CARLOS EDUARDO REAL PEREIRA

Lawsonia intracellularis: Macrophages permissibility, mechanism of cell entry and antimicrobial susceptibility

Belo Horizonte

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Lawsonia intracellularis: Macrophages permissibility, mechanism of cell entry and antimicrobial susceptibility

Tese apresentada ao Programa de Pós-Graduação em Ciência Animal da Escola de Veterinária da Universidade Federal de Minas Gerais como requisito parcial para obtenção do grau de Doutor em Ciência Animal

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Orientador: Prof. Roberto Maurício Carvalho Guedes

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Tese submetida à banca examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIA ANIMAL, como requisito para obtenção do grau de DOUTOR em CIÊNCIA ANIMAL, área de concentração em PATOLOGIA ANIMAL.

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Escola de Veterinária

Dedicada aos meus pais, João Carlos e Eliana,

a minha irmã Natália, a minha esposa

Isabella e ao meu filho Frederico.

“Se eu não for por mim, quem o será?

Mas se eu for só por mim, que serei eu?

Se não agora, quando?” (Hilel, o Ancião).

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Summary

Tables List

Figures List

Resumo
A enteropatia proliferativa, causada por *Lawsonia intracellularis*, é uma doença entérica com distribuição mundial que afeta várias espécies animais, principalmente suínos e equinos. Nesta tese foi realizada a avaliação *in vitro* do mecanismo de endocitose da *L. intracellularis* em células IPEC-J2, e foi evidenciado que *L. intracellularis* pode ser internalizada por mecanismos ativos (dependente de bactéria viável) ou passivos (dependente da clatrina). Foi demonstrado também a capacidade de sobrevivência da *L. intracellularis* em fagolisossomos de macrófagos suínos. Além disso, foi isolada uma segunda cepa brasileira de *L. intracellularis* provenientes de suíno afetado pela enteropatia proliferativa. Duas cepas brasileiras foram adicionadas a cepas de outros países e realizado o perfil de susceptibilidade antimicrobiana, bem como a avaliação de susceptibilidade de isolados provenientes de equinos.

Palavras-chave: *Lawsonia intracellularis*, patogênese, ileíte, diarreia, susceptibilidade antimicrobiana

Abstract
Proliferative enteropathy is a widespread enteric disease caused by *Lawsonia intracellularis* that infects a variety of animal species, most notably pigs and foals. The mechanism of endocytosis of *L. intracellularis* in IPEC-J2 cells was evaluated *in vitro*, in which it was evidenced that *L. intracellularis* can be internalized by active (live bacteria-dependent) and passive mechanisms (clathrin-dependent). In addition, the survival capacity of *L. intracellularis* in phagolysosome of macrophages was demonstrated. Furhtermore, a second Brazilian *L. intracellularis* strains was isolated from a pig. Along with the two Brazilian isolates, isolates from other countries, including two equine *L. intracellularis* isolates, were evaluated regarding their antimicrobial susceptibility profile.

Keywords: *Lawsonia intracellularis*, pathogenesis, ileitis, diarrhea, antimicrobial susceptibility

Introduction

Proliferative enteropathy (PE) is an infectious intestinal disease characterized by thickening of the intestinal epithelium. *Lawsonia intracellularis* is the etiologic agent of PE (McOrist et al., 1995). The major clinical sign described is mild to severe diarrhea in many different animal species (Lawson and Gebhart, 2000). Since the 1990s, PE has been endemic in swine herds and has been occasionally reported in various other species, including foals, non-human primates, rabbits, hamsters etc (Cooper et al., 1997; Pusterla et al., 2008).

This thesis comprises a literature review with an overview about *L. intracellularis* pathogenesis including teories related to mechanisms of endocytosis, phagocytosis, and bacterial intracellular traffic, as well as treatment of the disease. In Chapter 1, pig macrophages from peripheral blood monocytes were quantified and characterized concerning function, morphology and interaction with *Salmonella enterica* serovar Thyphimurium, thus establishing a reliable model for further studies with *L. intracellularis*. Chapter 2 describes the interaction and permissibility of macrophages to *L. intracellularis*. Studies presented in Chapter 3 evaluated the endocytosis mechanisms involved in *L. intracellularis* internalization *in vitro* in IPEC cells. In Chapter 4 the first two Brazilian *L. intracellularis* strains obtained from pigs were used to determine their antimicrobial susceptibility profile. Finally, Chapter 5 was the first study to evaluate the antimicrobial susceptibility profile of equine *L. intracellularis* strains.

Hypotheses

- *Lawsonia intracellularis* uses zipper mechanism to be internalized in eukaryotic cells.
- *L. intracellularis* can survive inside of macrophages.

Objectives

- To characterize (phenotypically) peripheral blood monocyte-derived swine macrophages.
- To evaluate the endocytosis process using by *L. intracellularis* internalization.
- To evaluate the survival capacity of *L. intracellularis* inside porcine macrophages.
- To isolate the second Brazilian strain of porcine *L. intracellularis*.
- To determine the *in vitro* minimum inhibitory concentration (MIC) of different antimicrobials against two cell-cultured Brazilian porcine isolates of *L. intracellularis*.
- To determine the *in vitro* MIC of antimicrobials against two *L. intracellularis* isolates obtained from equine.

Mini Review

Lawsonia intracellularis - Overview

Lawsonia intracellularis is a Gram-negative, intracellular obligatory, microaerophilic, motile bacterium, which is the causative agent of proliferative enteropathy (PE) (Lawson and McOrist, 1993). PE is better described and characterized in pigs, although it may affect many animal species including horses, mice, hamsters, rabbits, dogs, monkeys, among others (Lawson and Gebhart, 2000; Dauvillier et al., 2006). *L. intracellularis* infection occurs via the fecal-oral route, with affected pigs shedding large amounts of *L. intracellularis* (up to 10^8 *L. intracellularis* per gram of feces) for a long period (Guedes and Gebhart, 2003; Stege et al., 2004). Infection may occur after exposure to just a small amout of the bacterium, thus contributing to the high prevalence of the disease in swine herds around the world.

In pigs, PE has two clinical forms of presentation. The chronic form, known as porcine intestinal adenomatosis, is most commonly observed in pigs between 6 and 20 weeks of age. It is characterized by anorexia, diarrhea, and reduced weight gain. The acute form, also known as hemorrhagic proliferative enteropathy, affects older pigs, between 4 and 12 months of age, and it is characterized by blackened feces (melena) and sudden death (Lawson and Gebhart, 2000). There is also the subclinical form of the disease, in which no clinical signs are observed, although this form has great epidemiological importance, due to silent dissemination of the agent. In equine, the disease is characterized by diarrhea, fever, weight loss, and hypoproteinemia (Lavoie et al., 2000). Proliferative enteropathy in equine primarily affects younger horses, especially recently weaned foals, but it may occasionally affect older animals (Pusterla and Gebhart, 2013)

Macroscopically, lesions characteristic of PE are usually observed in the ileum, although, less frequently, they can be found in the cecum, colon, and jejunum. In the acute form of PE, lesions are characterized by edema and hyperemia of the mesentery and thickening of the intestinal mucosa. The mucosa present evident folds and hemorrhagic content can be observed in the intestinal lumen. The chronic form consists of edema of mesentery, corrugated surface of the intestinal serosa, thickening of the intestinal mucosa and a necrotic membrane adhered to mucosa (Ward and Winkelman, 1990).

In general, chronic and acute forms of PE are histologically similar. Proliferation of crypt epithelial cells is observed in the small and/or large intestines. The bacteria can be observed at the apex of intestinal epithelial cells by using special staining techniques such as immunohistochemistry or silver stain. The villi are atrophied with decreased number of goblet cells (Bengtsson et al., 2015). Inflammatory is mild or absent (McIntyre et al., 2003). A distinction between the acute and chronic forms of PE is the observation of marked mucosal hyperemia and accumulation of blood in the intestinal lumen in acute cases (Guedes et al., 2017).

Diarrhea observed in breeding and finishing animals is poorly specific, as it can be caused by a variety of enteric agents, frequently in association. Therefore, sampling for complementary tests is required to determine the cause of the clinical signs. As clinical samples for *ante-mortem* diagnostics, feces or serum samples can be evaluated by PCR and immunoperoxidase monolayer assay (IPMA), respectively (Gebhart et al., 1991; Guedes et al., 2002). These are highly specific tests for, respectively, detection of specific region presents in the genome of the bacterium, and of systemic IgG antibodies against *L. intracellularis*.

Pathogenesis

After successful *in vitro* culture and propagation of *L. intracellularis* (Lawson et al., 1993), studying mechanisms involved in the *L. intracellularis* pathogenesis became more feasible. *In vitro* examination of eukaryotic cells infected with *L. intracellularis* has shown the ability of the bacterium to be rapidly internalized, being observed intracellularly within the first hour after infection of susceptible cells (Lawson et al., 1995). Until recently, bacterial entry in eukaryotic cells appeared to require specific interactions with the host cell (McOrist et al., 1997). McOrist et al (1995) showed that *L. intracellularis* interacts closely with the host cell membrane and it is internalized within cytoplasmic vacuoles. Three hours after inoculation, bacteria can be observed, still inside the vacuoles, initiating their propagation in the cytoplasm of the cells. From the second to the sixth day

after incubation it is possible to observe free bacteria in the cytoplasm multiplying by binary fission. *L. intracellularis* genome sequence indicates that this bacterium may possess a type III secretion system, commonly found in Gram-negative pathogenic bacteria. This secretion system could assist the bacterium during cell invasion and evasion of the host immune system, releasing protein factors directly into the cytoplasm of eukaryotic cells (Alberdi et al., 2009). This mechanism may be an important pathogenicity factor of the bacterium (Kroll et al., 2005), but it remains uninvestigated to date.

Lawson and colleagues (1995) observed inhibition of the *L. intracellularis* proliferation when using cycloheximide and colquicin, substances that act to inhibit cell division, in *in vitro* cultures of *L. intracellularis*. It was hypothesized that the proliferation of the bacterium depends intimately on the metabolic profile of the host cells, more specifically in the apparatus that is active during mitosis.

Proliferative enteropathy has been successfully reproduced *in vivo*. Pure cultures of *L. intracellularis* or mucosal homogenate of affected intestines used as inoculum in susceptible pigs will develop typical clinical signs and lesions similar to naturally infected pigs (Guedes and Gebhart, 2003). Macro and microscopic lesions begin in the second week after infection (Guedes et al, 2017), but experimental studies have shown that the peak of the disease is between three and four weeks after inoculation. Fecal shedding of the bacteria is detected for four weeks and can reach up to 10 weeks (McOrist et al., 1996; Smith and McOrist, 1997; Guedes et al., 2017).

Recent studies have demonstrated, through experimental evaluation, the interaction between the bacterium and enterocytes approximately 12 hours after oral inoculation (Boutrup et al., 2010a). It should be noted, however, that this period maybe was abbreviated, since the model of infection in that case was based on intestinal loops with direct intraluminal inoculation of *L. intracellularis* (ligation-loop), resulting in interaction at about three hours post-inoculation (Boutrup et al., 2010b).

Similar to the *in vitro* events, two to six days after inoculation, it is possible to observe the multiplication of the bacteria in the cytoplasm of eukaryotic cells. This event will later determine the rupture of the cytoplasmic membrane of the cells and release of the microorganisms to the extracellular medium, where they will be capable of infecting new neighboring cells (Lawson and Gebhart, 2000).

In many cases, low intensity inflammatory reactions are observed in intestines affected by PE (McOrist et al., 1996). However, inflammation is not a primary feature of the disease. In an experimental study using hamsters, Jonhson and Jacoby (1978) observed that immature enterocytes from the proliferated ileum reaches the tip of villi within 14 days after infection. Cellular hyperplasia, associated with the presence of *L. intracellularis*, could be observed for more than 40 days after challenge using a PE-diseased intestinal mucosal homogenate, using hamsters as a model (Frisk, 1976; Jacoby, 1978). Guedes et al. (2017) and Huan et al. (2017) have demonstrated increased apoptosis rate during *L. intracellularis* infection, ruling out the possibility that the increase in the number of cells during the disease was due to the inhibition of this process of cell death. There is a need for future studies to more accurately assess cell proliferation during *L. intracellularis* infection.

In some cases of PE, *L. intracellularis* antigen can be observed in mesenteric lymph nodes and tonsils. However, apparently there is no bacterial proliferation at these sites, suggesting that those findings are a result of inactivation and degradation of the microorganism by macrophages (Jensen et al., 2000). On the other hand, Boutrup et al. (2010a), using the fluorescence *in situ* hybridization (FISH) technique, observed the presence of viable *L. intracellularis* in the intestinal lamina propria and inside mononuclear cells. Considering that macrophages play an important role in the pathogenesis of other enteric pathogens, it is relevant to better understand the involvement of macrophages in *L. intracellularis* pathogenesis.

For maintenance and propagation of the *L. intracellularis in vitro*, permissive cell lines are grown weekly. Upon reaching 100% confluence, bacterium is transferred into a new culture flask with low confluency. Such a procedure may be a factor in decreasing the pathogenicity of the bacterium by adapting it to *in vitro* conditions, caused mainly by genetic mutations or changes in the regulation of gene expression (Fux et al., 2005). An *in vivo* study demonstrated significant differences between the number of bacteria eliminated in the feces, intensity of immune response, clinical signs and lesions when compared to pure culture inoculation with *L. intracellularis* at 10, 20 and 40 *in vitro* passages. It should be noted that with 10 and 20 *in vitro* passages PE was reproduced, which was not observed in the pigs inoculated with *L. intracellularis* after 40 passages *in vitro* (Vannucci et al., 2013). In

addition, in another study, Vannucci and colleagues (2012) compared the transcription capacity of pathogenic homologues (10 passages) and nonpathogenic (60 passages) homologues. Differences in gene functional categories that were more remarked in adaptation, stress response, cell membrane, motility, membrane transport, biosynthesis of micro and macromolecules, and cell division categories were observed. In all of these categories, the pathogenic strain had the highest number of expressed genes. These results indicate that the mechanisms involved in the pathogenicity of *L. intracellularis* appear to be more complex than previously speculated.

Bacterial Endocytosis

Intracellular parasitism by bacterial microorganisms is a mechanism for escaping from the host defenses. In addition, some species are able to utilize the intracellular environment for nutrient acquisition and replication (Pizarro-Cerda and Cossart, 2006). Within eukaryotic cells, pathogenic bacteria reduce exposure to various immune mechanisms produced by the host, and develop various mechanisms for their survival. Nevertheless, the host uses several bactericidal mechanisms, even intracellularly, for eliminating pathogens (Pizarro-Cerda and Cossart, 2006). The mechanism of entry of the bacterium into the host cell, called endocytosis, will depend on cellular characteristics (phagocytic and non-phagocytic), prokaryotic species, and individual variations, since individuals of the same species interacting with the same cells eukaryotes can be internalized by different mechanisms (Cossart and Helenius, 2014).

Zipper mechanism

The zipper mechanism of internalization is related to proteins or structures of the outer membrane of the bacterium that interact directly with specific receptors of the host cells. In this process, there is a clustering of these eukaryotic cell receptors, associated with changes in the actin cytoskeleton resulting in the coating of the microorganism by the plasma membrane (Pizarro-Cerda and Cossart, 2004; Cossart and Helenius, 2014). From there, several mechanisms are activated and result in endocytosis of the pathogen. Bacterial structures and proteins often mimic host compounds to better exploit receptor properties (Cossart and Helenius, 2014).

In the zipper process, there is the involvement of a clathrin-dependent machinery (Figure 1.1). Clathrin is a protein involved in formation of vesicles during endocytosis of exogenous material. The involvement of this machinery occurs at the initial stage of the endocytosis process, soon after the adhesion of the bacterium to the surface of the host cell (Bonazzi et al., 2011). Eto et al. (2008) have evaluated the role of clathrin in the process of *E. coli* (UPEC) adhesion and internalization in bladder transitional epithelium cells, concluding that the inhibition of clathrin was determinant to decrease the *E. coli* internalization, but had no effect on the bacterial adhesion process.

On the host cell surface, there are several adhesion molecules, especially in coat cells because of their juxtaposition nature. Some pathogenic bacteria, such as *Listeria monocytogenes*, mimic these molecules to penetrate host cells. E-cadherin is an adhesion molecule that is only expressed in some epithelial cells, being mimicked by *L. monocytogenes* by the expression of internalin 1A responsible for the initial stage of adhesion and penetration of this microorganism in the host (Menguad et al., 1996; Pizarro-Cerda et al., 2012). For the penetration into other cells, *L. monocytogenes* uses other mechanism including another internalin (1B) that activates specific receptors (Met) in eukaryotic cells (Bierne and Cossart, 2002). Similarly, *Yersinia pseudotuberculosis* expresses invasins on its surface that bind to the cellular receptor called β-integrin (Isberg and Leong, 1990; Van Nhieu et al., 1996). These interactions induce a cascade of reactions that result in polymerization of actin, closure of the vesicle coated by the plasma membrane and envelopment of the aforementioned microorganism into the cell (Cossart and Sansonetti, 2004).

Other genera of bacteria such as *Staphylococcus* sp. and *Streptococcus* sp., use integrins in the internalization process that is mediated by clathrin (Almeida et al., 1996; Ozeri et al., 2001; Kreikemeyer et al., 2004). In these circumstances, the internalization mechanism is completed by myosin VI which interacts with actin and transports the bacterium into the cell through actin rearrangements by polymerization waves with recruitment of different enzymes (Ireton et al., 1996).

