyde in the insert and bottom well, respectively. After fixation, the two-dimensional enteroids were washed two times with 200 µL of PBS. The membranes were then cautiously removed with a scalpel blade, placed on biopsy molds and covered with Histogel. Histogel blocks for the two-dimensional swine enteroids were stored in 70% ethanol at room temperature until routine processing and paraffin embedding was done. Paraffin-embedded blocks were cut into 4 µm thick sections and placed on charged glass slides and were used for immunohistochemistry for detection of *L. intracellularis* (Guedes and Gebhart, 2003b) and evaluated by bright field microscopy.

Results

Lawsonia intracellularis propagates in two-dimensional swine enteroids

The medium covering the swine enteroid transwells was used to demonstrate *L. intracellularis* propagation by real-time PCR. Experiments E1 and E2 were inoculated with low concentrations of *L. intracellularis* (1.99×10^3 *L. intracellularis*/mL and 9.72×10^2 *L. intracellularis*/mL, respectively) and real time-PCR results were negative for the media samples in all time points, indicating nondetectable levels of *L. intracellularis* propagation. E3 received the highest inoculum concentration (1.04×10^7 *L. intracellularis*/mL) and E4 received an intermediate amount of bacteria (5.49×10^4 *L. intracellularis*/mL). Media collected from the samples of E3 and E4 were positive by real time-PCR for *L. intracellularis* at 3, 5, and 7 dpi (Figure 1), indicating that *L. intracellularis* propagated in the two-dimensional swine enteroids. Importantly, for both E3 and E4, there was an increase in the *L. intracellularis* concentration in the media with time. Bacterial propagation was further demonstrated by the detection of *L. intracellularis* in the media collected at 7 dpi, since the media was completely replaced at 5 dpi and any bacteria detected at 7 dpi would be due to the release of bacterial organisms propagating in the cells to the medium between 5 and 7 dpi.

The presence of *L. intracellularis* in the cytoplasm of swine enteroid cells was detected by immunohistochemistry in samples from E1, E3, and E4 but not in samples from E2 (inoculated with the lowest concentration of *L. intracellularis*). The highest amount of *L. intracellularis* antigen in the cytoplasm of swine enteroid cells was observed in samples from E3, in accordance with the higher numbers of organisms obtained by *L. intracellularis* real-time PCR. High amounts of *L. intracellularis* antigen in the cytoplasm were observed both at 3 and 7 dpi in samples from E3, suggesting that *L. intracellularis* inoculum in high concentration might be necessary for effective bacterial propagation.

Discussion

Our previous studies have shown that intestinal epithelial cell lines, such as Caco-2, IPEC-J2 and IEC-18 do not increase proliferation when infected by *L. intracellularis* (Resende et al., 2019b), and that the amount and location of *L. intracellularis* antigen in mouse three-dimensional enteroids (Resende et al., 2019a) differs from that normally seen in vivo (Vannucci and Gebhart, 2014). We sought to determine whether using two-dimensional swine enteroids derived from three-dimensional cultures would allow easier access of *L. intracellularis* to the apical region of the cells and better modeling of the infection than previously observed by microinjecting mouse enteroids Zhang et al., 2014; Wilson et al., 2015). By using the two-dimensional model, we provided direct access of *L. intracellularis* to the apical membrane of the epithelial cells, and we were able to use the media from the apical side to monitor *L. intracellularis* concentration overtime, which would not be feasible with three-dimensional enteroids.

When *L. intracellularis* is interacting with the host intestinal epithelium in vivo, there is hyperplasia of the intestinal crypts (Guedes et al., 2017), decreased numbers of goblet cells (Bengtsson et al., 2015), and increased apoptosis (Guedes et al., 2017). The mechanisms involved in the epithelial changes observed during *L. intracellularis* infection in the swine intestine are mostly unknown. Until now, the relationship between *L. intracellularis* and the intestinal epithelial cells has been investigated using single cell line cultures that do not present the different signaling pathways or cell diversity in the intestine as enteroid cultures do.

Swine enteroids have numerous advantages as in vitro models to investigate host pathogen interactions over other cell and tissue culture models. The feasibility of maintaining the enteroid culture in laboratory conditions decreases the need for euthanasia