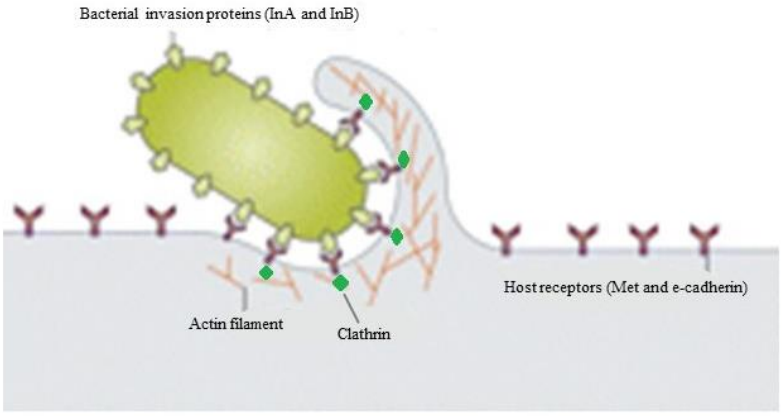

Figure 1.1 – *Listeria* endocytosis: *Listeria* uses the internalin A and/or B proteins to interact with Met and E-cadherin receptors in cell surface. Then, elicits downstream signaling pathways that culminate in recruitment of clathrin and actin (host cells proteins) (Modified from Haglund and Welch, 2011).

Trigger mechanism

After adhesion to the surface of the host cell, the microorganism uses the secretion system, mainly type 3, to transfer proteins to the host cell cytosol. From this, abrupt rearrangements are initiated in the cytoskeleton resulting in the coating and internalization of the pathogen by the plasma membrane (Cossart and Helenius, 2014).

The main bacterial genera that use this pathway of internalization are *Shigella* and *Salmonella*. In the case of *Salmonella*, the first type 3 secretion system (3TSS-1) and fimbrial adhesin are essential for triggering the process (Misselwitz et al., 2011). Using *Salmonella* as example, after the adhesion, the bacterial T3SS-1 is responsible for translocating the following proteins: Sop E, Sop E2, Sop B, Sip C, and Sip A (Stender et al., 2000; Zhou et al., 2001; Patel and Galan, 2006) that will then initiate the actin polymerization and stabilization (Figure 1.2). Subsequently, there is a sudden generation of rearrangements in the cytoskeleton that involves the microorganism with plasma membrane-coated vacuole (Caim et al., 2008).

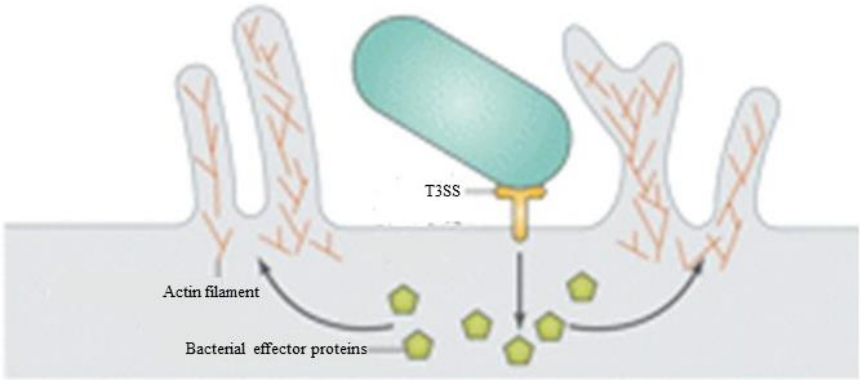

Figure 1.2 - *Salmonella* endocytosis: *Salmonella* bind to a cell receptor and uses the T3SS to inject effector proteins that culminate in actin cytoskeletal rearrangement (Modified from Haglund and Welch, 2011).

Phagocytosis and intracellular traffic

Phagocytosis is a specific form of endocytosis, performed by professional phagocytes cells (neutrophils, dendritic cells and macrophages) that recognize and internalize the invading pathogen. In the interior of the cell, the phagosomes, mediated by the Rabs (GTPases) and their effectors (Zeigerer et al., 2012), are responsible for the death of the bacterium through exposure to an acid environment, synthesis of antimicrobial enzymes, oxidative stress and lysosomal hydrolases

(Flannagan et al., 2009). However, some bacterial genera are capable of inhibiting phagolysosome formation, or escape from the phagosome and remain free in the cytosol, or else resist the hostile environment within the phagolysosomes.

Intracellular traffic modification by pathogenic bacteria

Several bacterial genera have developed particular mechanisms and virulence factors to ensure their survival and proliferation within the host cells. For this purpose, microorganisms have the need to subvert the various defense mechanisms of the host.

Generally, the mechanism used by these bacteria is the inactivation of the Rabs and their effectors, which are essential for the phagosome maturation. There are several specific markers related to the maturation period of the phagosome that enable to the evaluation of the time of its interruption (environment in which the agent is able to survive and often replicate). Such a maturation may happen early (immature phagosome) as occurs in *Legionella*, *Mycobacterium* and *Listeria* infection; late, as in *Brucella* and *Salmonella* infections; or after fusion with lysosome, as in infections by *Coxiella* and *Rhodococcus equi* (Tang, 2015). The subversion of this machinery is performed by a set of virulence factors secreted by the secretory systems of the pathogen that targets several host proteins that regulate the intracellular traffic of the phagosome (Hayes et al., 2010).

Treatment

The main form of controlling PE outbreaks is based on antimicrobial therapy (Sampieri et al., 2014). Antimicrobial therapy with an effective antimicrobial agent is able to stop the progression of PE in a short period of time. Therefore, the choice of the appropriate antimicrobial is critical to obtain success in controlling the disease. Despite the importance of antimicrobial treatment, information on the sensitivity of *L. intracellularis* to antimicrobial compounds is limited and restricted to pig strains (McOrist and Gebhart, 1995; Wattanaphansak et al., 2009; Yeh et al., 2011). The main reason for this lack of information is due to the difficulty in isolating *L. intracellularis* from infected intestine, requiring experienced personnel and several months for the establishment of a pure *L. intracellularis* culture. Consequently, the *in vitro* sensitivity of *L. intracellularis* to antimicrobials is difficult to obtain in a timely fashion to treat a PE outbreak. Furthermore, the obligate intracellular nature of *L. intracellularis* does not allow the use of standard antimicrobial susceptibility testing methods, used for other bacterial species. Studies evaluating the antimicrobial susceptibility of *L. intracellularis in vitro* have demonstrated that susceptibility to antimicrobials varies according to the origin of the isolate. However, some active principles have demonstrated efficacy against most of the tested strains, such as valnemulin, tiamulin and carbadox. On the other hand, several *L. intracellularis* strains have shown resistance against the following principles: lincomycin, bacitracin, gentamicin, neomycin (McOrist and Gebhart, 1995; Wattanaphansak et al., 2009; Yeh et al., 2011). Variations of the antimicrobial resistance profile of the different strains show the need for further studies evaluating the isolates from different regions.

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CHAPTER 1 - Peripheral blood monocyte-derived macrophages from pigs: Phenotypic characterization and interaction with *Salmonella enterica* serovar Typhimurium (wild type and *invA* mutant)

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Abstract
Macrophages are critical mediators of the inflammatory process, playing a relevant role in the pathogenesis of *Salmonella* Typhimurium. The protocols for isolation, culture and differentiation of monocytes into macrophages and their interaction with *Salmonella* are well established in humans and murine models, but little information is available in swine. The aims of this study were to establish an efficient protocol for macrophage culture and to evaluate the interaction of the *invA* mutant strain and the wild type (WT) *Salmonella* Typhimurium with porcine macrophages. Peripheral blood monocyte-derived macrophages from pigs were obtained, separated by density-gradient centrifugation, and cultured in Teflon vials for 10 days. After the differentiation period, cultures consisted of 92.4% $CD14^+$ cells. In addition, these cells showed phagocytic ability, demonstrated by the presence of the same amount of WT and *invA* mutant *Salmonella* Typhimurium one hour after interaction with macrophages. The early cytotoxic effect was SPI-1-dependent, in which log-phase WT strains were more efficient ($P<0.01$) than the *invA* mutant strain at inducing the death of macrophages.

Keywords: *Salmonella* Typhimurium, pigs, macrophage, cytotoxicity.

Introduction

Macrophages play important roles in the innate and adaptive immune responses against invading pathogens, contributing to the elimination of microorganisms through bactericidal activity while also being able to present antigens to T cells. Conversely, macrophages may be targeted by intracellular bacteria that remain viable and proliferate within the intracellular environment, leading to persistence and chronic infections (Donné et al., 2005; Maria-Pilar et al., 2005).

A standardized protocol for primary culture of peripheral blood monocyte-derived swine macrophages is not available, although such a protocol would be highly desirable for advancing our knowledge on swine macrophage function. However, the low concentration of monocytes in the peripheral blood of pigs may pose a challenge in the isolation and primary culture of these cells (Eze et al., 2010; Adenkola et al., 2011).

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a facultative intracellular bacterium capable of infecting several animal species, including pigs, causing considerable economic losses and relevant public health problems (Berends et al., 1996). Pigs infected with *S.* Typhimurium become a source of infection for other animals, the environment and carcasses at the slaughterhouse (Poppe et al., 1998). The carrier status of infected animals may be due to the ability of this pathogen to survive within the macrophage (Santos and Baumler, 2004). After contact with enterocytes and macrophages, *S.* Typhimurium has the ability to be internalized, to survive and, in some cases, to proliferate in the intracellular environment (Buchmeier and Heffron, 1989; Santos et al., 2001). Invasion of intestinal epithelial cells is mediated by *Salmonella* pathogenicity island 1 (SPI-1), which encodes a type III secretion system (T3SS-1) (Misselwitz et al., 2011). Once *S.* Typhimurium adheres to epithelial cells, a process that is mediated by fimbrial adhesin, the T3SS-1 translocates effector proteins, including SopE, SopE2, SopB, SipC and SipA, into the cytosol of the host cells, leading to cytoskeletal changes that results in a process similar to endocytosis and consequent internalization of the bacteria (Patel and Galan, 2006; Caim et al., 2008; Misselwitz et al., 2011). The *inv* operon, which encodes T3SS-1 inner membrane proteins, is also essential for invasion of epithelial host cells. *InvA* mutant strains lose their ability to actively invade host cells; therefore, these strains can only be internalized by eukaryotic cell-dependent mechanisms such as phagocytosis (Galan et al., 1992; Lilic et al., 2010). Within immortalized murine macrophages, these mutant strains can survive and proliferate at wild-type levels, causing late cytotoxicity, in contrast to the wild type strain, which is able to induce very early cytotoxicity (van der Vanden et al., 2000; Franchi et al., 2006; Miao et al., 2006). Most of these studies have been performed using murine models. Importantly, the course of *S.* Typhimurium infection *in vivo* may differ between mice and pigs (Boyen et al., 2006a). Considering that the research on the interaction between *Salmonella* and porcine macrophages is scarce, it is necessary to evaluate the species-specific interaction mechanism between the pathogen and this host.

The aim of this study was to phenotypically characterize peripheral blood monocyte-derived swine macrophages and to evaluate the interaction of these cells with *S.* Typhimurium (WT and mutant *invA*) and to evaluate possible effects of the age of pigs on macrophage functions under experimental conditions *in vitro*.

Material and Methods

Blood Samples and Ethics Statement

Peripheral blood was drawn from four young female pigs (60 days of age – nursery pigs) and four adult female pigs (>1 year of age – sows) by puncture of the jugular vein and collected in tubes with anticoagulant (EDTA and heparin).

The study was conducted in accordance with the rules of the Ethics in Animal Use Committee at the Universidade Federal de Minas Gerais (CEUA/UFMG protocol number 249/2015).

Salmonella Typhimurium strains

Wild-type (WT) *Salmonella* Typhimurium (strain IR715; ATCC14028 – Stojiljkovic et al., 1995) and an isogenic *invA* mutant strain (Winter et al., 2009) were used in this study, cultivated in Luria-Bertani broth (LB) with shaking at 37°C overnight to reach the stationary phase. A similar procedure was carried out for 5 hours to obtain cultures in the logarithmic phase.

Leukogram

Whole blood samples collected in EDTA were used for global counts and differential leukocyte analysis in an automated Vet ABC Hematology Analyzer (Heska Corporation, Waukesha, WI, USA).

Peripheral blood mononuclear cells – isolation, culture, and *in vitro* macrophage differentiation

Peripheral blood mononuclear cells (PBMC) were isolated by differential centrifugation of whole blood samples on a Histopaque cushion. Briefly, 60 mL of heparinized whole blood was collected from each animal and diluted (1:1 proportion) in Dulbecco's modified Eagle's medium (DMEM – Gibco, Carlsbad, USA). In 50 mL polypropylene conical tubes (BD Bioscience, San Jose, CA, USA), the diluted whole blood sample was carefully layered onto Histopaque solution (Sigma-Aldrich, St. Louis, MO, USA) at a proportion of 1:2 v/v Histopaque:blood, after which it was centrifuged at 1,065 x *g* for 40 min at 18°C. The mononuclear cells were collected from the interface between the plasma and Histopaque with a Pasteur pipette and transferred to another tube, where they were resuspended in erythrocyte lysis buffer for 5 min at room temperature and washed twice with DMEM by centrifugation at 1,065 x *g* for 15 min at 4°C. Subsequently, the cells were transferred to Teflon flasks (Nalge Nunc, Rochester, USA) and then cultured for 10 days at 37°C with 5% CO_2 in 10 mL of RPMI-1640 supplemented with 10% fetal bovine serum, 200 mM L-glutamine, 7.5% w/v sodium bicarbonate, 10 mM non-essential amino acids, 10 mM sodium pyruvate, 50 IU/mL penicillin and 50 µL/100 mL streptomycin. Following 24 hours of incubation, the culture medium was replaced to remove non-adherent cells and antibiotics. The cultures remained under the same conditions for 10 days, and the medium was replaced every three days. After this time, the monocyte-derived macrophages were recovered for flow cytometric phenotypic characterization and transferred to 96-well plates to assess *in vitro S.* Typhimurium survival in monocyte-derived macrophages and the cytotoxic effect of *S.* Typhimurium to monocyte-derived macrophages.

Flow cytometric analysis of porcine circulating leukocytes, peripheral blood mononuclear cells and monocyte-derived macrophages

Aliquots of heparinized whole blood (50 µL aliquots), mononuclear cells (approximately 2.5 x 10^5 cells in 50 µL) and monocyte-derived macrophages (1.5 x 10^5 cells in 50 µL) were stained with 1 µL of anti-CD14 monoclonal antibody (TüK4 clone) labeled with Alexa Fluor 647 (Serotec Inc, Oxford, UK) for 30 min at room temperature in the dark. After incubation, red blood cells were lysed (in whole blood samples) and cells prefixed by the addition of 3 mL commercial lysing solution (FACS Lysing Solution, BD Biosciences, San Jose, CA, USA) with vortexing, followed by incubation for 10 min at room temperature. The cells were washed once with phosphate-buffered saline (PBS) by centrifugation at 400 x *g* for 7 min at room temperature. The stained cells were then fixed in 300 µL of Macs Facs Fix (10.2 g/L sodium cacodylate, 10.0 g/L paraformaldehyde, 6.63 g/L sodium citrate, pH 7.2) and maintained at 4°C for up to 24 h prior to acquisition with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The software CellQuest™ was used for data acquisition and storage. The software FlowJo version 7.6.5 (TreeStar, San Diego, CA, USA) was used for morphometric and immunophenotypic analysis by flow cytometry.

***Salmonella* Typhimurium infection and intracellular survival in porcine monocyte-derived macrophages**

Monocyte-derived macrophages were seeded in 96-well plates (50,000 cells/well) for evaluation of *S.* Typhimurium (WT and *invA*) internalization, survival and proliferation at 1, 6, and 24 h post infection (hpi) at an MOI of 50, in triplicate. Bacteria were inoculated, and the plates were centrifuged at 1.000 x *g* for 5 minutes to synchronize infection. The plates were then incubated for 30 minutes at 37°C with 5% CO_2. Subsequently, 50 µg/mL of gentamicin (Sigma-Aldrich) was added to remove extracellular bacteria, and the cells were then incubated under the same conditions for another hour. After this last procedure (T1), the wells were washed with PBS, and the cells were lysed with 0.1% Triton X-100 (Roche, Germany). The samples were diluted, and 100 µL of each dilution was plated on LB agar for CFU counting. The procedure was repeated at 6 (T6) and 24 (T24) hpi.

Additional macrophages were cultured without bacteria (negative control), and the culture medium with antibiotic was incubated with the bacteria (control for antibiotic efficacy).

Evaluation of the cytotoxic effect of *S.* Typhimurium on monocyte-derived macrophages

Macrophages were seeded in 96-well plates (50,000 cells/well) to evaluate the cytotoxic effect of *S.* Typhimurium (log-phase WT and *invA* mutant strains) with an MOI of 50 at 1 and 6 hpi (T1 and T6, respectively), in triplicate. The supernatant from the wells was frozen at -80°C. Lactate dehydrogenase (LDH) was measured by using a CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, USA) to evaluate the cytotoxic effect according to the manufacturer's instructions, and optical density (OD) values were measured with an ELISA reader.

Statistical analysis

Data were subjected to ANOVA followed by Student's t-test. The software Prism GraphPad version 5.0 was used for statistical analysis to compare means. Differences were considered significant if $p<0.05$. GraphPad Prism 5.0 and Microsoft Excel were used for graphic arts.

Results

Morphometric and immunophenotypic features of porcine whole blood leukocytes, peripheral blood mononuclear cells and monocyte-derived macrophages

Flow cytometric analysis was performed to evaluate the morphometric features (size and granularity) of peripheral blood leukocytes, isolated peripheral blood mononuclear cells and monocyte-derived macrophages (Figures 1A-C). Additionally, specific staining with anti-CD14 monoclonal antibody was used to characterize the distinct cell populations by flow cytometry. (Figures 1D-F). The differential stainability of porcine peripheral blood leukocytes with anti-CD14 monoclonal antibody (TüK4 clone) allowed the discrimination of distinct clusters of cells, including lymphocytes (LYM), monocytes (MON), neutrophils (NEU) and eosinophils (EOS), with MON presenting the highest and heterogeneous fluorescence intensity. Similar profiles were observed for nursery pig and sow samples.

The heterogeneous profile of monocytes, displaying distinct fluorescence intensity on anti-CD14 staining, may suggest the existence of monocyte subsets in porcine peripheral blood, as observed in humans.

The high performance of differential centrifugation of porcine whole blood on a Histopaque cushion could be observed by the morphometric profile, which showed the purity of the mononuclear cell suspension obtained. The immunophenotypic profile of peripheral blood mononuclear cells also revealed that the anti-CD14 monoclonal antibody (TüK4 clone) allowed the discrimination of distinct clusters of cells, including LYM and MON. In this case, MON exhibited a homogeneous staining profile.

The morphometric and immunophenotypic pattern of monocyte-derived macrophages demonstrated a homogeneous staining profile with anti-CD14 antibody but revealed great variation in granularity among the monocyte-derived macrophages. This feature may indicate the existence of distinct cell subsets or distinct statuses of cell activation among these cells.

Overall recovery of swine peripheral blood monocyte-derived macrophages

The results of monocyte quantification in whole blood and PBMC, as well as the overall recovery of monocyte-derived macrophages, are summarized in Figure 2. In all cases, the high performance of differential centrifugation of porcine whole blood on the Histopaque cushion to yield a pure mononuclear cell suspension contrasted with the recovery of monocytes at this step of the process (Figure 2). Moreover, macrophage differentiation upon *in vitro* culture of PBMC in Teflon flasks yielded a high purity profile of $CD14^+$ cells, demonstrating the efficiency of this approach that selects the monocytes by their plastic-adherent properties; the procedure yielded 97.5% and 89.3% $CD14^+$ cells in nursery pigs and sows, respectively (Figure 2C). During the PBMC separation, we recovered $36.5 \pm 4.5\%$ and $44 \pm 6.5\%$ from the monocytes in nursery pig and sow whole blood, respectively. The percentage of monocyte-derived macrophages recovered from the monocytes in

PBMC was 4.9 ± 0.7% and 6.2 ± 2.9% in nursery pigs and sows, respectively. The overall recovery of cell subsets is shown in Figure 2G, demonstrating that, from the total absolute counts of monocytes in whole blood, only 1.8 ± 0.3 and 4.2 ± 1.8 macrophages were recovered from nursery pigs and sows, respectively.

Young animals have higher concentrations of mononuclear cells in whole blood ($p<0.05$), but the age of the animals did not significantly influence the rate of recovery of monocyte-derived macrophages after *in vitro* differentiation.

Intracellular survival and cytotoxic effect of *Salmonella* Typhimurium

The survival results are shown in Figure 3. There was no significant difference between the strains (WT and *invA*), indicating that the bacteria were internalized by phagocytosis without variation in resistance or susceptibility according to age. Proliferation of both strains of *S.* Typhimurium was observed inside the macrophages 6 and 24 hpi.

The cytotoxic effect of the WT strain (100% cell death) was higher ($p<0.05$) than that of the *invA* mutant (24.75% cell death at 6 hpi) (Figure 3).

Discussion

The phenotypic characterization demonstrated the efficacy of monocyte purification, since immediately after PBMC separation the percentage of $CD14^+$ cells was 9.1% and at the end of the differentiation process the value was 93.2%, a characteristic of phagocytic cells. These results clearly demonstrate the effectiveness of PBMC selection, since $CD14^-$ cells do not adhere to Teflon vials. These results are similar to those observed in other animal species (Saldarriaga et al., 2003; Bueno et al., 2005). Morphologically, an increase in macrophage size and granularity (data not shown) was observed, and these characteristics are in agreement with the alterations undergone by this cell line in the differentiation process (Bueno et al., 2005).

The percentage of monocytes in the peripheral blood of pigs varies widely (Eze et al., 2010; Adenkola et al., 2011) and can reach extremely low values (Oluwole and Omitogun, 2016). As a result, highly efficacious techniques are needed to obtain this cell type. Interestingly, in the present study, the quantity of *ex vivo* monocytes was significantly higher in young animals ($P<0.05$), a result not found in other studies evaluating this variable (Aladi et al., 2008; Eze et al., 2010). However, the higher quantity of monocytes present in the whole blood of young animals did not result in a greater quantity of macrophages after 10 days of differentiation.

An important virulence factor of *S.* Typhimurium is related to its ability to survive and proliferate within macrophages. In this study, we used an *invA* mutant strain to evaluate the role of that gene – responsible for the internalization of *Salmonella* in the cells of the intestinal lining – in the phagocytosis of these cells. The *invA* mutants were internalized in similar quantities to the WT strain, which evidences the phagocytic capacity of the cultured cells. In addition, the proliferation capacity of the two strains was measured in the intracellular medium. The results agree with those already reported in the literature (Riber & Lin, 1999; Boyen et al., 2006b) that demonstrate the ability of *Salmonella* to subvert the lysosomal digestion process mediated by porcine macrophages. The subversion mechanisms are related to SPI-2, which encodes T3SS-2, responsible for secreting effector proteins (i.e., SopD2, SifA, SopB) that alter trafficking of the *Salmonella*-containing vacuole. It prevents endosome maturation and fusion with lysosomes by establishing a replication niche within the macrophages (Brumell & Grinstein, 2004; Figueira & Holden, 2012; McGourty et al., 2012; D'Costa et al., 2015).

In addition to the capacity to survive and multiply within macrophages, *S.* Typhimurium can induce the death of these cells through a specific process called pyroptosis (Mariathasan et al., 2004). The evaluation of the cytotoxic capacity of the two strains showed that the *invA* strain (log phase) has a lower and later cytotoxic effect than the WT strain (log phase). *Salmonella* growth to log phase increases the expression of SPI-1, which is capable of inducing rapid cell death (Lundberg et al., 1999). Later cytotoxicity caused by strains with less expression or decreased function (i.e., *invA*) of SPI-1 occurs because these strains do not release flagellin into the host cell cytosol, an event that is responsible for activating the mechanism of pyroptosis (Lee & Galan, 2004; Miao et al., 2006;

Franchi et al., 2006). However, it should be noted that the structural components of flagella present in these strains are recognized by Toll-5 receptors and may later induce the cell death process (Smith et al., 2003).

Conclusion

The methodology described in this study was efficient for obtaining and differentiating swine monocytes into CD14+ macrophages. The interaction between Salmonella and porcine macrophages demonstrated the phagocytic capacity of these cells. In addition, the early cytotoxic effects of SPI-1 have been demonstrated in swine macrophages. Together, our results clearly characterized peripheral blood monocyte-derived swine macrophages both phenotypically and functionally, thus supporting further studies on macrophage function and pathogen interactions**.**

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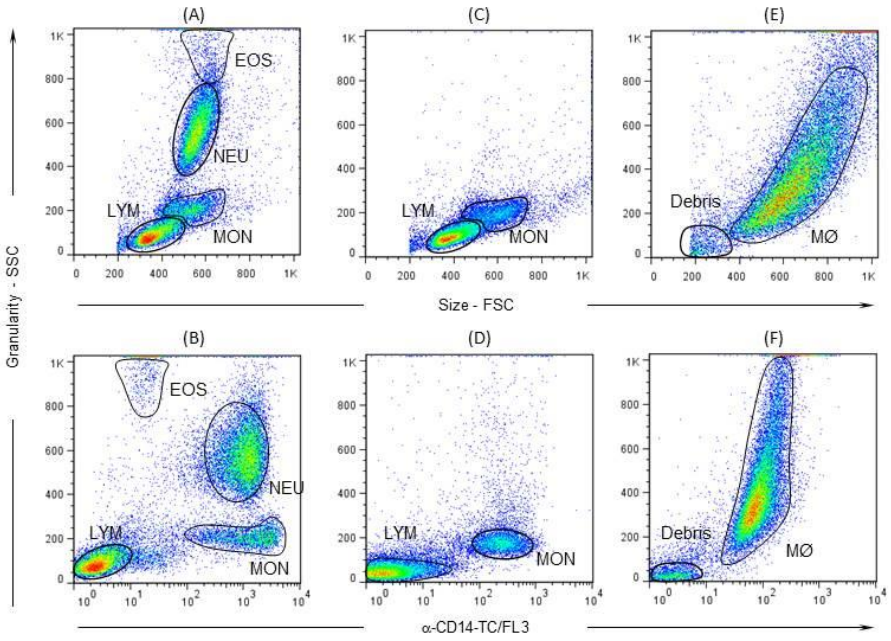

Figure 1

Figure 2.1 – Flow cytometric analysis of morphometric and immunophenotypic features of porcine whole blood leukocytes, peripheral blood mononuclear cells and monocyte-derived macrophages. Morphometric features (Size x Granularity) of (A) whole blood leukocytes [LYM, MON, NEU and EOS], (B) peripheral blood mononuclear cells [LYM and MON] and (C) monocyte-derived macrophages [debris and Mφ]. Immunophenotypic features ($CD14^+$ x Granularity of (D) whole blood leukocytes [LYM, MON, NEU and EOS], (E) peripheral blood mononuclear cells [LYM and MON] and (F) monocyte-derived macrophages [debris and Mφ]).

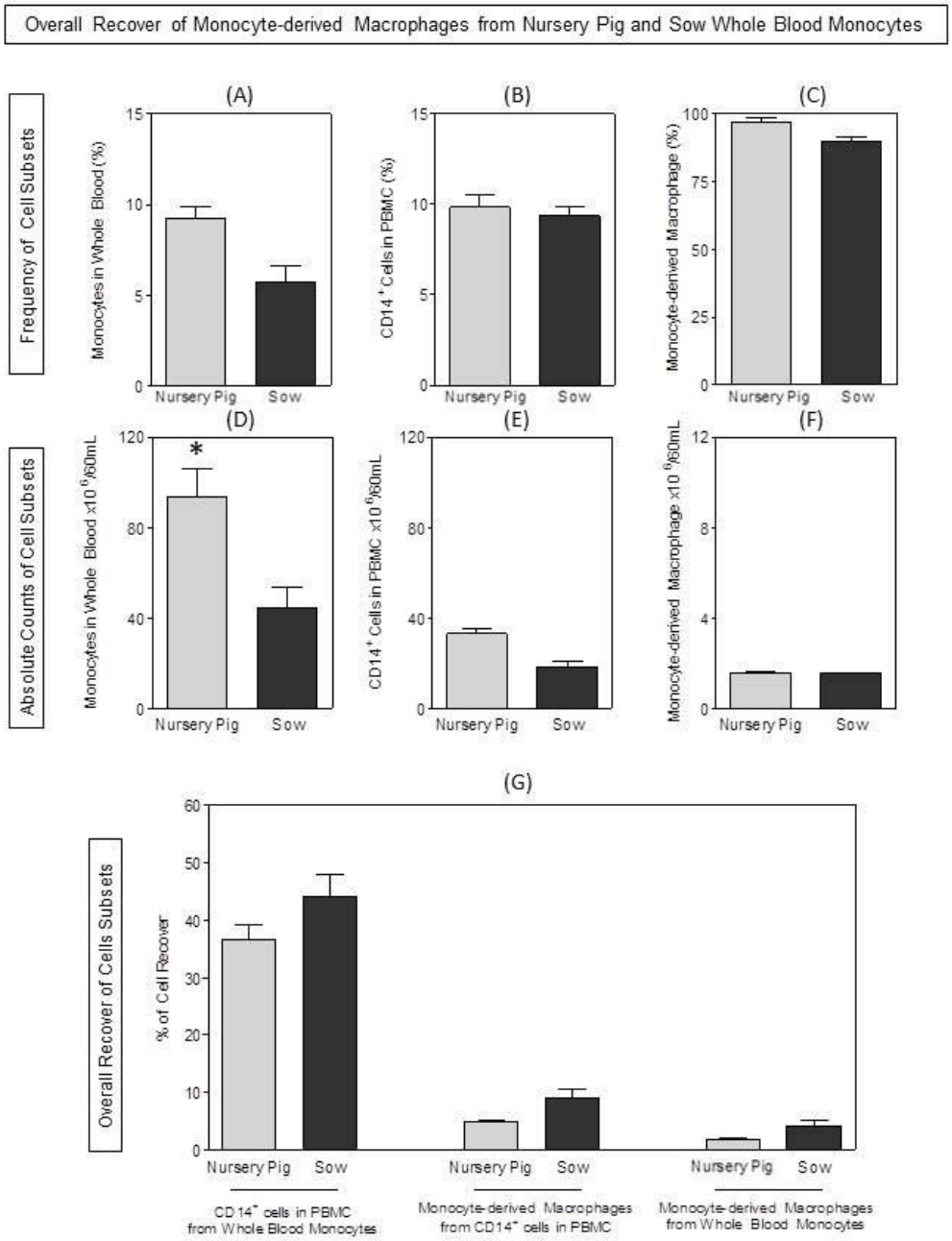

Figure 2

Figure 2.2 – Overall recovery of monocyte-derived macrophages from nursery pig and sow whole blood monocytes. (A) Percentage of monocytes in whole blood; (B) Percentage of $CD14^+$ cells (monocytes) among PBMC; (C) Percentage of $CD14^+$ after 10 days of cultivation (macrophages); (D) Absolute counts of monocytes in whole blood; (E) Absolute counts of $CD14^+$ cells (monocytes) among PBMC; (F) Absolute counts of $CD14^+$ after 10 days of cultivation (macrophages); (G) Recover of monocytes/macrophages. *, $p<0.05$.

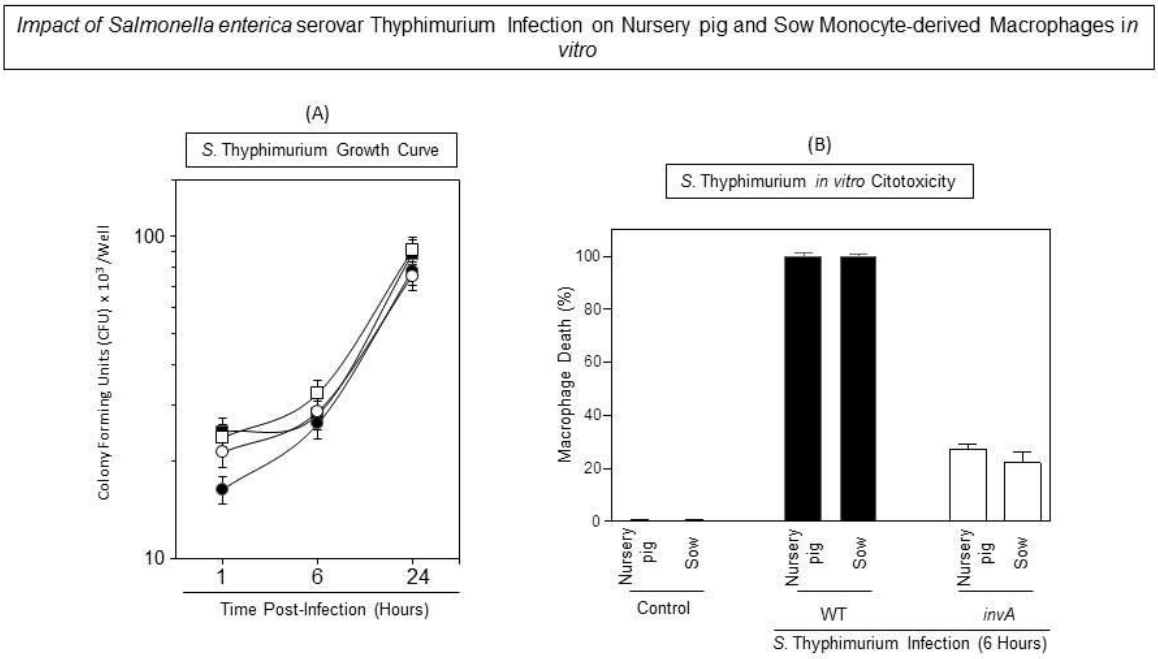

Figure 3

Figure 2.3 – Impact of *Salmonella enterica* serovar Typhimurium infection on nursery pig and sow monocyte-derived macrophages *in vitro*. (A) Time course of *Salmonella* intracellular survival in sow macrophages (■: *Salmonella* WT; □: *Salmonella invA* mutant) and nursery pig macrophages (●: *Salmonella* WT; ○:*Salmonella invA* mutant), at 1, 6 and 24 h post infection. (B) Comparison of cell death between nursery pig and sow macrophages 6 h post infection with *Salmonella* Typhimurium (wild type and *invA* mutant in the logarithmic phase).