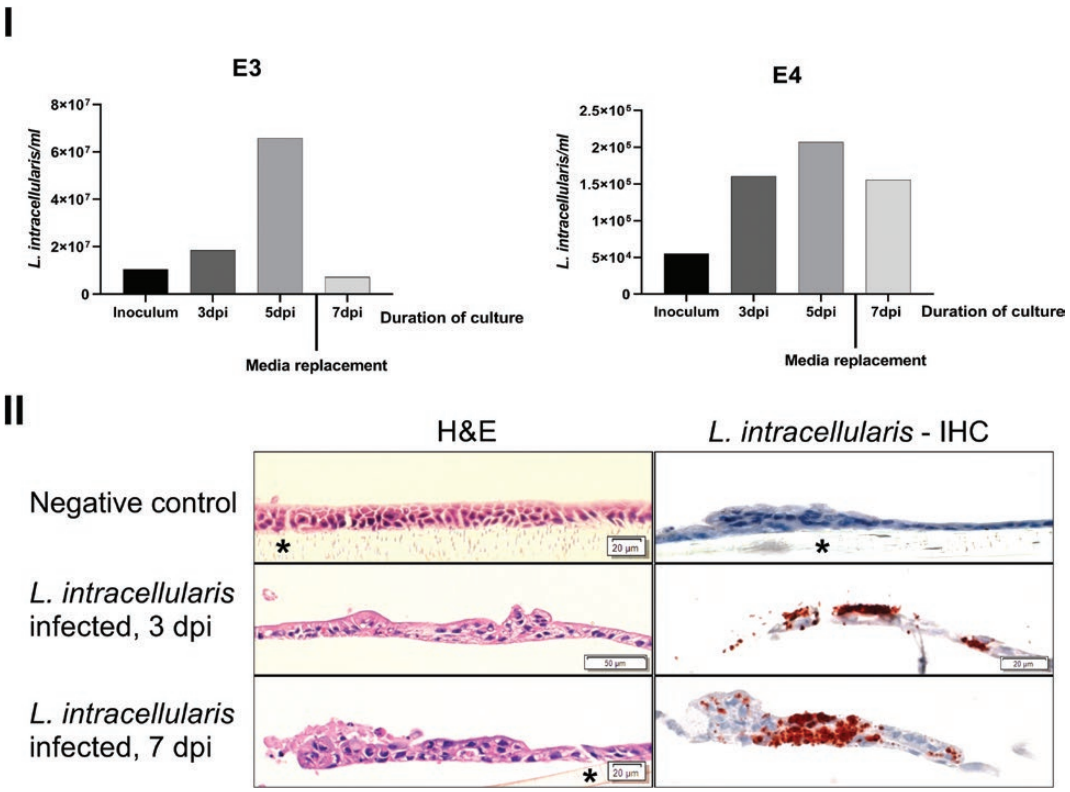

Figure 1. *L. intracellularis* propagates in two-dimensional swine enteroids. Panel I) The media covering two-dimensional swine enteroid in the transwells was used to investigate *L. intracellularis* propagation by real-time PCR. The media was collected at 3, 5 and 7 dpi. Results from the samples of experiment 3 (E3) and experiment 4 (E4) were positive at 3, 5 and 7 dpi, indicating that *L. intracellularis* was capable of propagation in the two-dimensional swine enteroids. An increase in the *L. intracellularis* concentration from 3 to 5 dpi was detected in E3 and E4, reflecting propagation of *L. intracellularis* organisms. The real-time PCR positive results for the media collected at 7 dpi are noteworthy, since the culture media was completely replaced at 5 dpi with fresh sterile media, indicating the release of bacterial organisms from the cells of the two-dimensional swine enteroids to the media in the apical side after 2 d of incubation in fresh media. Panel II). Cross-sections of two-dimensional swine enteroid.

of numerous animals to provide intestinal samples. Additionally, culturing swine enteroids without cell immortalization or genetic modification for high cellular proliferation allows for the assessment of the different signaling pathways of the epithelial cells affected by *L. intracellularis* infection.

The incubation period for *L. intracellularis* infection in pigs in vivo is about 11 days (Guedes et al., 2017), so we speculate that the length of the experiment (7 days) may not be sufficient to allow any long-term effects of *L. intracellularis* infection to develop in vitro, including the observation of hyperplasia. Future experiments will also account for this variable and will include transwells infected with *L. intracellularis* for longer than 14 days. Based on the results presented here, we defined that enteroids should be infected with *L. intracellularis* inocula at a concentration of about 10^7 *L. intracellularis* organisms/mL. Further analysis of the impact of *L. intracellularis* infection on the epithelial cell distribution and proliferation are needed. Long term, *L. intracellularis* infection of swine enteroids can be exploited in several ways such as to study 1) early host–pathogen events (endocytic process of *L. intracellularis* in intestinal epithelial cells); 2) phenotypic identification of the primary epithelial cells infected by *L. intracellularis*; 3) gene editing in swine enteroids to investigate the signaling pathways involved in *L. intracellularis*-induced proliferation; and 4) route of neighbor-cell infection during *L. intracellularis* propagation.

Our study provides proof-of-concept that swine enteroids allow *L. intracellularis* infection and propagation. We anticipate future studies that will assess the utility of two-dimensional swine enteroids as revolutionary models for the evaluation of novel therapeutics, vaccine candidates, and host–pathogen interactions involved during infection by *L. intracellularis*, accelerating the advancement of interventional strategies against this important pathogen in the swine industry.

Conflict of interest statement

None declared.

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