CHAPTER 2 - Survival of *Lawsonia intracellularis* in porcine peripheral blood monocyte-derived macrophages

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Abstract
Lawsonia intracellularis, an obligately intracellular enteric bacterium, is observed in intestinal epithelial cells and also in macrophages in the lamina propria of affected pigs. Macrophages play an important role in host defense against infectious agents, but the role of the macrophage in *L. intracellularis* infection is contradictory and not well understood. The aim of the current study was to evaluate whether macrophages are permissive to *L. intracellularis* infection *in vitro*. Pure culture of *L. intracellularis* was added to swine peripheral blood monocyte-derived macrophages. Viability of *L. intracellularis* was evaluated inside of macrophages at different time points by transmission electron microscopy (TEM). Potential replication of *L. intracellularis* in macrophage cytoplasm was also evaluated by qPCR. By TEM, an early interaction was observed between macrophages and *L. intracellularis* 1 hour post infection (hpi). *L. intracellularis* was also observed inside of phagolysosomes. The number of intracellular bacteria was determined at 1 hpi, 4 hpi, 1 day post infection (dpi), 2 dpi and 3 dpi by qPCR and compared to culture of the bacterium in McCoy cells. In both cell lines, the amount of *L. intracellularis* was decreased at 4 hpi, and increased at 1 dpi. Within the macrophages, the amount of *L. intracellularis* remained stable from 1 dpi to 3 dpi. Conversely, the amount of intracellular bacteria continued to increase in McCoy cells over time. This is the first study on the direct interaction between *L. intracellularis* and macrophages. In conclusion, *L. intracellularis* can survive a phagolysosomal environment in macrophages.

Key words: Macrophages, pathogenesis, proliferative enteropathy, phagolysosome survival, *Lawsonia intracellularis*

Introduction

Macrophages play an important role in innate and adaptive immune responses against invading pathogens. They contribute to the elimination of microorganisms and are capable of presenting antigens to T cells. In addition, macrophages may serve as an escape route for some bacterial species, as intracellular bacteria may remain viable and potentially proliferate and/or disseminate in the host (Donne et al., 2005; Maria-Pilar et al., 2005).

Lawsonia intracellularis is a microaerophilic, obligately intracellular bacterium that causes porcine proliferative enteropathy (PPE) (Lawson and McOrist, 1993). PPE is responsible for economic losses worldwide in the pig industry, not only due to diarrhea, but also due to reduced weight gain, treatment and control costs of the disease (Lawson and Gebhart, 2000). Other animal species are also susceptible to *L. intracellularis* infection. In horses, proliferative enteropathy is considered an emerging disease (McGurrin et al., 2007). In non-human primates, there are reports of outbreaks of the hemorrhagic form of proliferative enteropathy, causing sudden death in affected animals (Klein et al., 1999; Lafortune et al., 2004).

The pathogenesis of *L. intracellularis* is still poorly understood. Infection occurs via the fecal-oral route, and in the gastrointestinal tract the microorganism overcomes the hostile environment of the stomach by enzymatic and protein mechanisms (Vannucci et al., 2014). Arriving at the more aboral region of the small intestine (ileum), the bacterium uses a single polar flagellum (Lawson and Gebhart, 2000) to overcome the intestinal mucus barrier, allowing an intimate contact between *L. intracellularis* and the intestinal epithelial cells. Then, *L. intracellularis* gains entry into the host-cell cytoplasm by endocytosis. In the intracellular environment, *L. intracellularis* is able to proliferate by binary fission (McOrist et al., 1995).

The most likely theory for infection and *in vivo* propagation of *L. intracellularis* is that the bacteria infect only cells with high mitogenic potential, such as intestinal crypt cells (immature and undifferentiated enterocytes), and is dependent on host cell mitosis for its propagation (Lawson et al, 1993; Smith and Lawson, 2001; Gebhart and Guedes, 2010). It is believed that *in vivo* propagation of *L. intracellularis* occurs from enterocyte to enterocyte, by exfoliation and rupture of cells with high bacterial load, release of these microorganisms into the intestinal lumen and, consequently, infection of adjacent immature enterocytes (Jacoby, 1978; Johnson and Jacoby, 1978; Jasni et al, 1994). Contradictory and inconclusive observations have suggested a potential role of macrophages in the spread of *L. intracellularis* infection. Johnson and Jacoby (1978) and Umemura et al. (1982) have demonstrated, through electron microscopy, partially degraded bacteria within macrophages of the lamina propria in tissues of infected animals. On the other hand, Boutrup et al., (2010), using fluorescence *in situ* hybridization (FISH), observed the presence of viable *L. intracellularis* within the mononuclear cells of the lamina propria. This technique uses a fluorochrome-conjugated oligonucleotide probe that targets the 16s gene of ribosomal RNA (rRNA). The use of this target is justified by the fact that there is hybridization only in metabolically active microorganisms at the moment of tissue fixation. Consequently, only metabolically active organisms would present intact rRNA, permissive to binding reactions with the probe. In addition, since all eukaryotic cells require translational ribosomes, ribosomes are present in large quantities in all metabolically active cells (Amann and Fuchsou, 2008).

To our knowledge, there is no research that has unambiguously revealed the role of macrophages in *L. intracellularis* infection. To better understand this interaction, the present work aimed to evaluate the survival capacity of *L. intracellularis* inside macrophages derived from peripheral blood mononuclear cells (PBMC) of pigs.

Materials and Methods

Ethics Statement

The study was conducted in accordance with the rules of the Ethics in Animal Use Committee at the Universidade Federal de Minas Gerais (CEUA/UFMG protocol number 249/2015).

Pure culture propagation

L. intracellularis strain PHE/MN1-00 (ATCC PTA-3457), previously isolated from a pig with the hemorrhagic form of PPE, was used in passages ranging from 12 to 14. Pure culture of the bacterium was thawed and grown in McCoy cell culture, using Dulbecco´s modified Eagle medium (DMEM - Gibco, Carlsbad, US) supplemented with 1% L-glutamine (Gibco Invitrogen Corporation, 25030-081) and 7% fetal bovine serum (FBS, Sigma Chemical). The infected cell cultures were incubated in bags with an atmosphere of approximately, 6% oxygen and 8% carbon dioxide (Vannucci et al., 2012), for three consecutive passages to allow the bacterium to recover from the freezing condition. Subsequently, the culture supernatant was filtered through 0.8 μm filters (MerckMillipore, Darmstadt, Germany) to remove the McCoy cells prior to infecting the PBMC culture.

Isolation, culture and infection of macrophages

For PBMC isolation, 60 mL of blood were collected from a healthy pig, using heparin to prevent clotting. Then, the non-coagulated blood was diluted in DMEM medium (Gibco, Carlsbad, US) to a ratio of 1:1. The blood/DMEM mixture wasdiluted in Histopaque (Sigma) at the ratio of 1 Histopaque: 2 blood/DMEM and centrifuged at 1200 g for 40 min at 18ºC. The layer containing mononuclear cells was collected using a sterile pasteur pipette and then centrifuged at 1200 g for 15 min at 4ºC. The pellet was resuspended in lysis buffer for 5 minutes at room temperature, then added to DMEM (10 mL), and centrifuged at 1200 g for 15 min at 4ºC. The pellet was finally resuspended in 10 mL RPMI-1640 medium supplemented with 10% fetal bovine serum, L-glutamine (200 mM), sodium bicarbonate (7.5% w/v), non-essential amino acids (10 mM), pyruvate (10 mM), penicillin (50 IU/mL) and streptomycin (50 μl/100 mL). The number of cells in the suspension was counted in a Neubauer's chamber, transferred to Teflon flasks (NalgeNunc, Rochester, US), and incubated at 37°C with 5% CO_2. The medium was changed to remove dead and non-adherent cells and to remove the antibiotic at 24 hours after incubation. The culture was maintained in the same conditions for 10 days with medium changes every 3 days. After 10 days of culture, the macrophage cells were seeded in a 6 or 24-well plate for ultrastructural and quantitative evaluation of the interaction between the macrophages and *L. intracellularis*.

Transmission electron microscopy

In order to verify the presence of *L. intracellularis* inside the macrophages using transmission electron microscopy, wells were infected with MOI (multiplicity of infection) of approximately 10 bacteria to 1 macrophage. Then the cells were fixed and evaluated at two different time periods post-infection: 1 hour post infection (early infection) and 2 days post-infection (late infection). As a negative control, uninoculated macrophages were maintained in similar conditions to the inoculated flasks.

Inoculated macrophage cell cultures were fixed in a solution containing 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 4 hours at 4ºC. Then the macrophages were resuspended using a cell scraper, transferred to microtubes and centrifuged for 10 minutes at 1000 g at room temperature. Subsequently, a post-fixation protocol was applied where samples were incubated in 1% osmium tetroxide and 0.8% potassium ferrocyanide for 1 hour, and washed three times in 0.1 M cacodylate buffer. In the next step, cell cultures were washed in distilled water and immediately dehydrated using acetone in increasing concentrations of 30, 50, 70, 90 and 100% for 10 minutes at each concentration. After dehydration, the cells were slowly impregnated in epoxy resin for 6 hours. For polymerization and coinfection of the blocks, the material was kept in an oven at 60ºC for 48 hours.

Ultra-thin sections (70 nm) obtained with a diamond razor were contrasted with 5% uranyl acetate and lead citrate and observed in a transmission electron microscope (JEOL Ltd., Tokyo, Japan).

Quantification by qPCR

After 1 (T0) and 4 (T1) hours and 1 (T2), 2 (T3) and 3 (T4) days of culture, cells were washed three times with PBS, lysed and processed for bacterial DNA extraction using DNeasy Blood & Tissue Kit®, according to with instructions from the manufacturer (Qiagen, Valencia, CA, USA). The

extracted DNA was quantified by the qPCR technique as previously described (Wattanaphansak et al., 2010). As a positive control, McCoy cells infected with *L. intracellularis* were used to confirm the extraction and to create a bacterial growth curve.

Results

Transmission electron microscopy

Through transmission electron microscopy, it was possible to observe the presence of *L. intracellularis* in different stages of interaction with macrophages. Figure 1A shows an initial interaction between *L. intracellularis* and the macrophage cellular membrane prior to internalization (1 hpi). Figure 1B shows the presence of *L. intracellularis* in the host cytoplasm within a phagosome (1 hpi). In Figures 1C and 1D it is possible to note the bacteria inside phagolysosomes with the undamaged cell wall, indicating bacterial survival in this environment (2 dpi). In Figure 1E free bacteria are observed in the cytoplasm of the macrophages at 2 dpi. Figure 1F demonstrates the negative control.

qPCR

McCoy cells infected with *L. intracellularis* were used as a control for the bacterial growth (Vannucci et al., 2012) under conditions similar to those used for *L. intracellularis* propagation, starting with cell culture around 30% of confluence remaining until 3 days after the inoculation. In the McCoy cell culture, the amount of *L. intracellularis* decreased in the first hours after infection. At later stages of infection, an increase in the bacterial numbers can be observed (Figure 2). In the macrophage culture, there was less decrease in the amount of *L. intracellularis* in the first hours after infection, but the increase of the bacterial DNA was less throughout the time course (Figure 2), remaining constant from T2 to T4.

Discussion

Through the ultrastructural evaluation we could observe the presence of viable *L. intracellularis* inside phagolysosomes in the cultured macrophages. This result reinforces the hypothesis of the survival capacity of *L. intracellularis* inside the macrophages (Boutroup et al., 2010). However, the capacity to survive within the macrophages is not related to the capacity of the bacterium to disseminate in the organism, since only in fortuitous cases of *L. intracellularis* infection are extra intestinal lesions found (Segales et al., 2001). The presence of live bacteria in phagolysosomal compartments has already been observed in other species such as *Rhodococcus equi* and *Coxiella burnetii* (Beron et al., 2002; Toyooka et al., 2005), among other bacterial pathogens. *R. equi* blocks acidification of the phagolysosome, which allows its survival and proliferation in this environment (Toyooka et al., 2005). However, *C. burnetii* delays the fusion process between the phagosome and the lysosome (Beron et al., 2002), which may allow the synthesis of protective molecules in the hostile environment of the phagolysosomes (Romano et al., 2007).

Intracellular traffic of bacteria in macrophages begins with phagocytosis that result in the formation of a phagosome that will mature into phagolysosomes in a series of ordered events (Flannagan et al., 2012). After internalization on the cell surface, the phagosome rapidly acquires early endosome markers, so-called GTPases (e.g. Rab5). These GTPases stimulate endosome fusion and maturation processes, resulting in acidification of the intravacuolar medium (Kinchen & Ravichandran, 2008). After the initial maturation process, the GTPases are replaced: Rab5 gives rise to Rab7 and later, Rab7 is replaced by lysosomal markers (LAMP1 and LAMP2) until during phagosome and lysosome fusion, when the compartment acquires cathepsins and hydrolases and will reduce the pH to values around 4.5 (Kinchen & Ravichandran, 2008; Flannagan et al., 2012).

Several bacterial genera have developed mechanisms and virulence factors to ensure survival and proliferation within host cells. For this purpose, microorganisms must be able to subvert normal host defense mechanisms. Generally, the mechanism used by these bacteria is the inactivation of the Rabs (endossomal proteins) and their effectors that are essential for the phagosome maturation (Brumell and Scidmore, 2007). The various specific markers of the phagosome maturation period allow studies evaluating the time at which the phagosome maturation is interrupted (environment in

which the agent is able to survive and often to replicate), and may be early (immature phagosome) as it occurs in *Legionella*, *Mycobacterium* and *Listeria* infection (Prada-Delgado et al., 2001; Michard et al., 2015;Danelishvili and Bermudez, 2015), or late, as it occurs in *Brucella* and *Salmonella* infections (Brumell & Grinstein, 2004; Starr et al., 2012). There is also survival and proliferation after lysosome fusion as it occurs in infections by *Coxiella burnetti* and *Rhodococcus equi* (Toyooka et al., 2005; Beron et al., 2002). The process of subversion of this host machinery is performed by a set of virulence factors secreted by the secretory systems of the pathogen that targets several host proteins that regulate the intracellular phagosome trafficking (Hayes et al., 2010).

L. intracellularis survival within the macrophages may also be related to the persistence of the microorganisms in the host, which results in the chronic presentation of the disease. Therefore, the elimination of *L. intracellularis* for long periods, as previously demonstrated by Guedes and Gebhart (2002) and Stege et al., (2004), may be a source of dissemination of the pathogen in the herd.

To assess the bacterial numbers in the macrophage cultures over time, qPCR was performed. This technique detects DNA particles with good sensitivity and specificifiy but lacks the ability to differentiate metabolically active and inactive microorganisms. In addition, the chronological evaluation was performed by using samples from different wells, which can result in numerical variations from well to well. Nevertheless, from the results obtained, we can hypothesize that *L. intracellularis* has the capacity to proliferate at low levels within the macrophages, but other methods should be used in future studies to confirm this hypothesis. Our findings do not corroborate Lawson et al. (1995) findings, who have indicated that *L. intracellularis* needs mitotically active cells for its proliferation. *L. intracellularis* is a unique bacterial species, with several peculiarities obligately intracellular microorganism, requires specific microaerophilic atmosphere) that make it difficult to grow and manipulate in laboratory conditions. Therefore, evaluation of *L. intracellularis* survival and its quantification inside the cells is not possible using conventional assays used for studying other bacterial species (Buchmeier nd Heffron, 1989; Celli et al., 2003).

In conclusion, the present study has demonstrated the survival capacity of *L. intracellularis* within macrophages, more precisely, in the phagolysosomes.

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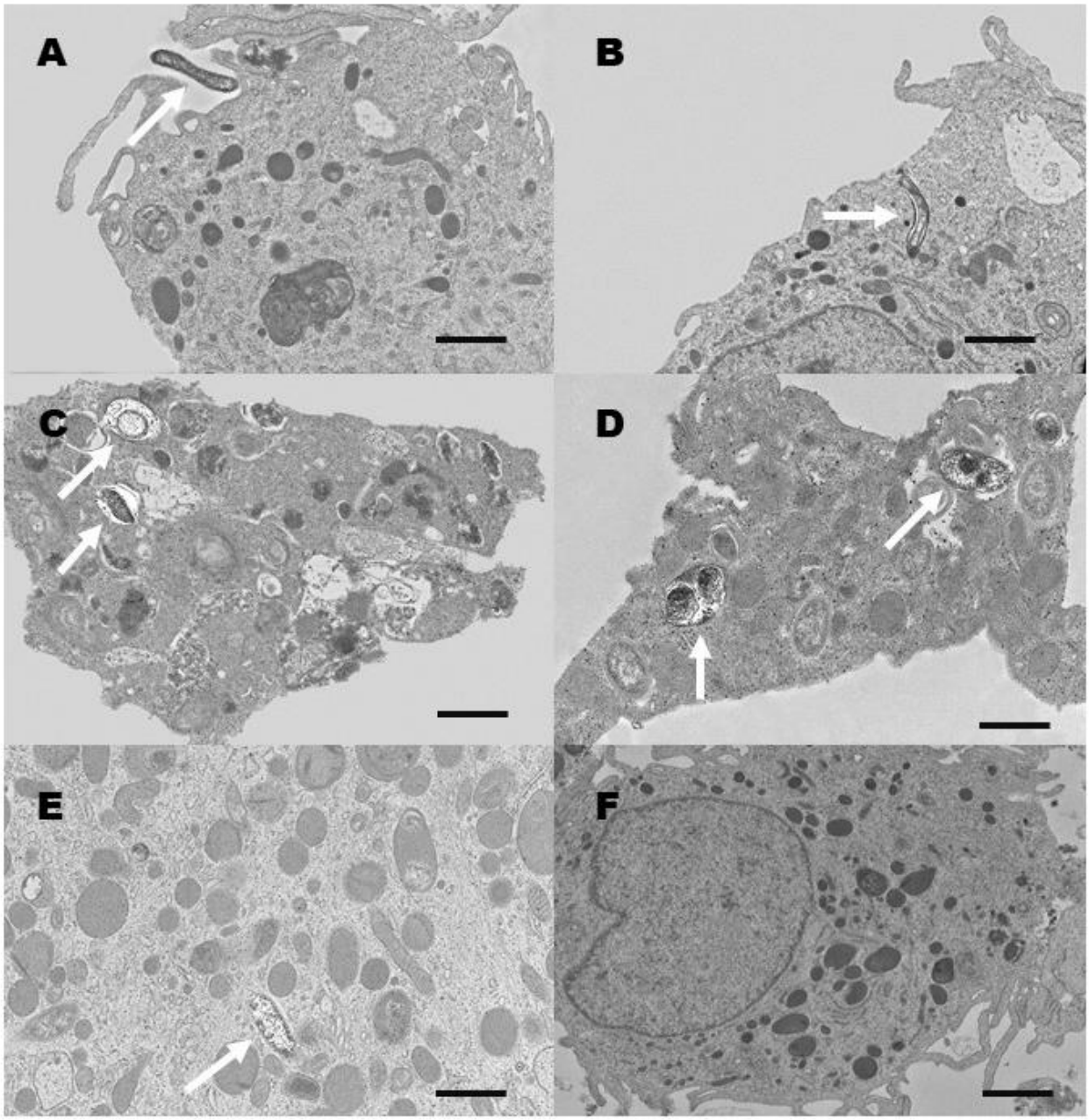

Figure 3.1: Interactions between macrophages and *L. intracellularis* demonstrated by TEM. (A): initial interaction between *L. intracellularis* (arrow) and the macrophage cellular membrane prior to internalization (1 hpi; bar = 1 µm). (B): presence of *L. intracellularis* (arrow) in the macrophage cytoplasm within a phagosome (1 hpi; bar = 1 µm). (C) and (D): *L. intracellularis* (arrows) inside phagolysosomes with undamaged double membrane, indicating bacterial survival in this environment (2 dpi; bar = 600 nm). (E): free bacteria (arrow) in the cytoplasm of the macrophages (2 dpi; bar = 800 nm) and (F): negative control (bar = 2µm).

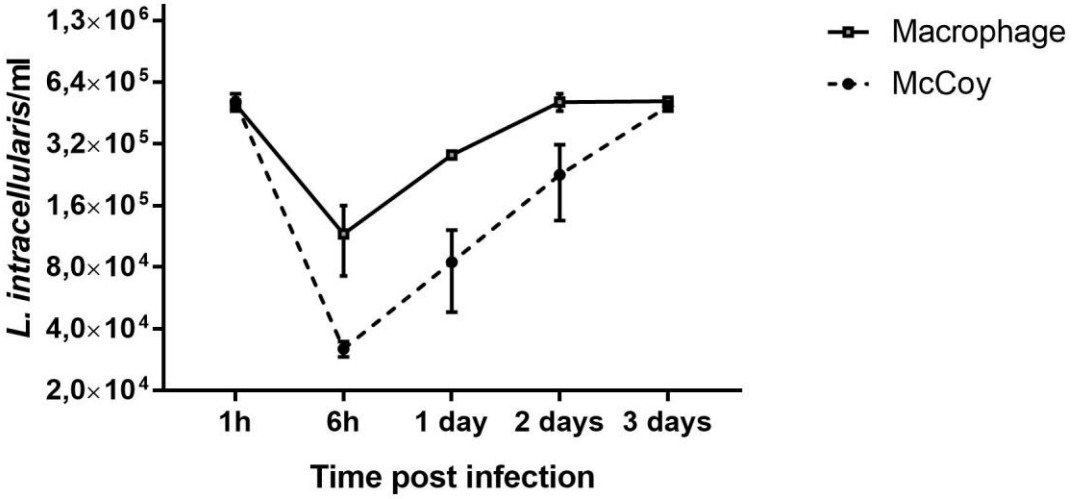

Figure 3.2 – *L. intracellularis* quantification by qPCR. Time course of *L. intracellularis* quantification in macrophages and McCoy cells at 1 h, 6 h, 1, 2 and 3 days post infection and ± standard deviation.

CHAPTER 3 - Evaluation of the endocytic machinery responsible for the *Lawsonia intracellularis* internalization

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Abstract
Lawsonia intracellularis is an obligate intracellular bacterium and causative agent of proliferative enteropathy. The pathogenesis of *L. intracellularis* is still poorly understood, beginning with understanding of the mechanisms involved in the process of bacterium endocytosis into the host cell. In this study, we evaluated the mechanisms involved in endocytosis of *L. intracellularis* in cultured intestinal porcine epithelial cells. The zipper endocytosis process, which is mediated by clathrin, was investigated as the route of *L. intracellularis* endocytosis. Confocal microscopy was used to co-localize *L. intracellularis* and clathrin. Clathrin gene knock down was then applied to verify whether *L. intracellularis* endocytosis is totally clathrin dependent. Finally, we tested if *L. intracellularis* endocytosis is dependent on the microorganism viability or if its entrance is only host cell-dependent *L. intracellularis* bacteria were observed co-localized with clathrin by confocal microscopy but the amount of *L. intracellularis* internalized in cells (with and without clathrin knock down) did not differ statistically. The internalization of nonviable *L. intracellularis* showed a decrease in the internalization in cells with less clathrin synthesis ($P<0.05$). In conclusion, we demonstrated the dependence on clathrin for *L. intracellularis* internalization in host cell; however, *L. intracellularis* also has active mechanisms of internalization independent of clathrin. We have also confirmed the independence of bacterial viability for host cell internalization.

Keywords: Clathrin, endocytosis, *Lawsonia intracellularis*, pathogenesis, proliferative enteropathy, intracellular bacteria

Introduction

Lawsonia intracellularis is an obligate intracellular, microaerophilic, Gram-negative bacterium that causes proliferative enteropathy (PE) (Lawson et al, 1993). This disease is endemic in swine herds worldwide and is occasionally reported in other species, including non-human primates, horses, rabbits, wild mammals, and birds (Cooper et al., 1997; Lafortune et al., 2004).

The pathogenesis of *L. intracellularis* is still poorly understood. *L. intracellularis* infection occurs via the fecal-oral route. In the gastrointestinal tract, the microorganism overcomes the hostile environment of the stomach through enzymatic and protein mechanisms (Vannucci et al., 2014). Arriving in the aboral region of the small intestine (ileum), the bacterium uses a single polar flagellum (Lawson and Gebhart, 2000) to cross the intestinal mucus barrier, allowing intimate contact with the enterocytes and then invades the host cell.

Among the bacterial endocytosis processes, the zipper mechanism does not require metabolically active invaders for its initiation. The zipper endocytosis process is associated with proteins or structures of the outer membrane of the bacterium that interact directly with specific host cells receptors. In this process, there is a clustering of eukaryotic cell receptors, associated with changes in the actin cytoskeleton resulting in the microorganism being coated by the cytoplasmic membrane (Pizarro-Cerda and Cossart, 2004; Cossart and Helenius, 2014). Then, several downstream mechanisms are activated resulting in pathogen endocytosis. In the zipper endocytosis process, there is involvement of clathrin-dependent machinery, associated with the formation of vesicles from the endocytosis of exogenous material. The involvement of this machinery occurs in the initial stage of the endocytosis process, shortly after the adhesion of the bacterium to the surface of the host cell (Bonazzi et al., 2011).

Another method used by bacteria for internalization into cells is the so-called trigger endocytosis mechanism. This process is dependent on bacterial effector proteins that are introduced into the host cells, generally through the type III secretion system. These proteins are responsible for mobilizing the actin for rearrangement of the cytoskeleton and pathogen internalization (Misselwitz et al., 2011). Immediately after internalization, the endosome is transported within the cell through proteins called Rab (Bhuin and Roy, 2014). Rab5 is one of the main proteins involved in the initial trafficking of the endosome and is used as a marker of this vesicle in the initial phase, soon after endocytosis (Mendoza et al., 2014).

The mechanisms involved in the endocytosis of *L. intracellularis* are not yet fully understood. Lawson et al. (1995) have demonstrated that *L. intracellularis* can be internalized even after its inactivation. This finding indicates that the process of *L. intracellularis* internalization in the host cells is dependent on the recognition of bacterial membrane proteins by the host. Therefore, the objective of the present study was to evaluate the involvement of clathrin in the endocytosis process of *L. intracellularis*, as well as to determine whether the bacterium uses other mechanisms for its internalization in the host cell.

Materials and Methods

Cell cultures and *in vitro* infection

Intestinal porcine epithelial cell (IPEC-J2) cells (Berschneider, 1989) were cultured in T25 flasks with Dulbecco's Modified Eagle Medium (DMEM)/ F12, supplemented with 5% fetal bovine serum, 5 ng/mL epidermal growth factor (E9644, Sigma-Aldrich), and 5 ng/mL mixture of insulin, transferrin and selenium (BD Biosciences), without antibiotics, at 37°C in a humidified atmosphere with 5% CO_2, as previously described (McOrist et al., 1995). *L. intracellularis* strain PHE/MN1-00 (ATCC PTA-3457), previously isolated from swine with the hemorrhagic form of proliferative enteropathy, was used at passage 12 to 14 to infect the IPEC-J2 cells. Pure culture of the bacterium was thawed and grown in McCoy cell culture incubated in a bag with an atmosphere of approximately, 6% oxygenand 8% carbon dioxide (Vannucci et al., 2012), for three consecutive passages to allow the bacterium to recover from the freezing condition. Subsequently, the culture supernatant was filtered (0.8 μm, Millipore) to remove McCoy cells, and inoculated into 24-well plates containing IPEC-J2 at approximately 30% of confluence. Experiments were performed three times, each time with triplicates.

The experimental groups were: a) IPEC-J2 knock-down for clathrin (described below) infected with viable *L. intracellularis*; b) IPEC-J2 knock-down for clathrin (described below) inoculated with heat-killed *L. intracellularis* (65ºC for 30 minutes); c) IPEC-J2 not transfected (control) infected with live *L. intracellularis* and d) IPEC-J2 not transfected (control) inoculated with heat-killed *L. intracellularis*. The cell culture was allowed to be in contact with *L. intracellularis* microorganisms for one hour. After that period, the supernatant was removed and the cell monolayer was washed three times with PBS and fixed with 4% paraformaldehyde.

RNA silencing

The process of gene silencing through siRNA was performed to evaluate the requirement for clathrin-dependent endocytosis for *L. intracellularis* internalization. The knock down of the gene responsible for the synthesis of this protein was performed via silencing RNA (siRNA) followed by the inoculation of the bacterium (Veiga and Cossart, 2005).

IPEC-J2 cells were transfected using the following oligonucleotide sequencesfor clathrin synthesis: clathrin heavy chain sense, 5'-GGCCCAGGUGGUAAUCAUUTT-3'; clathrin antisense heavy chain, 5'-AAUGAUUACCACCUGGGCCTG-3' (Veiga and Cossart, 2005). Two days before infection, IPEC-J2 cells were incubated for 15 minutes with 2.5 μg of siRNA, using lipofectamine 3000 (Invitrogen, USA), according to the manufacturer's instructions. Then, the media was replaced with fresh IPEC-J2 media without the oligonucleotides and the cell culture was infected with *L. intracellularis*.

Western blot

IPEC-J2 cells transfected with siRNA and non-transfected IPEC-J2 cells were washed three times with PBS and lysed with M-PER (Invitrogen, USA) for 5 minutes. The lysate was purified by centrifugation at 12,000 g for 10 minutes. Protein lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane and labeled with anti-clathrin antibody (P1663 - Cell Signaling, Danvers, MA, USA). β-actin detection was used as the internal control witth anti-β-actin antibody (C4 – sc47778, Santa Cruz Biotechnology) and quantified using imageJ software.

Confocal microscopy

IPEC-J2 cells were grown on coverslips in 24-well plates and infected with *L. intracellularis* at an MOI of 10 at 37°C for one hour. Subsequently, the cells were washed three times with PBS and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Thereafter, further washing with PBS was carried out to remove excess paraformaldehyde. Fixed cells were incubated for 30 minutes at 37ºCwith the following primary antibodies: anti-clathrin (P1663 - Cell Signaling, Danvers, MA, USA; source: rabbit), anti Rab5 (ab18211 – Abcam; source: rabbit), and anti-*L. intracellularis* (Guedes and Gebhart, 2003; source: mouse). After washing with PBS, the secondary antibodies Goat anti-rabbit IgG conjugated to Cy3 (ab97075 - Abcam) and goat anti-mouse IgG conjugated to AlexaFluor 488 (ab150113 - Abcam) were added and incubated for 30 minutes at 37ºC. Finally, the coverslips were mounted on glass slides with mounting media containing DAPI (ProLong Diamond Antifade Mountant - P36971 - ThermoFisher, USA) and viewed under a confocal microscope (FV500, Olympus).

Quantification (qPCR) of *L. intracellularis*

After 1 hour of culture, the IPEC-J2 cells were washed three times with PBS, lysed and processed for bacterial DNA extraction using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), according to instructions from the manufacturer. The extracted DNA was quantified by the qPCR technique as previously described (Wattanaphansak et al., 2010).

Statistical analysis

Quantitative data were presented with mean and standard deviation. Statistical analysis was performed using Student's t-test and considered significant when $P < 0.05$. The software Prism GraphPad version 5.0 was used for statistical analysis.

Results

Confocal microscopy

The co-localization of *L. intracellularis* and clathrin was observed by confocal microscopy and indicates an activation of the clathrin-dependent endocytosis mechanism to internalize *L. intracellularis* (Figure 1). In addition, the co-localization of *L. intracellularis* and Rab5 (Figure 2) was also observed, which demonstrated that *L. intracellularis* bacteria are associated with early endosomes.

siRNA, Western Blot and qPCR

The success of clathrin knock down by siRNA transfection was demonstrated by the western blot technique (Figure 3A) and quantified by imageJ software and relative protein levels had been reduced by 68.9 ± 2% (Figure 3B). Quantification of bacterial internalization was evaluated by cell culture lysis (control and siRNA) and bacterial extraction and quantification through qPCR. We observed that viable *L. intracellularis* had a slightly higher internalization capacity than heat-killed *L. intracellularis* in cells able to synthesize normal levels of clathrin ($P > 0.05$). Conversely, internalization of heat-killed *L. intracellularis* was significantly decreased ($P < 0.05$) in clathrin knock down IPEC-J2 cells (Figure 4), compared to non-treated cells.

Discussion

In the present study, the levels of internalization were significantly lower ($P < 0.05$) when infecting IPEC-J2 cells under a clathrin gene silencing condition with heat-killed *L. intracellularis*. This result is evidence that *L. intracellularis* can be internalized by clathrin-dependent mechanisms without losing the property of being endocytosed by other active processes. Additionally, we determined that Rab5 is involved in *L. intracellularis* entry into IPEC-J2 cells. Rab5 iswidely used as an early endosome marker (Mendoza et al., 2014), and its co-locatization with *L. intracellularis* indicates the early phase of internalization. Further studies are needed to evaluate the intracellular traffic of *L. intracellularis* within epithelial cells.

The importance of *L. intracellularis* as a pathogen capable of causing disease of economic importance in the swine industry is well acknowledged. Nevertheless, little information on the pathogenesis and, more specifically, on the mechanisms of entry of the bacterium into eukaryotic cells, is available. In a previous study, Lawson et al. (1995) have observed that dead *L. intracellularis* could be visualized inside cells, suggesting that the process of endocytosis would be dependent on the eukaryotic cell only. In the present study, we evaluated the mechanisms involved in the endocytosis of *L. intracellularis* and confirmed, by confocal microscopy, that *L. intracellularis* can be internalized through clathrin-dependent mechanisms. However, the amount of *L. intracellularis* organisms recovered from transfected and non-transfected IPEC-J2 cells did not differ statistically. Thus, the capacity of *L. intracellularis* to be internalized by active mechanisms was confirmed.

The main mechanisms involved in bacterial endocytosis are the zipper and the trigger processes. The zipper process is related to the presence of proteins or outer membrane compounds in the bacterium surface that interact directly with host receptors and induce a cascade reaction that culminates with bacterial endocytosis (Pizzarro-Cerda and Cossart, 2004, 2006). The zipper mechanism has been demonstrated in *Listeria monocytogenes* endocytosis. *L. monocytogenes* expresses two proteins involved in the internalization mechanism (Internalins A and B - InlA and InlB). Those proteins mimic some host cell proteins and initiate the process of clathrin mediated endocytosis (Veiga et al. 2007; Bonazzi et al., 2011). In our study, the presence of clathrin, topographically co-localized with *L. intracellularis* as demonstrated by confocal microscopy, proved that the *L. intracellularis* internalization process is clathrin-dependent.

IPEC-J2 cells with decreased clathrin synthesis after treatment with specific siRNA had similar amounts of internalized *L. intracellularis* when compared to non-treated cells. This finding indicates the possibility that *L. intracellularis* could also be internalized by other non-clathrin-dependent mechanisms. To evaluate this, IPEC-J2 cell cultures (control and knock down for clathrin) were infected with dead *L. intracellularis*. This experiment resulted in a decreased internalization of heat-killed bacteria in cells with diminished clathrin, supplying further evidence that *L. intracellularis* can be internalized through self-dependent mechanisms, possibly through the process of endocytosis

called trigger. In the trigger process, there is an initial pathogen-host interaction and, from there, bacterial proteins are secreted into the eukaryotic cell cytosol through the type III secretion system, an apparatus that has already been shown to be present in *L. intracellularis* (Alberdi et al., 2009). The secreted bacterial proteins (also called effector proteins) are responsible for host cytoplasmic organization by rearrangements in the actin cytoskeleton (Cossart and Helenius, 2014). This trigger mechanism is well-described in bacteria of the genus *Salmonella. Salmonella* adhere to the cells through a fimbrial adhesion and then, through the type III secretion system 1 (T3SS-1), synthesize effector proteins responsible for actin polymerization and bacterial envelopment (Cain et al., 2008). It has been demonstrated (Mijouin et al., 2012) that *Salmonella* can also be internalized by the zipper mechanism mediated by the Rck membrane protein, in addition to the trigger mechanism.

As demonstrated by the present study, *L. intracellularis* can be endocytosed by a clathrin-dependent mechanism and/or by mechanisms that require its viability. Further studies are needed to determine which outer membrane proteins present in *L. intracellularis* have the ability to interact with host cell receptors to initiate the endocytosis process. Moreover, it is necessary to evaluate whether the type III secretion system, present in *L. intracellularis*, is involved with the internalization process and to determine the likely effector proteins synthetized by *L. intracellularis*.

In conclusion, the present study has demonstrated the involvement of clathrin in the endocytic process of *L. intracellularis* and has also confirmed that *L. intracellularis* can be internalized through mechanisms that depend on its viability.

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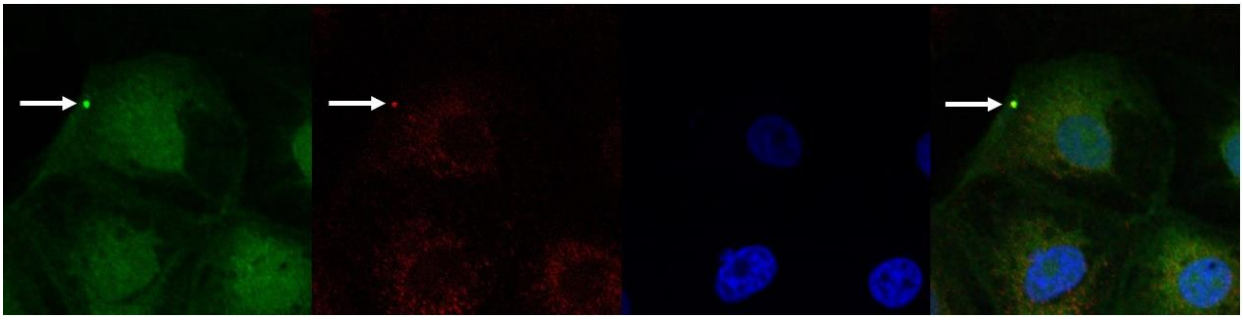

Figure 3.1 – Confocal microscopy. Cell nuclei (blue), *L. intracellularis* (arrow - green), clathrin (arrow- red), co-localization *L. intracellularis* and clathrin (arrow - yellow) in overlay image.

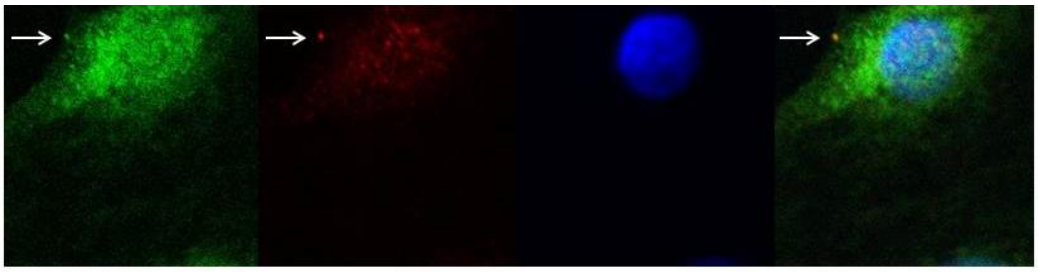

Figure 3.2 – Confocal microscopy. Cell nucleus (blue), *L. intracellularis* (arrow - green), Rab5 (arrow-red), co-localization *L. intracellularis* and Rab5 (arrow - yellow) in overlay image.

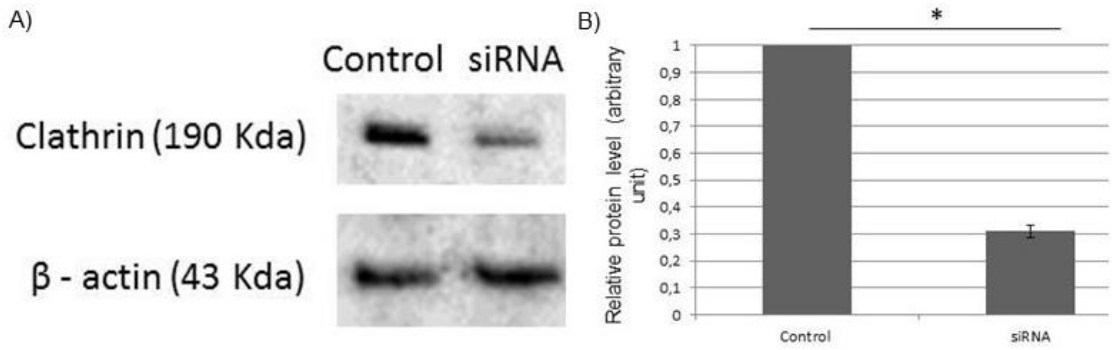

Figure 3.3 – (A) Effect of clathrin knock down using Western blot. The expression of clathrin in culture of IPEC-J2 infected with *L. intracellularis* demonstrated lower expression rate of clathrin in the transfected group (siRNA). β - actin was used as endogenous control. (B) The relative protein levels analyzed by ImageJ and standard deviation (SD).

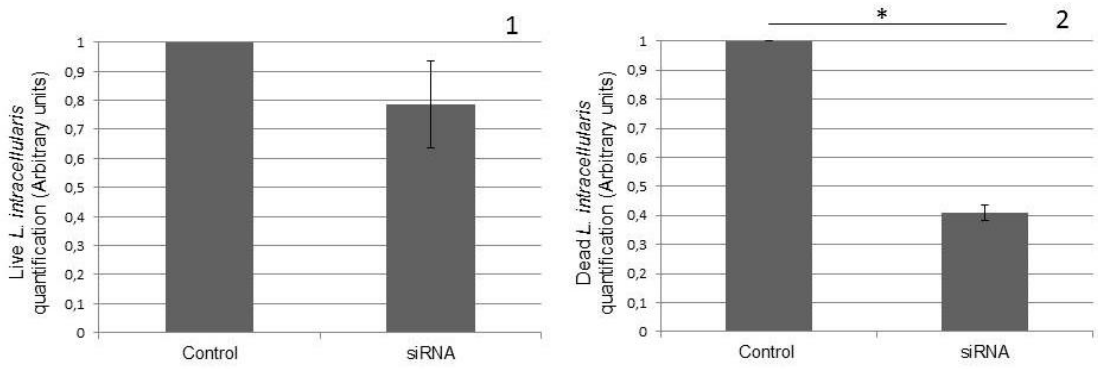

Figure 3.4 – The evaluation of the amount of *L. intracellularis* endocytosis in the different groups was performed using qPCR. Results are presented as the means ± SD of three independent experiments, using control groups (IPEC – J2 infected with *L. intracellularis*) and siRNA groups (IPEC – J2 knock down to clathrin and infected with *L. intracellularis*). Live *L. intracellularis* amount did not differ statistically in the control and transfected group (1) and heat killed *L. intracellularis* had a reduced internalization rate (*P <0.05) in the transfected group when compared to the control (2).

CHAPTER 4 - Isolation and *in vitro* antimicrobial susceptibility of porcine *Lawsonia intracellularis* from Brazil and Thailand

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Abstract
The goals of this study were to isolate South American and Southeast Asian strains of *Lawsonia intracellularis* and to determine the *in vitro* antimicrobial activity against these isolates. Tested antimicrobials included: chlortetracycline, lincomycin, tiamulin, tylosin and valnemulin (against both Brazilian and Thailand strains) and additionally, amoxicillin, zinc-bacitracin, carbadox, enrofloxacin, gentamicin, sulfamethazine, trimethoprim, spectinomycin and a combination (1:1) of spectinomycin and lincomycin were also tested against the Thai isolates. The minimum inhibitory concentration (MIC) was determined by the antimicrobial activity that inhibited 99% of *L. intracellularis* growth in a cell culture as compared to the control (antimicrobial-free). Two strains from Brazil and three strains from Thailand were isolated and established in cell culture. Each antimicrobial was evaluated for intracellular and extracellular activity. Pleuromutilin group (valnemulin and tiamulin) and carbadox were the most active against *L. intracellularis* strains tested. Tylosin showed intermediate activity, chlortetracycline had variable results between low and intermediate activity, as well as spectinomycin, spectinomycin and lincomycin, amoxicillin, sulfamethazine and enrofloxacin. *L. intracellularis* was resistant to lincomycin, gentamicin, trimethoprim, colistin and bacitracin in *in vitro*. This is the first report of isolation of *L. intracellularis* strains from South America and Southeast Asia and characterization of the antimicrobial susceptibility patterns of these new strains.

Keywords: Antimicrobial susceptibility**,** ileitis**,** *Lawsonia intracellularis*, MIC, Proliferative enteropathy, Pigs

Introduction

Proliferative enteropathy (PE), or ileitis, is one of the most important enteric bacterial infectious diseases in grower and finisher pigs. PE was first recognized in the North American swine industry in the early 1930s. Since then, PE has become a major enteric health concern for swine production in the United States and around the world. The etiological agent of this disease is an obligate intracellular and Gram-negative bacterium named *Lawsonia intracellularis*.

When a PE outbreak occurs in a herd, antimicrobial therapy is often used to control the disease. Antimicrobial therapy with an effective antimicrobial agent is able to stop the progression of the PE outbreak in a short period of time. Therefore, antimicrobial selection is critical for achieving the best possible outcome for the herd. Despite the importance of the antimicrobial treatment for PE, little information about *in vitro* sensitivity results against *L. intracellularis* for antimicrobial selection is available (McOrist et al., 1995; Wattanaphansak et al., 2009; Yeh et al., 2011). The main reason for this lack of information is due to the difficulty in isolating *L. intracellularis* from infected intestine or fecal samples, requiring experienced personnel and several months for the establishment of a pure *L. intracellularis* culture. Consequently, the *in vitro* sensitivities of *L. Intracellularis* for antimicrobials are difficult to obtain in a timely fashion to treat a PE outbreak. Furthermore, the obligate intracellular nature of *L. Intracellularis* prohibits the use of standard antimicrobial susceptibility testing methods. Instead, a complicated tissue culture system has been used to evaluate antimicrobial activity against some isolates of *L. intracellularis* originated from the United States and two other countries (McOrist et al., 1995; McOrist & Gebhart, 1995 Wattanaphansak et al., 2009; Yeh et al., 2011).

A previous study found that isolates of *L. intracellularis* can have different antimicrobial sensitivities (Wattanaphansak et al, 2009). Therefore, selection of antimicrobials for which most isolates showed good response would yield a better treatment success. So far, there is no information about *in vitro* sensitivities of *L. intracellularis* isolated from Latin America and Southeast Asia, where swine production is an important industry and there is documented high prevalence of proliferative enteropathy in these areas (McOrist, 2005; Wattanaphansak et al, 2011; Viott et al, 2013; Resende et al, 2015). In order to expand the limited information on *in vitro* antimicrobial sensitivity against *L. intracellularis*, additional primary isolates of *L. intracellularis* from Brazil and Thailand must be obtained, propagated *in vitro* and then evaluated.

The overall aims of this investigation were to obtain new isolates of *L. intracellularis* from Latin America and Southeast Asia and to determine the *in vitro* minimum inhibitory concentration (MIC) of antimicrobials against these new isolates for use as a guideline for antimicrobial selection in the treatment and control of PE.

Materials and Methods

L. intracellularis isolation

Brazilian isolates

L. intracellularis isolates were obtained from pigs affected with the acute form of PE. For the first isolate, BRPHE01_E5, ileum was obtained from afinishing pig from amulti-site commercial farm in the metropolitan region of Belo Horizonte, Minas Gerais state, Brazil, in 2011. The second isolate, BRPHE02_E8, was obtained from a finishing pig from a herd located in São Paulo state, Brazil, also in 2011. Affected intestines were submitted to the Veterinary Pathology Laboratory at the Universidade Federal de Minas Gerais, for immunohistochemistry confirmation of *L. intracellularis* infection.

Thailand isolates

Three PE affected swine intestines were used to obtain *L. intracellularis* isolates. One intestine had the acute form of the disease, proliferative hemorrhagic enteropathy (PHE) and two hadthe chronic form of the disease, porcine intestinal adenomatosis (PIA). The intestines were collected from three distinct herds in the Western region of Thailand. The PHE strain, CUPHE01_SW13, was obtained from a gilt that died suddenly with acute bloody diarrhea in a breeding herd in Kanchanaburi province in 2013. Both of the PIA intestines, CUPIA01_SW13 and CUPIA02_SW13, were collected from finishing pigs at the slaughter house in Nakornpathom province in 2012. All three affected intestines were submitted to the Veterinary Diagnostic Laboratory

at the Chulalongkorn University, Nakhonpathom, for PCR confirmation of *L. intracellularis* infection. All three infected intestine samples were PCR positive for *L. intracellularis*.

Isolation protocol

Infected intestines were cut into several pieces of approximately 5 cm and kept at -80°C until the initiation of the bacterial isolation process. The primary isolation of *L. intracellularis* from the infected intestines was modified from a previous study (Lawson et al. 1993). Briefly, the mucosa from 5 cm of infected intestines was scraped and blended by using a tissue grinder. The blended mucosa was suspended in 40 mL of sterile phosphate buffered saline (PBS). The suspension was centrifuged at 500 g for 20 minutes and the supernatant was filtered sequentially through 70 μm, 5 μm, and 0.8 μm filters. The filtered suspension was then centrifuged at 5,000 g for 20 minutes. The pellet was re-suspended in fresh culture medium containing: 50 μg/mL gentamicin and 10 μg/mL vancomycin. The bacterial suspension was transferred to 1-day-old McCoy cells and incubated in sealed bags with 10:10:80 CO_2:H_2:N_2 gas mixture, respectively (Vannucci et al., 2012). The culture medium was removed and replaced daily with the same concentration of antimicrobials for one week. The bacteria were harvested after 7 days of incubation and each subsequent passage was performed as previously described (Wattanaphansak et al., 2009). The bacterial growth was monitored using immunoperoxidase staining with specific rabbit polyclonal antibody as described previously (Guedes and Gebhart, 2003, Wattanaphansak et al., 2009). *L. intracellularis* was maintained in the McCoy culture until the number of heavily infected cells (HIC) was 90% to 100%.

After the establishment of a pure culture, each *L. intracellularis* isolate was used to quantify the inoculum for the antimicrobial MIC assay using a staining protocol as previously described (Guedes and Gebhart, 2003a; Wattanaphansak et al. 2009). Briefly, a series of ten-fold *L. intracellularis* dilutions, from 10^0 to 10^{-5}, was prepared in PBS. Then, 10 μL of each dilution was applied into 15-well glass slides as duplicates and the slides were allowed to dry at 37°C. After fixing with cold acetone for 1 minute, the slides were stained with the modified immunoperoxidase monolayer assay (IPMA) protocol as described by Guedes et al. (2002) using rabbit polyclonal antibody (Guedes and Gebhart, 2003b). The lowest dilution that had a *L. intracellularis* quantity between 50-500 bacteria/well was counted using a light microscope with 40X objective lens, and the initial concentration was calculated.

Source and preparation of antimicrobials

For the Brazilian isolates the following antibiotics were used: Chlortetracycline hydrochloride, lincomycin hydrochloride, and tylosin tartrate obtained as pure chemicals from Sigma Aldrich (St. Louis, MO, USA). Tiamulin hydrogen fumarate and valnemulin hydrochloride were supplied as pure chemicals from Novartis Animal Health (Switzerland, Basel). For the Thailand isolates, amoxicillin, Zinc-bacitracin, carbadox, enrofloxacin, gentamicin sulfate, polymyxin B (colistin), spectinomycin dihydrochloride, sulfamethazine and trimetroprim were also used and obtained as pure chemicals from Sigma Aldrich (St. Louis, MO, USA). The combination of lincomycin-spectinomycin was prepared as a 1:1 ratio for determination of the combined activity. The working solutions of tested antimicrobials were prepared as previously described (Wattanaphansak et al, 2009). Briefly, the antimicrobial stock solutions were prepared to a final concentration of 2,560 μg/mL and were filtrated through 0.2 μm-pore size filters. All stock solutions were aliquoted and kept at -20°C. A series of two-fold dilutions of this 10X concentration were made and diluted 1:10 with culture medium. The final concentrations of tested antimicrobials were 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 μg/mL. Each concentration of antimicrobial was tested in triplicate and each strain of *L. intracellularis* was tested twice from two independent bacterial preparations.

Antimicrobial sensitivity testing

The MIC assays were performed as described by Wattanaphansak et al (2009). The antimicrobials used for each strain were chosen according to the use in the pig industry of each country. Briefly, the MICs of antimicrobials against *L. intracellularis* were expressed as both intracellular and extracellular activities. The intracellular MIC was defined as the effect of antimicrobials on *L. intracellularis* after these intracellular organisms had infected the enterocytes.

One hundred μL of bacterial solutionwas added into 1-day-old McCoy cells, seeded in 96 well plates. After 24 hours of incubation in a sealed bag (Vannucci et al., 2012), the bacterial suspension was removed and replaced by 100 μL of fresh culture medium, containing various concentrations of antimicrobials. The antimicrobial suspension was replaced every day for 3 consecutive days post inoculation.

The extracellular MIC testing was performed in order to measure the effect of antimicrobial on *L. intracellularis* when the bacteria were freely circulating in the gut lumen. For this, a series of two-fold dilutions of stock antimicrobial solutions were diluted 1:10 with culture medium which contained *L. intracellularis*. The suspension was incubated at 37°C in a bag for 2 hours, allowing the bacteria to be exposed directly to the antimicrobials. After incubation, 100 μL of the bacterial suspension was transferred to one-day-old McCoy cells. The medium was removed 24 hours after incubation (under microaerophilic conditions) and replaced with 100 μL of new culture medium without any antimicrobials for 3 consecutive days. Each test plate contained control culture, containing no antimicrobials.

After 5 days of incubation, supernatant from the infected plates was removed and the cell culture monolayer was fixed with 50 μL of cold 50% acetone and 50% methanol for 1 minute. To assess the inhibitory effect of each antimicrobial on *L. intracellularis* proliferation, the infected plates were stained using a modified immunoperoxidase monolayer assay staining method as described previously (Wattanaphansak et al, 2009). Briefly, the fixed plates were re-hydrated with PBS for 30 minutes. The water was discarded and 50 μL of rabbit polyclonal antibody diluted in skim milk buffer to 1:10,000 was added. After 30 minutes of incubation at 37°C, the plates were then washed four times with PBS. Fifty μL of anti-rabbit IgG horseradish peroxidase conjugate diluted 1:5,000 in skim milk buffer was added to each well. After incubation for 30 minutes, the plates were washed four times with PBS. One hundred μL of chromogen solution (500 μL of 3-amino-9-ethyl-carbazol, 9.5 mL of acetate buffer, 5 μL of 30% hydrogen peroxide) were applied and incubated for 20 minutes. Finally, the stained plates were washed with distilled water and allowed to air-dry.

The number of heavily infected cells (HIC) in each well was counted using an inverted microscope with a 20X objective lens. The infected cells were considered to be HIC if the number of *L. intracellularis* inside the host cells had proliferated to greater than 30 bacteria per cell (McOrist et al., 1995). A comparison was then made where the number of HICs in each well was expressed as a percentage compared to the average HIC of controls. The intracellular and extracellular MIC endpoints of antimicrobials were defined as the lowest antimicrobial concentration that inhibited 99% of *L. intracellularis* proliferation in the McCoy cells.

Results

L. intracellularis strains BRPHE01_E5, BRPHE02_E8, CUPHE01_SW13, CUPIA01_SW13, and CUPIA02_SW13 were isolated as pure cultures from swine intestines affected by PE. The number of cells heavily infected by each isolate, which is an indicator of viable bacteria, dramatically increased to approximately 100% around passage 5. Moreover, all isolates were continuously maintained and propagated *in vitro.*

L. intracellularis isolates were tested for antimicrobial MICs at passages up to 15. The final concentration of inoculum was approximately between 10^6 and 10^7 *L. intracellularis*/mL for all isolates.

Brazilian isolates

The value of extracellular and intracellular MICs of all tested antimicrobials against the Brazilian *L. intracellularis* isolates are shown in Table 5.1. Compared to the antimicrobial-free control, the MIC endpoints for each antimicrobial were the concentrations that were able to inhibit 99% of *L. intracellularis* proliferation. An example of HIC of *L. Intracellularis* growth at different concentrations of antimicrobials in the McCoy cells is shown in Figure 5.1. The difference in MIC median of two independent preparations for each isolate was within two-fold dilutions.

For intracellular MIC testing, tiamulin and valnemulin had the highest activity against the Brazilian *L. intracellularis* isolates, with MICs ranging from 0.125 to 2 μg/mL. Tylosin had moderate activity against the *L. intracellularis* isolates with MICs ranging from 2 to 8 μg/mL. Chlortetracycline

had lower activity with MICs ranging from 8 to 64 μg/mL. Lincomycin had the lowest activity against the *L. intracellularis* isolates with MICs greater than 128 μg/mL.

For extracellular activity, the results showed that tiamulin and valnemulin had highest activity against the *L. Intracellularis* isolates with MICs from 0.125 to 2 μg/mL. Chlortetracycline had moderate antimicrobial activities against *L. intracellularis* with MICs ranging from of 32 to 64 μg/mL. Tylosin had different results between the two strains, with moderate activity against BRPHE02_E8 (16-32 ug/mL) and low activity against BRPHE01_E5 (>128 ug/mL). Lincomycin had lowest antimicrobial activities with the MIC of >128 μg/mL for both isolates.

Thailand isolates

The median value of extracellular and intracellular MICs for all tested antimicrobials against three Thailand *L. intracellularis* isolates is shown in Table 5.2. For intracellular MIC testing, carbadox, tiamulin and valnemulin displayed the highest activity against all three Thailand *L. intracellularis* isolates with MICs of 0.125 to 1 μg/mL. Amoxicillin, enrofloxacin, and tylosin had moderate activity against all three *L. intracellularis* isolates with MICs rangingfrom 2 to 32 μg/mL. Zinc-bacitracin, chlortetracycline, colistin, gentamicin, lincomycin, spectinomycin, lincomycin:spectinomycin (1:1), sulfamethazine, and trimethoprim showed the lowest activity against all three *L. intracellularis* isolates with MIC ranging from 64 to >128 μg/mL.

For extracellular activity, the results showed that carbadox, tiamulin, and valnemulin had highest activity against the three *L. intracellularis* isolates with MICs from 0.25 to 8 μg/mL. Spectinomycin, lincomycin:spectinomycin (1:1), and tylosin had moderate antimicrobial activities against *L. intracellularis* with MICs of from 2 to 32 μg/mL. Amoxicillin, bacitracin, chlortetracycline, colistin, enrofloxacin, gentamicin, sulfamethazine and trimethoprim had lowest antimicrobial activities, with the MICs ranging from 64 to 128 μg/mL.

Discussion

Although outbreaks of PE occur globally, it has been estimated that less than 25 *L. intracellularis* isolates have been successfully cultured and maintained *in vitro* worldwide. Of these, only 15 *L. intracellularis* isolates have been tested for their antimicrobial susceptibilities: 3 United Kingdom isolates (McOrist et al, 1995; McOrist and Gebhart, 1995); 1 Danish, 6 North American isolates (Wattanaphansak et al, 2009); and 2 Korean isolates (Yeh et al, 2011). Our study was the first to culture *L. intracellularis* from PE field cases and to evaluate its antimicrobial susceptibility in Brazil and Thailand. Five *L. intracellularis* isolates were successfully established in pure culture, two from Brazil and three from Thailand. Like other continents, a high level of *L. intracellularis* infection had been found in swine herds in South American and Southeast Asian countries. It was estimated that the herd prevalence of PE in pigs was 77% in Vietnam, 85% in China, 86% in the Philippines, 94% in Japan, and 100% in Korea, Malaysian and Thailand (McOrist, 2005). Serological studies conducted in Brazil and Thailand found that, in absence of herd vaccination, 100% of commercial herds were seropositive for *L. intracellularis* infection indicating direct contact with the pathogen (Wattanaphansak et al., 2011; Resende et al, 2015).

Studies describing *L. intracellularis* susceptibility to antimicrobials are limited. Published data have shown MIC endpoints for diverse isolates expressed as both extracellular and intracellular MICs using a tissue culture system (McOrist et al., 1995; McOrist and Gebhart, 1995; Wattanaphansak et al., 2009; Yeh et al., 2011). Both MIC endpoints were designed to mimic the pattern of *L. intracellularis* infection *in vivo*. The bacteria would be exposed to antimicrobials before and after invasion into intestinal cells (extracellularly and intracellularly, respectively). Similar to previous studies, our MIC endpoints for the two independent bacterial preparations (intracellular and extracellular) consistently fell within two-fold dilution, indicating the reproducibility of the assays.

Our extracellular and intracellular MIC results showed that carbadox, tiamulin and valnemulin were the most active compounds against the Brazilian and Thai isolates, inhibiting extracellular and intracellular activities with concentrations of 0.125-8 μg/mL. Since the use of carbadox is not permitted in Brazil, this component was not tested for the Brazilian isolates. Tylosin had intermediate activity against all the isolates with MICs ranging from 2 to 64 μg/mL for intracellular and extracellular activities, except for one Brazilian isolate, which had an MIC of >128

μg/mL for extracellular activity. Lincomycin was the least active compound against the Brazilian and Thai *L. intracellularis* isolates with an MIC of >128 μg/mL. This might be due to the fact that this antimicrobial has been used more intensively with high dosages for control of other endemic pathogens in swine farms or by the difficulty of extrapolating the *in vitro* results to the *in vivo* scenario.

When compared to other data, the MIC values for the Brazilian and Thai *L. intracellularis* isolates tended to have higher MIC endpoints than the North American, European and Korean isolates (Wattanaphansak et al, 2009; Yeh et al. 2011). For intracellular MIC results, valnemulin and tiamulin demonstrated the highest activity against the Brazilian and Thai *L. intracellularis* isolates, similar to the results previously published for North American, European, and Korean isolates (Wattanaphansak et al, 2009; Yeh et al. 2011).

Chlortetracycline showed intermediate activity against Brazilian isolates (8 - 64 μg/mL), whereas Thai isolates less sensitive (64 - >128 μg/mL). This was the only antibiotic with substantial differences between the Thai and Brazilian isolates, with the exception of the Thaistrain CUPIA02_SW13, which had similar results to the Brazilian isolates. Thai isolates were also resistant to colistin, gentamicin, trimethoprim and bacitracin (64 - >128 μg/mL).

Previous reports have shown that extracellular MICs for all tested antimicrobials were higher than the intracellular MICs (Wattanaphansak et al., 2009; Yeh et al., 2011), and our results were similar. The difference between intracellular and extracellular MICs may be due to the period of time *L. intracellularis* was exposed to the antimicrobial agent in each of the preparations. Extracellular MICs were designed to have a 24 hour incubation, while intracellular preparationswere incubated with *L. intracellularis* for three consecutive days. Moreover, it is likely that the effect of antimicrobials accumulated inside the cells overtime. This accumulation of intracellular antimicrobial concentration suggests that a one-time antimicrobial treatment may be insufficient to inhibit the growth of *L. intracellularis in vitro*.

In conclusion, our *in vitro* data expand the information regarding antimicrobial susceptibility of *L. intracellularis*. We confirm that Brazilian and Thai *L. intracellularis* isolates have a unique antimicrobial sensitivity pattern, in relation to other regions. Since it is impractical to culture *L. intracellularis* and perform an antimicrobial sensitivity test during a PE outbreak, our data serve as a guideline for the range of antimicrobial activities against *L. intracellularis*.

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Conflict of interest

Despite the fact of the project had been partially funded by Novartis Animal Health, the company had no involvement in conducting the study.

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Table 5.1. Extracellular and intracellular MIC endpoints for five antimicrobials against two Brazilian *L. intracellularis* isolates. The bacteria were prepared independently and tested twice. The endpoint was obtained from three replicates of each passage. Data is expressed as Minimum Inhibitory Concentration (MIC) μg/mL.

Antimicrobials	Minimum Inhibitory Concentration (MIC) μg/mL			
	BRPHE01_E5 Passages 13-14		BRPHE02_E8 Passages13-14	
	Intracellular activity	Extracellular activity	Intracellular activity	Extracellular activity
1. Chlortetracycline	32-64	32-64	8-16	64
2. Valnemulin	0.125	0.125	0.125	0.125
3. Tiamulin	0.125	1-2	1-2	0.5
4. Lincomycin	>128	>128	>128	>128
5. Tylosin	2-8	>128	2	16-32

Table 5.2. Extracellular and intracellular MIC endpoints for 15 antimicrobials against three Thailand *L. intracellularis* isolates. The bacteria were prepared independently and tested twice. The endpoint was obtained from the median value of three replicates of each passage.

Antimicrobials	Minimum Inhibitory Concentration (MIC) μg/mL					
	CUPHE01_SW13 Passages 6-7		CUPIA01_SW13 Passages 8-9		CUPIA02_SW13 Passages 5-6	
	Intracellular activity	Extracellular activity	Intracellular activity	Extracellular activity	Intracellular activity	Extracellular activity
1. Chlortetracycline	>128-64	>128	>128	>128	32	32
2. Carbadox	0.125	8	0.125-0.25	0.5-1	0.125-0.25	0.5
3. Valnemulin	0.125	0.5-1	0.125	0.5-0.25	0.125	0.25
4. Tiamulin	1	8	1	4	0.125-0.25	0.5
5. Lincomycin	>128	>128	>128	>128	>128	>128
6. Spectinomycin	128	32	>128	8-4	>128-128	4-2
7.Lincomycin+ Spectinomycin	128-64	16-32	>128	8-4	64-128	2
8. Gentamicin	>128	>128	>128	128->128	>128	64-128
9. Amoxicillin	8-16	>128	8	>128	16-32	64
10. Sulfamethazine	128	>128	4-8	>128	32-64	>128
11. Trimethoprim	64	>128	>128-128	>128	>128	>128
12. Enrofloxacin	8	>128	4-8	64	16	32-64
13. Colistin	>128	>128	>128	>128	>128	>128
14. Tylosin	8-16	32	4	64	2	8
15. Zinc-Bacitracin	>128-128	>128	>128	>128	>128	>128

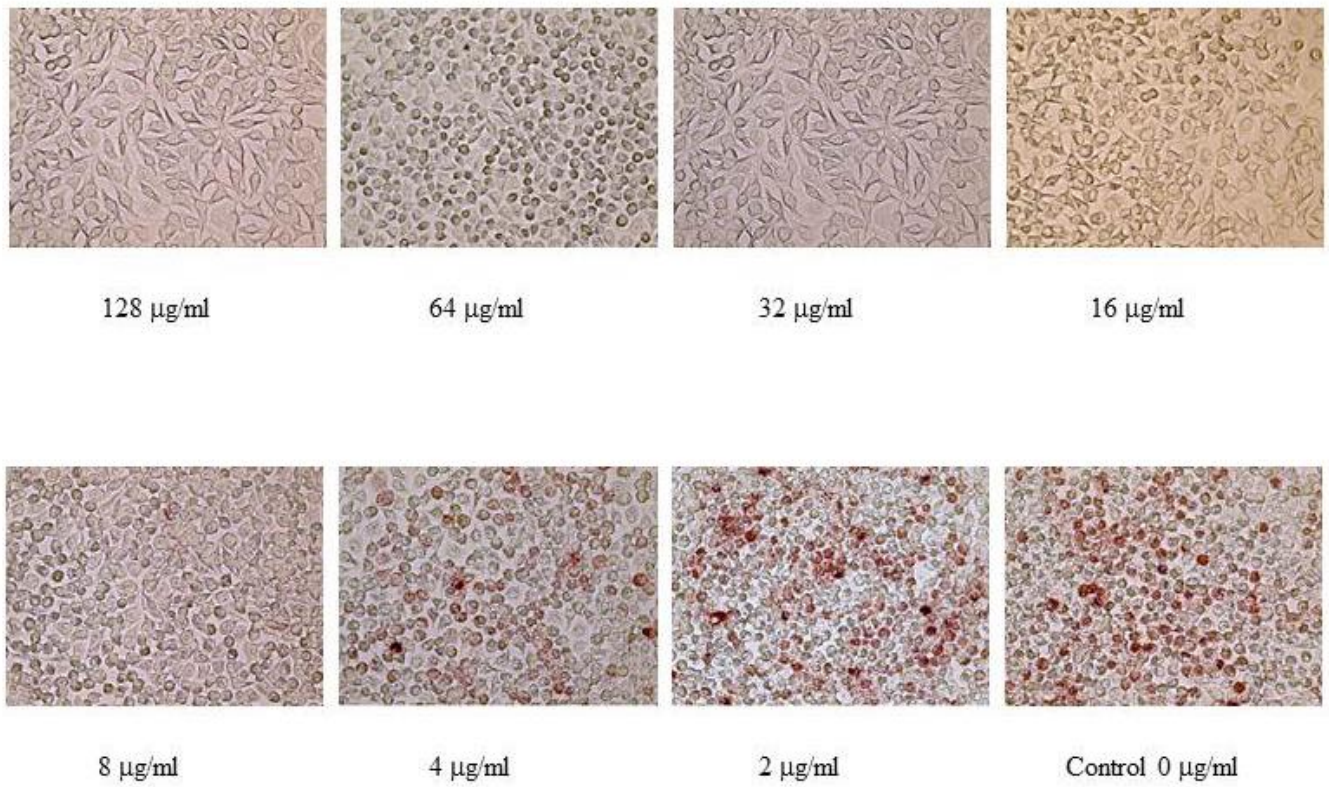

Figure 5.1. An example of an MIC endpoint for tiamulin against *L. intracellularis* strain CUHE01_SW13 at passage 6. Photographs of McCoy cells infected with *L. intracellularis* growing in the presence of tiamulin with concentrations ranging from 2 µg/mL to 128 µg/mL. There was no *L. intracellularis* growth in cells treated with tiamulin at concentrations ranging from 16 µg/mL to 128 µg/mL. The numbers of HICs dramatically increased at the concentration of 4 µg/ml (>1% compared to control). Therefore, the MIC of tiamulin for this *L. intracellularis* strain is 8 µg/mL (<1% compared to control).

CHAPTER 5 - *In vitro* antimicrobial activity against equine *Lawsonia intracellularis* isolates

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Abstract
Lawsonia intracellularis is the etiologic agent of equine proliferative enteropathy (EPE). This disease considered emerging in horses, leads to diarrhea, severe protein loss and can result in death if left untreated. Timely treatment of EPE is critical for recovery from the disease, and hence, information about antimicrobial susceptibilities of equine *L. intracellularis* strains to antimicrobials used in equines is needed. However, *L. intracellularis* is an obligately intracellular bacterium and so must be isolated and maintained in cell cultures. The objective of this study was to determine the *in vitro* antimicrobial activities of 14 antimicrobials against two equine *L. intracellularis* strains. Tested antimicrobials included metronidazole, minocycline hydrochloride, erythromycin, cephalothin sodium salt, combination (4:1) of sulfamethazine and trimethoprim, chloramphenicol, rifampicin, penicillin, ampicillin, doxycycline hydrochloride, cefazolin sodium salt, clarithromycin, ceftiofur hydrochloride and enrofloxacin. The minimum inhibitory concentration (MIC) was based on intracellular and extracellular activity that inhibited 99% of *L. intracellularis* growth in cell culture as compared to the antimicrobial-free control. Rifampicin and clarithromycin were the most active antimicrobials against the two *L. intracellularis* strains tested, with MICs of 0.125 when tested both intracellularly and extracellularly. Doxycyline, minocycline, erythromycin, chloramphenicol and enrofloxacin showed high to intermediate activity, especially when evaluating intracellular activity Sulfamethazine/trimethoprim showed variable results. Ampicillin, penicillin and metronidazole showed moderate to low activity. *L. intracellularis* was resistant to cefazolin, cephalothin and ceftiofur in *in vitro* conditions. This is the first report of antimicrobial susceptibility patterns for equine *L. intracellularis* strains.

Keywords: Antimicrobial susceptibility**,** *Lawsonia intracellularis*, MIC, equine proliferative enteropathy.

Introduction

Equine proliferative enteropathy (EPE) is an emerging disease primarily affecting younger horses, primarily recently weaned foals, but may occasionally affect older animals (Pusterla and Gebhart, 2013). The disease is characterized by diarrhea, fever, weight loss and hypoproteinemia (Lavoie et al., 2000). *Lawsonia intracellularis*, the etiological agent of EPE, is a Gram-negative, obligately intracellular, motile bacterium (Pusterla and Gebhart, 2013). *L. intracellularis* causes similar disease in other animal species and has significant economic importance in the pig industry (Vannucci and Gebhart, 2014).

The main method of control for EPE is based on antimicrobial therapy (Sampieri, et al., 2014). The timely use of effective antimicrobial agents generally stops the progression of the disease and recovery is common. Therefore, the choice of appropriate antimicrobial is critical to obtain success in treating the disease. Despite the importance of antimicrobial treatment, information on the *in vitro* sensitivity of *L. intracellularis* to antimicrobial compounds is limited and restricted to pig strains (McOrist and Gebhart, 1995; Wattanaphansak et al., 2009; Yeh et al., 2011). This is due to the fact that very few equine strains have been established in cell culture and the difficulty of maintaining the organism in laboratory conditions. We have established two equine strains of *L. intracellularis* in cell culture for use in determining antimicrobial MICs *in vitro.*

The aim of this study was to determine the *in vitro* minimum inhibitory concentration (MIC) of antimicrobials against two cell-cultured equine strains of *L. intracellularis* (Foal/96 and e40504). Each antimicrobial was evaluated for both intracellular and extracellular activity. Tested antimicrobials included metronidazole, minocycline hydrochloride, erythromycin, cephalothin sodium salt, chloramphenicol, sulfamethazine/trimethoprim, rifampicin, penicillin, ampicilin, doxycycline hydrochloride, cefazolin sodium salt, clarithromycin, ceftiofur hydrochloride and enrofloxacin.

Materials and Methods

Lawsonia intracellularis culture

Two *L. intracellularis* strains obtained from EPE-affected foals, designated foal/96 and e40504, were tested. Both strains were obtained from the intestines of foals that were euthanized due to severe lesions of proliferative enteropathy, foal/96 was isolated in 1996 and e40504 in 2014. Each strain of *L. intracellularis* was isolated and maintained in murine fibroblast-like McCoy cells (CRL 1696, American Type Culture Collection, Virginia, United States) as described previously (Guedes and Gebhart, 2003; Vannucci et al., 2012) and stored at -80ºC until use. The infected cell cultures were monitored weekly, using immunoperoxidase staining with *L. intracellularis*-specific rabbit polyclonal antibody as described previously (Guedes and Gebhart, 2003). *L. intracellularis* was maintained in cell cultures for three consecutive passages before antimicrobial susceptibility testing, to allow the bacterium to recover from the frozen condition.

After establishment of optimum growth in pure culture, each *L. intracellularis* strain inoculum was quantified for the antimicrobial MIC assay using a qPCR assay, as previously described (Wattanaphansak et al., 2010).

Source and preparation of antimicrobials

The following antimicrobial agents were obtained as pure chemicals: metronidazole, minocycline hydrochloride, erythromycin, cephalothin sodium salt, chloramphenicol, sulfamethazine/trimethoprim (4:1), rifampicin, penicillin, ampicillin, doxycycline hydrochloride, cefazolin sodium salt, clarithromycin, ceftiofur hydrochloride and enrofloxacin (Sigma-Aldrich, Missouri, United States). The working solutions of tested antimicrobials were prepared as previously described (Wattanaphansak et al, 2009). Briefly, the antimicrobial stock solutions were prepared to a final concentration of 2,560 μg/ml and were filtrated through 0.2 μm-pore size filters. All stock solutions were aliquoted and kept at -20ºC until use. A series of two-fold dilutions of this 10X concentration were made and diluted 1:10 with culture medium. The final concentrations of tested antimicrobials were 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 μg/ml. Each concentration of

antimicrobial was tested in triplicate and each strain of *L. intracellularis* was tested twice from two independent bacterial preparations.

Antimicrobial sensitivity testing

A cell culture system was modified from previous antimicrobial MIC studies of porcine *L. intracellularis* strains (McOrist and Gebhart 1995; Wattanaphansak et al., 2009) to determine the MIC of each antimicrobial against *L. intracellularis*. For intracellular testing, 100 µl of each bacterial suspension containing approximately 10^6–10^7 organisms/ml was inoculated onto one-day-old McCoy cells 24 h before exposure to the antimicrobials. This allowed sufficient time for *L. intracellularis* to penetrate the host cells prior to antimicrobial exposure. After incubation, the bacterial suspension was removed and replaced with 100 µl of fresh culture medium containing two-fold concentrations of various antimicrobials (0.125 to 128 µg/ml) at 1, 2, and 3 days post inoculation, followed by fresh culture media on day 4 with no antimicrobial, as previously described (Wattanaphansak et al., 2009). For extracellular testing, both strains of *L. intracellularis* were exposure in two-fold concentrations of all antimicrobials for 2 hours at 37ºC in a bag. After exposure, 100 µl of bacteria suspension was transferred onto one-day-old McCoy cells. The medium was change without any antimicrobials for three consecutive days. After 5 days of incubation, supernatant from the infected plates was removed and the cell culture monolayers were fixed with cold 50% acetone and 50% methanol for 1 min. To assess the inhibitory effect of each antimicrobial on *L. intracellularis* growth, the infected plates were stained using a modified immunoperoxidase monolayer assay (IPMA) staining method with primary antibody from a rabbit hyperimmunized with *L. intracellularis* whole cell antigen (Guedes and Gebhart, 2003). *L. intracellularis* growth was evaluated by counting the number of heavily infected cells (HIC) (McOrist and Gebhart, 1995) in each well using an inverted microscope (Olympus, Tokyo, Japan) with 20X objective lens. Cells were considered to be HIC if the number of intracellular *L. intracellularis* had proliferated to greater than 30 bacteria per cell. The number of HICs in each well was then expressed as a percentage compared to the average HIC of the control wells. The intracellular MIC endpoints for each antimicrobial in this study was defined as the lowest antimicrobial concentration that inhibited 99% of *L. intracellularis* proliferation in the McCoy cells after 5 days of incubation. These inhibitions were indicated by the percentage of HIC of each antimicrobial concentration as compared to the antimicrobial-free control (Wattanaphansak et al., 2009).

Results

L. intracellularis strains were tested for antimicrobial MICs when between passages 15 and 17 for e40504, and between passages 26 and 27 for foal/96. The final concentration of inoculum was approximately between 10^6 and 10^7 *L. intracellularis*/ml for both strains in all repetitions.

The value of extracellular and intracellular MICs for all tested antimicrobials against the equine *L. intracellularis* strains are shown in Table 1. MIC endpoints for each antimicrobial were the concentrations that were able to inhibit 99% of *L. intracellularis* proliferation when compared to the antimicrobial-free control. The difference in MIC median of two independent preparations for each strain was within two doubling dilutions.

For both intracellular and extracellular MIC testing, rifampicin and clarithromycin had the highest activity against both equine *L. intracellularis* strains, with MIC values being 0.125 µg/ml as *L. intracellularis* did not grow in the lowest concentration used.

Doxycyline, minocycline, erythromycin, chloramphenicol and enrofloxacin had good activity against both *L. intracellularis* strains when testing intracellular MICs, with the MIC values ranging from 0.125 to 0.5 µg/ml. Enrofloxacin also had good extracellular activity, with MICs ranging from 0.25 to 1 µg/ml. Doxycycline, minocycline, erythromycin and chloramphenicol) had moderate to low activity ranging from 1 to 32 µg/ml with the exception of erythromycin for the foal/96 strain which was 0.5 µg/ml and doxycycline for the e40504 strain which was 0.125 to 0.5 µg/ml.

Ampicilin, penicilin and metronidazole showed moderate activity against both *L. intracellularis* strains when testing intracellular MICs, ranging from 1 to 32 µg/ml, but showed low activities when testing extracellular MICs, ranging from 2 to >128 µg/ml. Sulfamethazine/trimethoprim (4:1) showed variable intracellular MIC values for each strain with the

foal/96 strain MICs ranging from 0.5 to 2 μg/ml and the e40504 strain MICs ranging from 128 to >128 μg/ml. The extracellular activity of sulfamethazine/trimethoprim (4:1) for both strains ranged from 64 to >128 μg/ml.

Cefazolin, cephalothin and ceftiofur showed the lowest activities against both *L. intracellularis* strains. Both intracellular and extracellular MICs for those antimicrobials ranged from 32 to >128 μg/ml.

Discussion

This study represents the first evaluation of antimicrobial susceptibilities for *L. intracellularis* strains obtained from EPE-affected foals. Although outbreaks of proliferative enteropathy occur in multiple species globally, few *L. intracellularis* strains from have been successfully cultured and maintained *in vitro* worldwide, and those are primarily porcine. Among these *L. intracellularis* strains, only two have been obtained from foals. While 15 swine *L. intracellularis* strains have been tested for their antimicrobial susceptibilities (McOrist and Gebhart, 1995; Wattanaphansak et al., 2009; Yeh et al., 2011), there is no report in the literature about the antimicrobial susceptibilities of horse strains.

We used extracellular and intracellular assays *in vitro* to mimic the antimicrobial effects of each antimicrobial before and after *L. intracellularis* is internalized in the host cell. In the swine industry, antimicrobials are commonly used in low dosages in a diet which diminishes the severity of infections and may even prevent the pigs from being infected. Hence, the extracellular assay is the approach that best represents the dynamics of proliferative enteropathy in swine herds. In horses, antimicrobial compounds are used in individual therapeutic treatment, usually by intra-venous administration. Therefore, the intracellular assay is the approach that better mimics the clinical treatment of horses affected by EPE. Nevertheless, since *L. intracellularis* can spread within the intestine of the host by infecting neighboring intestinal cells during the course of the disease, the extracellular activity of antimicrobials for horse *L. intracellularis* strains may also be relevant.

Our intracellular and extracellular MIC results showed that clarithromycin and rifampicin were the most active compounds against both equine strains, inhibiting *L. intracellularis* growth at concentrations of 0.125 μg/ml. No other studies have evaluated those antimicrobial compounds for equine, or even porcine, *L. intracellularis* strains. Rifampicin is commonly used in antimicrobial therapy in foals affected by this disease, often in combination with erythromycin (Lavoie, 2000). Erythromycin had high activities against both strains, mainly in the intracellular assay (0.125-0.25 μg/ml). Similar results were observed previously for porcine *L. intracellularis* strains (McOrist and Gebhart, 1995).

Doxycyline, minocycline, erythromycin, chloramphenicol and enrofloxacin also showed efficacies against both strains at low antimicrobial concentrations, with MICs ranging from 0.125 to 0.5 μg/ml for the intracellular assay. However, the same antimicrobials were less effective in the extracellular assay with MICs ranging from 1 to 16 μg/ml. The exceptions were enrofloxacin for both strains, erythromycin for the foal/96 strain and doxycycline for the e40504 strain, which also showed good activities in the extracellular assay. These results are in agreement with a study (Slovis, 2014) that documented successful treatment of EPE with macrolides (erythromycin or azithromycin), oxytetracyclines (oxytetracycline, doxycycline or minocycline) and chloramphenicol. In our study, these molecules showed high activity against equine *L. intracellularis* strains. Interestingly, enrofloxacin had low activity against porcine *L. intracellularis* strains (McOrist and Gebhart, 1995; Yeh et al., 2011).

Metronidazole, ampicillin and penicillin had moderate activities against both equine *L. intracellularis* strains, with higher MIC results observed for the intracellular assay than for the extracellular assay. The foal/96 strain was more susceptible than the e40504 strain to metronidazole. Ampicillin and penicillin were previously tested against porcine *L. intracellularis* strains (McOrist and Gebhart, 1995; Yeh et al., 2011), and also had moderate activities against *L. intracellularis*, as observed in the present study.

Sulfamethazine/trimethoprim showed variable antimicrobial activities when testing not only intracellular and extracellular activities but also between equine *L. intracellularis* strains. Cefazolin,

cephalothin and ceftiofur were the least active antimicrobial compounds against both equine *L. intracellularis* strains, with MICs ranging from 32 to >128 μg/ml.

In conclusion, our *in vitro* results generate information on the antimicrobial susceptibilities of *L. intracellularis* strains obtained from foals. Rifampicin and clarithromycin were the most active of the tested antimicrobials against equine *L. intracellularis* strains *in vitro*. Our results serve as a guideline for the treatment choice against *L. intracellularis* for EPE cases or during EPE outbreaks.

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Conflict of interest
Though this project was partially funded by Boehringer-Ingelheim, the company had no involvement in conducting or interpreting the results of the study.

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Table 6.1. Intracellular and extracellular MIC endpoints for 14 antimicrobials against two equine *L. intracellularis* isolates. The bacterial innoculums were prepared independently and tested twice. The endpoint was obtained from the median value of three replicates of each passage, in each experiment.

Antimicrobials	Minimum Inhibitory Concentration (MIC) µg/mL			
	foal/96 Passages 26-27		e40505 Passages 15-17	
	Intracellular activity	Extracellular activity	Intracellular activity	Extracellular activity
1. Rifampicin	0.125	0.125	0.125	0.125
2. Sulfamethazine + Trimethoprim	0.5-2	64->128	128->128	64->128
3. Doxycycline	0.125	2-4	0.125-0.25	0.125-0.5
4. Cefazolin	>128	>128	64->128	64->128
5. Ampicilin	4	8-16	4-8	128->128
6. Penicilin	2	2	2	32-128
7.Clarithromycin	0.125	0.125	0.125	0.125
8. Metronidazole	1-2	16-32	32	128->128
9. Minocycline	0.125-0.25	1-2	0.125	8-32
10. Cephalotin	32-64	>128	128->128	128->128
11. Erythromycin	0.125-0.25	0.5	0.125	4-16
12 Chloramphenicol	0.25-0.5	2-8	0.125-0.5	1-4
13. Ceftiofur	128->128	>128	128->128	32->128
14. Enrofloxacin	0.125-0.25	0.5	0.125	0.25-1

Conclusions

- The methodology described in Chapter 1 was efficient for obtaining and differentiating swine monocytes into $CD14^+$ macrophages. The interaction between *Salmonella* and porcine macrophages demonstrated the phagocytic capacity of these cells. In addition, the early cytotoxic effects of SPI-1 have been demonstrated in swine macrophages. Together, our results clearly characterized peripheral blood monocyte-derived swine macrophages both phenotypically and functionally, thus supporting further studies on macrophage function and pathogen interactions.
- Chapter 2 demonstrated the involvement of clathrin in the endocytic process of *L. intracellularis* and has also confirmed that *L. intracellularis* can be internalized through mechanisms that depend on its viability.
- Chapter 3 demonstrated the survival capacity of *L. intracellularis* within macrophages, more precisely, in the phagolysosomes.
- Based on our *in vitro* results (Chapter 4), we confirm that Brazilian and Thai *L. intracellularis* isolates have a unique antimicrobial sensitivity pattern.
- Our *in vitro* results generated information for the antimicrobial susceptibility of *L. intracellularis* isolates obtained from foals (Chapter 5).